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		Abstract Numbers
Symposia/Plenary Lecture		S 2 – S 6
Experimental Pharmacology		
1	Transport	1 – 9
2	Molecular Receptor Pharmacology	10 – 37
3	Cholinergic Mechanisms	38 – 39
4	Adrenergic Mechanisms	40 – 47
5	Serotoninergic Mechanisms	48 – 49
6	Glutamatergic Mechanisms	50
7	Purinergic Mechanisms	51 – 56a
8	Peptidergic Mechanisms, Opioids	57 – 61
9	Phospholipases, Eicosanoids	62 – 64
10	NO- and CO-mediated Mechanisms	65 – 78
11	Heterotrimeric G-Proteins	79 – 92
12	Small G-Proteins	93 – 115
13	Cyclic Nucleotides	116 – 128
14	Protein Kinases, Tyrosine Kinases	129 – 141
15	Calcium Channel Modulators, Calcium, Calmodulin	142 – 153
16	Potassium Channel Modulators	154 – 167
17	Renin-Angiotensin-System	168 – 172
18	Endocrine Pharmacology	173 – 179
19	Immunopharmacology, Cytokines	180 – 210
20	Central Nervous System	211 – 238
21	Heart	239 – 274
22	Circulation	275 – 282
23	Respiratory Tract	283 – 284
24	Gastrointestinal Tract	285 – 291
25	Kidney	292 – 294
26	Pharmacokinetics and Drug Metabolism in Animals	295 – 298
27	Miscellaneous	299 – 312

Abstract Numbers

Toxicology

		Abstract Numbers
28	Drug Metabolizing Enzymes	313 – 333
29	Drug Metabolism / Toxicokinetics	334 – 342
30	Organ Toxicity	343 – 368
31	Toxicological Aspects of Drugs	369 – 375
32	Toxicological Aspects of Natural Compounds	376 – 389
33	Apoptosis	390 – 393
34	DNA Modification	394 – 397
35	DNA Repair	398 – 411
36	Mutagenesis / Clastogenesis	412 – 417
37	Carcinogenesis	418 – 423
38	Signal Transduction	424 – 436
39	Genomics / Proteomics	437 – 442
40	Transport Mechanisms	443 – 449
41	Reactive Oxygen Species	450 – 453
42	In Vitro Test Systems	454 – 466
43	Biomonitoring / Hemoglobin Adducts	467 – 469
44	Food Toxicology	470 – 475
45	Environmental Toxicology	476 – 477
46	Regulatory Toxicology	478 – 480

Clinical Pharmacology

47	Molecular Clinical Pharmacology and Disease Models	481 – 484
48	Pharmacogenomics and Pharmacoepidemiology	485 – 490
49	Pharmacokinetics	491 – 494
50	Clinical Studies	495 – 498
51	Other Areas of Clinical Pharmacology	499 – 505

AUTHOR INDEX
Figures refer to the abstract number

Aasland D. 399
Abel J. 319, 320, 352, 425, 427, 428, 455, 456, 471
Abraham G. 45, 284
Abraham K. 376
Ackermann F. 306
Ackermann S. 158, 159
Adams F. 311, 495, 501
Adamska M. 327, 328, 329, 332
Adler H. 65
Adler M. 389
Aeby P. 454
Aeynichenko E. 255
Aggarwal M. 325, 344, 414
Agnoletto L. 83
Agostini H.T.A. 40
Aguilar-Bryan L. 157
Ahles A. 19
Ahmad M.H. 357, 359, 405
Ahmadian M.R. 86
Ahuja V. 346
Aigner A. 87, 373
Akool E.S. 191
Aktories K. 2, 83, 88, 89, 93, 444, 445
Alber J. 8
Albrecht C. 363, 426
Ale-Agha N. 388
Alexandre E. 438
Allescher H. 133
Altemöller M. 397
Altenhöfer S. 77, 183
Amann A. 283, 291
Amarenco P. 500
Amberg A. 365
Ambroziak K. 4
Ammer H. 57, 92, 476
Andrade A.J. 349
Angay O. 289
Angerer J. 468
Angioni C. 63
Appel K.E. 335, 349, 442
Appelt K. 54
Appenroth D. 292
Appl H. 29
Arand M. 327, 328, 329, 330, 331, 332
Artati A. 342
Arveiler D. 500
Auer V. 179
Augustin R. 105, 106

Baarlink C. 102
Bach F.W. 375
Bachmann M. 75, 189, 190
Bader M. 469
Baer W.U. 222
Bäumer W. 204, 205, 375
Bakhiya N. 447
Baktir G. 298
Baldus S. 277
Bali K. 227
Baltus D. 103
Banduhn N. 464
Bankstahl J.P. 234
Barakat W. 226
Baranowska U. 43
Barbara A. 318
Barbelivien A. 218
Bargiotas P. 232
Barreto F. 42, 171, 239, 252
Barreto-Pereira H.F. 41
Barth H. 100, 443, 446
Barthel A. 435
Bartsch H. 488
Barufe D. 124
Bauer A. 459
Bauer M. 400
Bauer-Marinovic M. 447
Bauersachs J. 273
Baugé E. 275
Baum M. 470
Baumann B. 226
Baumann M. 243
Baumann S. 491
Baumann-Wilschke I. 346
Baumgärtel-Allekotte D. 184
Baumgart P. 247
Bayer M. 208
Beavo J.A. 123
Bechara R. 215
Becherel O.J. 406
Becirovic E. 149
Beck A. 71
Beck G. 279
Beck K.F. 187
Becker A. 59
Becker C. 419
Becker D. 56
Becker M. 61, 307
Becker W. 63
Beer Stolz D. 458
Beetz N. 41, 82, 239
Behm C. 384, 414
Behrendt H. 314, 477
Beilfuß A. 503
Beining K. 502
Bellinghausen I. 197, 199
Ben Hamida S. 219
Bengel C. 486
Berger F. 470
Berger S. 171
Berlan M. 311
Bernauer U. 313, 335
Bernshausen T. 322, 323, 457
Bert B. 237, 305
Besche V. 196, 197, 198, 209
Besz D. 494
Bethmann K. 234
Beuerlein K. 282
Beyer M. 156
Bieber D. 51
Biedermann T. 182
Biel M. 82, 149, 213, 225, 259
Bieler C. 478
Bien S. 300
Billiet L. 275
Bingold T. 188
Birchmeier C. 420
Birkenfeld A.L. 311
Birnbaumer L. 84
Bitopoulos A. 308
Blaszkewicz M. 333
Bloch M. 175
Blömeke B. 350
Blume R. 176, 178
Blume U. 422
Bock I. 110
Bock M. 356
Bock U. 283, 291
Bockamp E. 419
Böckler A. 243
Böckmann C. 170
Böer U. 224
Boege F. 397
Böhm C. 169
Böhm V. 498
Boehmer S. 472
Boehn S.N. 457, 462
Boehncke W. 192
Boekhoff I. 90, 306
Bönisch H . 5, 48, 214, 223,
Boenisch H. 290
Börchers S. 178
Börner C. 200, 201
Bohr V. 410
Bohr V.A. 411
Boknik P. 240, 270
Bolay C. 436
Bolt H.M. 333, 463
Bonaterra G.A. 210
Boots A. 363
Boronowski D. 250
Borowiak M. 420
Borth H. 90
Bos J.L. 123
Boschmann M. 311, 495, 501
Bothe H. 320
Braam U. 56
Braeter M. 288
Braeuning A. 324
Bramer R. 468
Brand E. 247, 248, 482, 500, 502
Brand-Herrmann S.-M. 247
Brand-Herrmann S.M. 248, 482, 500, 502
Brandt D. 102
Braun M. 375
Brawek B. 220
Breit A. 60, 303
Breithardt G. 244, 255
Bremer S. 368, 461
Brenneis C. 63
Breuer D. 468
Brieger J. 353
Briegert M. 400
Brink A. 415
Brinkkötter P.T. 279
Broeckel U. 82
Bros M. 195, 196, 197, 198, 199, 209
Broszat S. 337
Brown R. 355
Brueggener L. 335
Brüning J. 257
Brüning T. 468
Brugger S. 12
Brune K. 228, 493, 494
Brunner F. 68
Brunner J. 331
Bryan J. 155, 157, 166
Bucher M. 294
Buchholz S. 146
Buchmann A. 420, 442
Buchwalow I.B. 243
Budde T. 254
Budziarek P. 311
Büch T. 87
Büchele B. 22, 275, 301
Büchter C. 380, 381
Bünemann M. 27, 31, 81, 117, 128
Bürkle A. 408, 409, 410
Bunney T.D. 108
Burgener J. 330
Burger S. 39
Burghard S. 9
Burk O. 486
Burkhardt S. 354
Buss H. 181
Bußmann A. 505
Buters J.T.M. 314, 477
Butt E. 123, 127
Butterweck V. 238

Calebiro D. 116
Cambien F. 247, 248, 500, 502
Carmichael P. 454
Caron S. 169
Cassel J.C. 217, 218, 219
Castoldi M. 273
Castor T. 197
Cavalié A. 146
Chagnon M.C. 450
Chahoud I. 349
Cheremina O. 493, 494
Chmill S. 343
Choi S.L. 452
Chopra M. 393
Chovolou Y. 377, 378, 379, 380, 381, 382, 383, 385, 429, 430
Christ T. 164, 165
Christalla P. 251
Christen S. 208
Christen U. 207, 208
Christensen A.E. 123
Christensen M.O. 397
Christian F. 129, 245
Christmann M. 391, 399, 411, 423
Christow C. 107, 108
Chubanov V. 147, 148
Clemenz M. 169
Cline J.E. 455
Closs E.I. 65, 66, 67
Closs E. 485
Coco C. 103
Copin C. 275
Coronel R. 244
Coste O. 63
Creutzenberg O. 355
Cronin A. 327, 332
Csanády G.A. 342, 452
Csicsaky M. 469
Cuaz-Pérolin C. 275
Cuny H. 225
Czapp M. 476
Czernecki R. 236
Czubayko F. 373
Czuczwar S.J. 236

Dai G. 272
Daiber A. 66, 76
Dally H. 488
Damm E. 60
Damrot J. 432
Dandapat S. 344
Dandekar T. 127
Dang N.-A. 196, 197, 198
Daniel C. 194, 206
Daskala I. 295
deBakker J. 244
Debbab A. 383

Decker K. 413
Decker M. 327, 329
Dedoussis G. 408
Degen G.H. 315, 325, 344, 347, 348, 384, 414, 463
Dekant W. 364, 365, 366, 389
Dempe J.S. 387
Dendorfer A. 37, 262
Deng S. 68, 241, 299
Di Giallonardo F. 327, 328
Diabaté S. 424
Diefenbach J. 409
Diel P. 347, 348
Dietel B. 416
Dieter H.H. 474
Dietrich A. 142
Dietrich C. 451
Dietrich N. 307
Dietrich S. 477
Dislian V. 504
Dittrich M. 127
Dittrich-Breiholz O. 139
Dobrev D. 165, 242
Doehring A. 489
Dördelmann C. 247, 500
Döring B. 7
Dörrenhaus A. 317
Doerrie A. 181
Dolga A.M. 119
Doller A. 191
Dominiak P. 37, 168, 262, 280
Donauer J. 433
Dorn S.B. 463
Dorsch S. 31
Doskeland S.T. 123
Doss X. 271
Draus E. 336
Dreger S.C. 96, 97, 109, 110
Dreieicher E. 187
Dreissigacker U. 312
Drewes G. 403, 422
Drews G. 157, 162
Driscoll P.C. 108
Drzewiecki K. 270
Düfer M. 157, 162
Dürk H. 332
Dürschmied D. 312
Dumont L. 267
Duraphe P. 136, 138

Ebelt H. 243
Eberhardt W. 75, 191
Eckers A. 435
Eckert G.P. 229
Eckhardt A. 345, 436
Eddiasty I.T. 296
Edler L. 488
Edwards R.J. 454
Egerer M. 2
Eich M. 392
Eichhorn B. 287, 288
Eisel U.L.M. 119
Eisele T. 34
Eisenblätter M. 346
Eisenbrand G. 431, 470
Eisinger D.A. 57
Eixarch H. 283
El-Armouche A. 240, 266, 274
El-Hawari Y. 326
El-Wahsch D. 30
Elbaz A. 500
Ellis E.C.S. 458
Elz S. 15
Emser A. 353
Endlich A. 67
Engelhardt S. 19, 116, 264, 273
Engeli S. 484
Engst W. 394
Enzenmüller S. 446
Epe B. 402, 404, 406, 407
Erdmann K. 285
Erdorf M. 126
Erkel G. 73
Ernst H. 355
Eschenhagen T. 46, 240, 251, 266, 274
Eschrich D. 334, 464
Eshkind L. 419
Esser C. 343
Essmann F. 180
Eucker T. 282
Evans A. 500
Everett K.L. 108

Fabian E.J. 322, 323, 457
Fabian J. 49
Fabisch C. 257
Fabritz L. 243, 244, 255
Fahrer J. 410
Fallah G. 30
Faust D. 451
Fazeli G. 396, 416
Fedorchenko O. 137, 138
Fedrowitz M. 3, 421
Fehr J. 90
Fehr M. 397
Feil R. 70, 71, 130, 141
Feil S. 70, 71
Feld J. 431, 470
Fernandes C. 39
Fernau N.S. 351
Fery Y. 321
Fester I. 83, 88
Feuerstein T.J. 218, 220
Fichtl B. 358
Fieblinger D. 412
Fiedler J. 273
Filser J.G. 342, 452
Fink H. 230, 237, 305
Fischer C. 134
Fischer E. 92
Fischer J. 248
Fischer J.W. 268, 272, 286
Fischer T. 273
Fischer W. 54
Fissler B. 335
Fleck C. 292
Fleckenstein D. 33, 271
Fleckenstein-Grün G. 267
Flick B. 460
Flockerzi V. 145, 146
Flohr-Beckhaus C. 407
Florian S. 447
Föller M. 70
Föllmann W. 315, 317, 384
Foellmann W. 414
Förster I. 363, 473
Förster K. 468
Förstermann U. 66, 67, 77, 183
Foest A. 370
Fonteles R. 92
Fortmüller L. 244, 255
Foryst-Ludwig A. 169, 175
Foth H. 357, 359, 405
Fraccarollo D. 273
Frank H. 394
Frank S. 173, 174
Franke G. 311, 495
Franke H. 54
Frantz S. 273
Freed W.J. 235
Frei E. 120, 152
Freichel M. 145, 146
Frère F. 331, 332
Freudenberger T. 268
Fricker G. 9
Friebe A. 65, 69, 78
Frisk A.L. 437
Fritsch R. 151
Fritsch S. 424
Fritsche E. 352, 454, 455, 456, 471
Fritz G. 96, 97, 202, 432
Fritz J. 397
Frölich J.C. 505
Fromm M.F. 483, 492, 499
Fromme H. 472, 477
Frost N. 169
Fuchs B. 142
Funcke M. 467
Furkert J. 129, 245
Fusser M. 406

Gaestel M. 97
Gallitz I. 428
Galuppo P. 273
Gamer A.O. 354
Gangadharan V. 141
Gansser S. 217
Gao H. 293
Gao W. 133
Garcia Moreno I. 371, 372
Gaßmann K. 455, 471
Gautier-Bertrand M. 500
Gavrilova O. 297
Gebhart M. 151
Geduhn J. 122, 125
Geiger E.V. 489
Geiger J. 127
Geisslinger G. 63, 133, 140, 481
Geng H. 181
Genieser H.G. 123
Genth H. 96, 97, 98, 99, 109, 110, 111
Genze F. 275
Gergs U. 243, 261, 265, 269
Gerhard R. 95, 112
Gerlach M. 416
Gerling A. 70
Gerloff K. 363
Gernert M. 216, 235
Gerstberger R. 39
Gerstmayr N. 436
Gerstner S. 449
Gestrich C. 411
Geyer J. 5, 6, 7, 39, 297
Gherbal R. 357
Ghofrani H.A. 142
Ghoreschi K. 70
Giannoglou G. 504
Giegold O. 192
Giehl K 104
Gier B. 157, 162
Gierden E. 467
Gierschik P. 79, 91, 107, 108, 113, 114, 115
Gierschik S. 115
Giesemann T. 2, 445
Gietzen K. 312
Gilsbach R. 41, 42, 171, 222, 239, 252
Glaeser H. 483, 499
Glahn F. 357, 359, 405
Glatt H.R. 394, 403, 447
Glöckner R. 316
Glowacki C. 197, 198
Glusa E. 44
Gminski R. 413
Goebel C. 454
Gödtel-Armbrust U. 68, 241, 299, 485, 486
Görnemann T. 44
Göthert M. 43
Göttel M. 450
Göttle M. 125
Götz M.E. 376
Gohla A. 84, 85, 136, 137, 138
Gold M. 87
Goldhaber J.I. 255
Goldstein M. 390
Golka K. 333
Gollasch M. 303
Golubev V. A. 277
Goren I. 173, 174, 189
Gorzelniak K. 484
Gosepath J. 353
Gothner M. 312
Gottschalk S. 495, 501
Grabbe S. 195, 196, 197, 198, 199, 209
Grandoch M. 33, 271, 503
Graulich E. 65
Grecksch G. 11
Grether-Beck S. 427
Grico N. 433
Grießmeier K. 225
Grimm M.O. 287
Grimminger F. 142
Grittner D. 160, 250
Groneberg D. 69, 78
Gross C. 273
Gross D. 28
Groß M. 393
Grosse R. 101, 102
Grosser G. 7
Grosser N. 276
Großer N. 285
Grote K. 349
Grote-Wessels S. 270
Grube M. 1, 46, 254, 490
Gruber L. 289
Gründemann S. 437
Grundmann O. 238
Grzelinski M. 87
Gudermann T. 60, 87, 90, 142, 147, 148, 303, 306
Guenther R. 434
Günther S. 258
Guerini D. 18
Guhl J. 337
Gumbel-Mako S. 449
Gundert-Remy U. 313, 335, 376
Guttenberg G. 445

Haag S. 21, 38
Haarmann-Stemmann T. 319, 320
Haas B. 72, 309
Haberlandt C. 223
Habermeier A. 65, 66, 67, 485
Haenisch B. 214, 223, 290
Häussinger D. 84
Hagedorn C. 247, 500
Hahn H. 241
Hahn J.-U. 468
Halabi-Cabezon I. 99
Halbhuber K.-J. 292
Halemann J.H. 440
Haltner-Ukomadu E. 283, 291
Hamann M. 231, 233
Hambrock A. 158, 159
Hameyer D. 419
Hammer C. 48
Hampel K. 129, 245

Han L. 62
Hanafy A. 296
Haney J. 254
Hansen F.C. 266
Hansen J.B. 160
Hansen T. 355
Harati H. 218
Harders A. 359
Hardt K. 192
Harorli T. 345
Harris R. 108
Harrison M.D. 82
Harst A. 53
Harteneck C. 143, 144
Hartmann L. 56a
Hartung T. 368
Hasenfuss G. 68, 240
Hasenkamp S. 482
Hatzistilianou M. 504
Haubold M.H. 40
Hauptmann S. 269
Hausmann R. 30, 34
Hecker D.C. 361
Heidbreder M. 262
Heim K.H. 268
Heimes K. 49
Hein L. 41, 42, 82, 171, 239, 252
Hein L.H. 40
Hein P. 27
Heine K. 100
Heinen C. 293
Heinrich A. 224
Heinrich-Hirsch B. 313
Heinrichs J. 455
Heinz C. 413
Heise T. 459
Hell J.W. 32
Hempel S. 316
Hengstler J.G. 458, 459
Henke B. 143
Henkel J. 106
Hensel A. 49
Hensel M. 446
Herberg F.W. 129, 245
Herbst F. 461
Herden C. 235
Hermanns I. 356
Hermes M. 36
Herrero M. 194
Herrero San Juan M. 206
Herrero San Juan M.H. 193
Herzig S. 239, 256, 257
Herzog M. 202
Hescheler J. 150, 271
Heß A. 228
Hesse D. 106
Heußel C.P. 488
Hewitt P. 438, 440
Hickel R. 314, 339, 340, 341
Hiemke C. 281
Hilberg P. 498
Hiller K.-A. 345, 436
Hiller S. 158, 159
Hilmenyuk T. 197, 199
Hinds T.R. 123
Hinkelmann U. 276, 285
Hintermann E. 207, 208
Hinz B. 64, 493, 494
Hippe H.-J. 80
Hippius M. 498
Hirsch B. 109
Hirsch-Ernst K.I. 448
Hobler C. 349
Hoda J.C. 151
Höbel S. 373
Höcherl K. 294
Höfer N. 347, 348
Höger S. 279
Hoegl S. 189
Höllt V. 10, 11, 58, 59, 200, 201
Hörnlein S. 498
Höschele D. 371, 372
Hösl E. 260
Hövelmeyer N. 209
Hoffmann A. 137, 138, 490
Hoffmann C. 25, 27
Hoffmann D. 364, 366
Hoffmann K. 52, 234
Hofinger A. 453
Hofmann A. 309
Hofmann F. 70, 71, 120, 130, 131, 132, 133, 152, 179, 213, 259, 441
Hofmann T. 148
Hofstetter C. 189
Hogendijk M. 244
Hohlfeld A. 383
Hohlfeld T. 20, 211, 503
Holdener M. 207

Homburg S. 332
Hommel A. 105, 106
Hommers L.G. 81
Homuth G. 490
Hoopmann M. 467
Horke S. 77, 183
Hotz-Wagenblatt A. 487
Hrach J. 438
Hu K. 273
Hua J.H. 40
Huang D.Y. 293
Huber A. 133
Huber O. 105
Huber S.M. 70
Hübenthal U. 352
Hübschle T. 67
Huelsenbeck J. 96, 97, 99, 109
Huelsken J. 324
Huener H.-A. 462
Huppmann R. 467
Huster M. 120, 152

Ignatius R. 346
Illes P. 50, 54, 55, 56a
Immig H. 245
Inga A. 418

Jackisch R. 217, 218, 219
Jackson P. 355
Jacob G. 281
Jacobs S. 116
Jäger B. 488
Jäger D. 24
Jährling F. 196
Jahreis G. 417
Jaite J. 408
Jakimenko A. 448
Jakobs K.H. 62
Jakobs S. 431
Jank T. 2
Janke J. 484, 495
Jaschke A. 105
Jaschke S. 478
Jeanclos E. 85, 138
Jedlitschky G. 1
Jennissen K. 72
Jobi K. 304
Jöhren O. 37
Jöst E. 374
Johnson E.F. 207
Jonkheijm P. 136
Joost H.-G. 84
Joost H.G. 106
Jordan J. 281, 311, 484, 495, 501
Josephs M.B. 108
Joshua-Tor L. 310
Juergens K.U. 38
Juergens U.R. 21
Just I. 95, 96, 97, 98, 99, 109, 111, 112, 441
Just L. 188
Just S. 273

Käfferlein H.U. 468
Kaelble S. 310
Kästner L. 160
Kästner S. 256
Kahl R. 377, 378, 379, 380, 381, 382, 429, 430
Kaina B. 374, 390, 391, 392, 398, 399, 400, 401, 411, 419, 423, 432, 451
Kaiser E. 443
Kaldis P. 181
Kalwa H. 87, 142
Kamp H.G. 462
Kampkötter A. 377, 378, 379, 380, 381, 382, 383, 385, 429, 430, 435
Kamyschnikow A. 393
Kappert K. 135
Kasinathan R.S. 70
Katan M. 108
Kato E. 56a
Katus H.A. 266
Kaufmann T. 462
Kebig A. 26
Kehe K. 339, 340, 341, 356
Kelber O. 210, 238, 289
Keller S. 417
Kellert M. 415
Kepura F. 60
Kessler W. 452
Kettner M. 481
Kettner-Buhrow D. 310
Khan M.D. 342
Khobta A. 402, 404
Kiedron O. 401
Kietzmann M. 204, 205, 302, 375
Kilian A. 260
Kinscherf R. 210
Kintscher U. 169, 175

Kirchhefer U. 258
Kirchhof P. 244, 255
Kirkpatrick C.J. 356
Kitsera N. 402, 404
Kitzing T. 102
Kitzing T.M. 101
Klein C. 256
Kleine-Katthöfer P. 247
Kleinert H. 67, 73, 74, 76
Kleinsasser N. 340
Klement K. 84
Klenk J.C. 17
Kleppisch T. 132, 213
Kleschyov A. L. 277
Kletke A. 180
Kleuser B. 205
Kleyer S. 496
Klimpel C. 178
Klingel K. 254
Klink B. 137
Klinke A. 277
Klintzsch S. 297
Klöckner U. 243, 258
Kloor D. 36
Klotz K.-N. 31, 51
Klotz L.O. 351, 388, 435
Kloz U. 409
Klug S. 460
Klugbauer N. 94, 134, 153
Kluge M. 496
Klugmann M. 11
Klussmann E. 129, 245
Knaapen A. 426
Knepel W. 224
Knerr S. 393
Kneuer C. 45
Knobbe C. 137
Knöfel L.F. 488
Knopp C.A. 37
Knust Z. 93
Koch M. 315, 462
Koch T. 10, 11
Koczan D. 458
Köberle B. 398
Köck K. 1
Köhle C. 324, 461
Köhler H.R. 367
König A. 312
König B. 122, 125
König J. 492, 499
König P. 69
Koesling D. 69, 78, 121
Kojda G. 268
Koka S. 70
Kolodziej A. 58, 59
Korth M. 163, 167
Koschak A. 151
Kostopanagiotou G. 295
Kouvelas D. 308, 504
Kovacs F. 221
Kracht M. 139, 181, 310
Krätzner R. 448
Kraus J. 200, 201
Krause C. 375
Krause E. 442
Krause G. 61
Krippeit-Drews P. 157, 162
Kroemer H. 46
Kroemer H.-K. 254,
Kroemer H.K. 1, 300, 490
Krüger D. 289
Krug H.F. 424
Kruger A. 68
Krysiak O. 418
Kuban J. 100
Kucerová D. 270
Kühnel S. 315, 317
Kühner P. 154, 155, 166
Küper J. 160
Kuhlow D. 403
Kuhn U. 498
Kulawik A. 385
Kuner R. 12, 141, 227
Kunzelmann K.H. 340
Kunzmann A. 408
Kuppinger O. 92
Kupsch A. 495
Kurejova M. 141
Kurig B. 85, 89
Kurowski C. 75
Kurz C.M. 43
Kuteykin-Teplyakov K. 4
Kutschenko A. 176

Laakmann S. 244
Ladwein M. 99
Lafontan M. 311
Lallas A. 308

Lam R. 182
Lampen A. 376
Lamy E. 386
Landsiedel R. 322, 323, 354, 457, 462
Lang A.E. 444
Lang F. 70, 182
Lang T. 485
Lange N. 231
Langen J.S. 367
Langer T. 115
Langhammer M. 307
Langlotz S. 210
Laumonnier Y. 22, 185, 203
Lavin M.F. 406
Leemhuis J. 212
Lehmann A.C. 484
Lehmann L. 417
Leisen C. 296
Leiss V. 131, 179
Leisse M.C. 495
Lektarau Y. 414
Lemoine H. 160, 250
Lenarz T. 375
Lenkei Z. 212
Leppänen O. 135
Letzel S. 353
Leuner K. 143
Leven M. 238
Li H. 66, 67
Li Q. 22, 203, 342
Li X. 186
Lichte L. 10
Lichter J. 350
Liebl B. 475
Lilienblum W. 469
Limtanyakul J. 345
Linder S. 130
Linker K. 73, 74, 76
Linsel N. 24
Lipp A. 495
Lips S.L.K.S. 39
Liubarskyi I. 503
Lobsien E. 495
Lochnit G. 139
Löscher W. 3, 4, 234, 235, 421
Lösel R.M. 279
Lötsch J. 481, 489
Lohmueller B. 153
Lohse M.J. 17, 19, 25, 27, 31, 81, 82, 116, 117, 128, 246, 253
López de Jesús M. 62
Loppnow H. 265
Lorenz D. 129, 172, 245
Lorenz K. 51, 246
Lorenz K.L 253
Loske R. 417
Lother A. 171
Lu Q. 66
Lubjuhn J. 232
Luch A. 376
Ludwig A. 213, 260
Ludwig R.J. 192
Lüpertz R. 429
Lüpke N.-P. 464
Luft F.C. 281, 311, 484, 501
Luiten P.G.M. 119
Lukowski R. 130, 131
Luna Tortos C. 3
Luo C. 141
Lupp A. 316, 498
Luszczki J.J. 236
Lutz S. 103, 167
Lutz U. 491
Lutz W.K. 415, 491

Ma-Hock L. 354, 457
Mader R. 259
Magina S. 47
Maguy A. 242
Mahmud H. 182
Mahran L.G. 296
Maier M. 360
Maier-Peuschel M. 25, 116
Maier-Peuschel M.M. 253
Majchrzak M. 218
Malavolta M. 408
Malek A. 373
Malfertheiner P. 483
Malik J.K. 325, 344, 414
Malinowska B. 43
Mally A. 364, 365, 366, 389
Mandery K. 483, 499
Mang C. 67
Mangerich A. 409
Mann W. 353
Manns M.P. 207
Marczynski B. 468
Marklein G. 290
Marko D. 397
Markwardt F. 56
Marowsky A. 330
Marquardt C. 424
Martin H.-J. 326
Marx-Stoelting P. 420
Marzoll A. 268
Maser E. 326, 336, 337
Matar N. 150
Mathar I. 145
Matt L. 213
Mattern D. 433
Matthes J. 256, 257
Matus M. 249, 263
Matzner N. 182
Maul B. 172
May K. 490
May M. 98, 111
Mayer P. 53, 268
McKnight S.G. 32
Mederos y Schnitzler M. 147, 303
Meerkamp K. 6, 7, 297
Mehringer R. 80
Meibers S. 303
Meier J. 125
Meissner M. 145
Menendez D. 418
Menke A. 104
Mensing T. 468
Menzel K. 498
Merk H.F. 334
Merkle S. 264
Merrill M.A. 32
Mersch-Sundermann V. 386, 413
Messari J. 295
Meszaros J. 256
Mészáros J.G. 239
Metges C.C. 307
Metz J. 210
Metzger A. 68, 241
Metzler M. 387, 449
Meyer D. 90
Meyer D.K. 134, 212
Meyer Popken O. 353
Meyer-Kirchrath J. 20, 211
Meyer-Kirchrath J.M. 35
Michalakis S. 149, 213, 259
Michalopoulos G.K. 458
Michel M.C. 288
Michels G. 79, 91
Mielke H. 376
Miesel A. 280
Milius D. 56a
Milosevic J. 55
Mittal R. 139
Mizuno K. 62
Mocchegiani E. 408
Moeller R. 350
Moepps B. 79, 91, 107, 114, 115
Moeser C. 140
Mohr K. 24, 26
Mohr-Andrä M. 24, 26
Molderings G.J. 290
Moldovanova I. 281
Monien B.H. 394, 447
Montell C. 148
Moors M. 455
Morad S.A.F. 301
Morena M.S. 52
Morisawa T. 276
Moro C. 311
Morrison C. 500
Moser M. 105
Mostertz J. 490
Moura D. 47
Moura E. 42
Moura J.E. 41
Muchova L. 276
Muckenthaler M. 273
Mückter H. 475
Mühl H. 75, 188, 189, 190
Mühleisen A. 461
Muehlenstaedt C. 441
Müller C. 159, 394, 417, 466
Mueller D. 438
Müller E. 174
Müller F.U. 249, 255, 263, 270, 278
Müller H. 168, 280, 282
Müller J. 312
Müller K. 389
Müller M. 143, 469
Müller O.J. 266
Müller P.J. 487, 488
Müller R. 134
Mueller S.O. 321, 438, 439
Müller W.E. 143, 229
Müllershausen F. 18
Münzel T. 66
Muetzelburg M.V. 441
Munzel T. 277
Muszynski A. 255
Muthig V.M. 40
Muttray A. 353

Nagel G. 423
Nagel R. 367
Nance M. 103
Napiwotzki J. 250
Naraharisetti S.B. 325, 344
Nattel S. 242
Naumann S.C. 391, 401
Nave K.A. 11
Nestler S. 486
Nestorov P. 71
Neuber C. 266
Neuhuber W. 133, 163
Neumann H. 300
Neumann J. 243, 258, 261, 265, 269
Neurath G. 478
Neurath M.F. 419
Nicaud V. 500
Nichols B. 32
Nicolay J.P. 70
Niederberger E. 140
Niedorf F. 302, 375
Niehaus-Rolf C. 464
Nienhaus G.U. 113
Niesler B. 48
Niess R. 296
Niestroy J.L. 318
Nijholt I.M. 119
Nikolaev A. 419
Nikolaev V.O. 25, 116, 117, 128
Nikolova T. 401
Niroomand F. 80
Nörenberg W. 54, 55
Nolte M.W. 235
Nordheim A. 163
Noutsias M. 254
Novakovic A. 84
Nürnberg B. 84, 85, 86, 89, 136, 137, 138, 180, 256

Oberemm A. 459
Odersky A. 136, 137, 138
Oeder S. 477
Oehler C. 292
Oehme S. 284
Oehring H. 292
Oelze M. 76
Oertel B.G. 481
Oesch F. 433, 451
Oesterle D. 452
Östman A. 135
Oetjen E. 176, 178
Offermanns S. 163
Ohler S. 377, 378, 383
Ok M. 127
Okpanyi S.N. 210
Okyar A. 298
Oliveira J.F. 50
Olling A. 112
Oppermann M. 268
Oppermann U. 326
Oros-Peusquens A.M. 211
Orth J. 83, 88
Orth J.H. 89
Osman B. 191
Osswald H. 36, 158, 159, 293
Ottenjann N.K. 49
Oude Weernink P.A. 62
Oxynos A. 314, 341

Paal K. 442
Pahlke G. 397
Palavinskas R. 335
Palinkiewicz W. 46
Pamberg A.-M. 228
Pankow K. 61, 172
Papastergiou P. 115
Papazisis G. 308
Papsdorf G. 172
Parolaro D. 222
Passreiter C. 385
Paul C. 132
Paul M. 247, 248, 500, 502
Pautz A. 73, 74, 76
Pearl L.H. 108
Pease C.K. 454
Pellizzer C. 368
Pertz H.H. 44
Pesch B. 468
Peters I. 229
Petzinger E. 6, 7, 8, 39, 297
Peuschel H. 427
Pfau W. 478
Pfeifer A. 72, 309

Pfeiffer E. 387, 449
Pfeiffer R. 352
Pfeilschifter J. 75, 173, 174, 187, 188, 189, 190, 191, 194, 206
Pfeilschifter J.M. 192, 193
Pfreimer M. 114, 115
Philipson K.D. 255
Pich A. 441
Pichler B. 70
Piée-Staffa A. 398
Pieh J. 439
Piekorz R. 84, 256
Piekorz R.P. 180
Pieper B. 156
Pilka E. 326
Pinkenburg O. 87
Pissarek M. 211
Pitterle K. 22, 203, 301
Plant T. 60
Platzek T. 412
Plöttner S. 315, 317
Podlech J. 397
Pönicke K. 269
Pohl C. 356
Poleganov M.A. 190
Popovic M. 185
Popp L. 140
Popp O. 409
Poppe H. 123
Porter A. 299
Potschka H. 476
Pott C. 255
Poulos Ch. 308
Preuß I. 83, 89
Proksch P. 377, 383
Püschel P.G. 106
Pütz C. 452

Qi X. 242
Qiu H. 486
Quast U. 154, 155, 161, 166
Queisser N. 395, 416
Quiros S. 401
Quitterer U. 253

Raasch W. 168, 280
Rabausch B. 272
Rached E. 364, 365, 389
Racké K. 21, 38
Radeke H. 206
Radeke H.H. 192, 193, 194
Ramer R. 64
Ranganathan V. 325
Rankovic M. 10
Rao G.S. 325
Rappold G. 48
Raquet N. 369
Raschke M. 437
Rauch B.H. 20, 184, 304
Rauch G.-J. 80
Rauch S.J. 184
Rauhaus K. 250
Raulf-Heimsoth M. 468
Ravens U. 164, 165, 242, 287, 288
Rehmann H. 123
Reichel V. 9
Reichl F.X. 314, 339, 340, 341
Reidegeld S. 49
Reifenberger G. 137
Reimer J. 300
Reines I. 205
Reisinger K. 334
Reissig S. 209
Renné C. 481
Renner B. 494
Rentsch R. 476
Renzing A. 196
Resch P. 472
Reske-Kunz A.B. 195, 196, 197, 198, 199, 209
Resnick M.A. 418
Retlich M. 107, 108, 113
Reum T. 480
Reuter U. 230
Reuther S. 254, 490
Rex A. 230, 237, 305
Richert L. 438
Richter A. 231, 233
Richter C. 193, 206
Richter I. 332, 415
Ridilla M. 103
Riedinger K. 215
Riefke B. 437
Riegert C. 219
Riehle R. 36
Riester M.B. 342
Rignall B. 442
Rimmbach C. 300
Risch A. 487, 488
Ristow M. 403, 422
Ritz V. 448
Rochais F. 19
Rockel T.D. 455
Rodrigues-Lima F. 108
Roe S.M. 108
Röcker C. 113
Röser C. 41
Rohr-Udilova N. 453
Rohrig R. 377, 378, 385
Rolle F. 460
Romanin C. 151
Rood A. 160, 250
Roos P.H. 315, 317, 318
Roos W.P. 374, 390, 391, 392, 401
Rosefort C. 30
Rosenau T. 453
Rosenkranz A.C. 184, 304
Rosenkranz S. 135
Rosenthal W. 16, 23, 129, 245
Rosenwald A. 273
Ross R. 195, 196
Rossbach B. 353
Roßbach K. 204
Rosskopf D. 62, 300
Rothmaier A.K. 218, 219
Rottbauer W. 80, 273
Rottner K. 99
Rouis M. 275
Rubini P. 55
Rubino T. 222
Rüdell G. 448
Rüßmann C. 248
Ruetten H. 67
Ruhl S. 377, 378, 385
Ruhnau J. 312
Ruiz P. 244
Runge D. 458
Runge D.M. 458
Rupps G. 100
Russ U. 154, 155, 161, 166
Russwurm C. 121
Russwurm M. 121
Rustenbeck I. 177
Ruth P. 133, 162, 163, 167, 182, 293
Rutz S. 218, 219
Ruwiedel K. 352, 454
Rybalkin S.D. 123

Sachinidis A. 271
Sack M. 380, 382
Saklatvala J. 181
Salber D. 211
Saloga J. 197, 199
Salzig C. 361
Sander S. E. 233
Sandin A. 135
Sandrock K. 94
Saranteas T. 295
Sarkar S.N. 344
Sausbier M. 133, 163, 182, 293
Sausbier U. 133, 163, 167, 182, 293
Sautter I. 476
Schäfer K.H. 291
Schäferling M. 118
Scheel J. 464
Scheiermann P. 188, 189
Scheinhof M. 230
Schemann M. 289
Scherthan H. 409
Scheurer P. 282
Schiefelbein D. 173
Schins R.P.F. 363, 425, 426, 473
Schirling M. 367
Schirp J. 505
Schlatter J. 415
Schlicker E. 43
Schlossmann J. 120
Schmalenbach C. 24
Schmall F. 353
Schmalz G. 345, 436
Schmalzing G. 30, 34, 56
Schmechel A. 46
Schmid M. 301
Schmid U. 395, 396
Schmidberger H. 374
Schmidt A. 23, 68, 241
Schmidt C. 294
Schmidt G. 93, 94, 96, 109, 110
Schmidt H.H.H.W. 282
Schmidt M. 33, 62, 119
Schmidt N. 73, 74, 76, 237
Schmidt P.H. 481
Schmidt R. 63
Schmidt W.J. 215
Schmidt-Petersen K 500, 502
Schmidtko A. 133
Schmitt C. 440
Schmitt J.P. 246, 253
Schmitteckert E.M. 116, 253
Schmitz B. 248
Schmitz H.-J. 369
Schmitz L.M. 181
Schmitz S. 56
Schmitz W. 249, 255, 263, 270, 278
Schmitz-Salue C. 448
Schneider E. 13
Schneider H. 139
Schneider K. 474
Schneider L. 180
Schneider T. 150
Schnell D. 14
Schnetzke U. 279
Schnizler K. 32
Schober W. 477
Schöberl F. 132
Schönfelder G. 367, 418, 491
Schoentaube J. 95
Scholich K. 63, 481
Scholz P. 460
Schoos C. 20, 211
Schradi K. 94
Schramm N. 211
Schreiber S.C. 104
Schreiber T. 455, 456
Schrenk D. 321, 361, 369, 393, 450
Schroeder C. 281
Schroeder D. 290
Schröder H. 276, 285
Schröder K. 334
Schrör K. 20, 35, 184, 268, 271, 304, 503
Schülein R. 16, 23
Schürmann A. 84, 105, 106
Schürmann C. 174
Schüssler P. 496
Schütz G. 171
Schug M. 459
Schuhholz J. 91
Schuhmacher-Wolz U. 474
Schuler B. 70
Schulte J. 255, 263
Schulte J.S. 249
Schultheiss H.-P. 254
Schulz F. 97, 98, 111
Schulz M. 156, 322
Schulz N. 265
Schulz R. 92
Schulz S. 11, 116, 276, 285
Schulze-Osthoff K. 180
Schunk J. 54
Schupp M. 169
Schupp N. 395, 396, 416
Schuster H. 312
Schwaninger M. 226, 232
Schwanstecher C. 156
Schwanstecher M. 156
Schwartz A. 239
Schwarz J. 55
Schwarz M. 324, 368, 420, 442, 461
Schweda F. 145, 396
Schwede F. 123
Schweigler T. 164
Schweikl H. 345, 436
Schweinfurth H. 437
Schwind K.-R. 462
Scott-Algara D. 275
Sebastiani J. 356
Seehase S. 112
Seidel A. 394
Seidl M. 249, 263
Seidl M.D. 278
Seifert R. 13, 14, 15, 28, 29, 118, 122, 125, 126
Seifert S. 241
Seiler A. 135
Seiss M. 314, 339, 340, 341
Selinski S. 333
Sen\' V. D. 277
Seuwen K. 18
Shakibaei M. 370
Shamsinejad M. 485
Shankaranarayanan A. 103
Shashidharan P. 231
Shibeshi W. 45
Shumilina E. 182
Shutov L.P. 32
Shymanets A. 85, 86
Siebelmann M. 170
Sieber M. 365, 366
Siebolts U. 135
Siedschlag V. 218
Siegmund W. 490
Siems E. 300
Siems W.-E. 61, 172, 307
Simmet Th. 22, 185, 186, 203, 275, 301
Simon A. 65, 212
Singh A. 151

Sinnegger-Brauns M.J. 151
Skidgel R.A. 172
Smital P. 120
Snipe J. 418
Sobiesiak M. 182
Sobottka H. 54
Solbach T.F. 492
Sommer C. 423
Song H. 279
Sotoud H. 274
Spahl W. 340
Spahn-Langguth H. 296, 298
Spangler C. 118
Spangler C.M. 118
Spanier G. 66
Sparwel J. 135
Spickenheuer A. 468
Spiegelhalder B. 488
Spielmann H. 479
Spranger J. 495
Sprenger R. 485
Sprossmann F. 163
Staels B. 169
Staessen J.A. 482
Stahl J. 302, 375
Stahlmann R. 346, 370
Stampfl A. 360
Stanchev D. 56a
Stark H. 204
Steffen C. 480
Steger-Hartmann T. 437
Steiger A. 496
Steinberg P. 403, 422, 465, 466
Steinbrink K. 65
Steingräber A.K. 278
Stets R. 358
Stevenson D.K. 276, 285
Stichtenoth D.O. 497
Stieber J. 259, 260
Stindt D. 257
Stobbe N. 379
Stoiber T. 463
Stoll M. 82, 500
Stolze K. 453
Stopper H. 395, 396, 416
Storch U. 303
Strack S. 32, 434
Straehle U. 434
Strand D. 77, 485
Straßer A. 15
Strauss V. 354
Striessnig J. 151
Strobel H. 67
Strom S.C. 458
Strugala G. 288
Stümpel F. 270
Stumm R. 11, 58, 59
Stummann T. 461
Su Z. 115
Suchenwirth R. 467
Suckow-Schnitker A.K. 385
Sudowe S. 196
Sun X. 172
Sur H. 249, 263
Sur T.M.H. 278
Sutter A. 196
Suzuki T. 67
Sydlik U. 425, 427, 428
Sydykov A. 142
Syrovets T. 22, 185, 186, 203, 275
Syttkus B. 309
Szabo B. 212, 221, 222
Szaszak M. 129, 245

Tang W. 125
Tatge H. 112
Taugner F. 447
Teetz M. 113
Tegeder I. 12, 133, 140, 141
Teiber J.F. 77
Telgmann R. 247, 248, 482, 500, 502
Tempel K. 262
Terlau H. 37
Ternes T. 475
Teschner C. 160
Tesmer J.J.G. 103
Tesseromatis C. 295
Thiefes A. 139
Thierbach R. 403, 422, 465
Thomas M. 488
Thomas P. 340
Thum T. 273
Thunemann M. 70
Tiburcy M. 251
Tigges J. 352
Tigka E. 295
Timpel C. 380, 382
Töllner K. 216
Tönnies M. 313
Tomicic M.T. 399, 411
Torky A. 357, 359, 405
Track N. 339, 341
Tränkle C. 24
Traidl-Hoffmann C. 130
Traut U. 210
Treiber G. 483
Triebskorn R. 367
Trojandt S. 197, 199
Tuengerthal S. 488
Türeli A.E. 291
Tuschl G. 438
Twarock S. 272, 286
Tzvetkov M. 224

Ugele B. 6, 7
Uhlemann F. 103
Uhr M. 496
Uhrig J. 92
Ullmann K. 422, 466
Ullrich A. 458
Unfried K. 425, 427, 428
Ungemach F.R. 45, 284
Unger E. 463
Unger T. 169, 175
Unsöld B. 240
Urban M. 62
Urbanski M.J. 82, 212, 221
Usachev Y.M. 32
Usanova S. 398
Usmani S.M. 283
Utku E. 167

Valtcheva N. 71
van Berlo D. 426
van der Hoeven F. 409
van Kanegan M.J. 32
van Ravenzwaay B. 322, 323, 457, 462
van Schooten F.J. 426
Vatter P. 107, 108
Vennekens R. 145
Verspohl E.J. 49, 170
Vettel C. 103
Vieira-Coelho M.A. 41, 42, 47
Vieth B. 376
Vischer P. 502
Völkel M. 505
Völkel W. 338, 472
Vogel J. 70
Vogel R. 479
Vogelbein S. 16
Voigt J.-P. 237, 305
Voigt N. 165, 242
Vollmar S. 211
von Ameln-Mayerhofer A. 215
von Hayn K. 117, 128
von Hein C.P. 497
von Herrath M.G. 207
von Kries J.P. 16, 245
von Kügelgen I. 52, 53, 290

Wäring J. 147
Wätjen W. 377, 378, 379, 380, 381, 382, 383, 385, 429, 430
Wagner A. 90
Wagner S. 338, 365
Wahl M. 434
Wahl-Schott C.A. 225, 259
Waisman A. 209
Wait R. 181
Waldmann H. 136
Walijew A. 440
Walitza S. 416
Walliser C. 107, 108, 113
Walstab J. 48
Walter P.L. 435
Walther S.C. 472
Walther T. 61, 307
Walther U.I. 358
Wang P. 172
Warneke W. 305
Warnken M. 21, 38
Weber A.-A. 33, 271, 503
Weber H. 335
Wedekind F. 219
Wedlich D. 104
Wegener J.W. 120, 152
Wehland M. 460
Wehling M. 279
Weichenmeier I. 477
Weidinger M. 213
Weiergräber M. 150
Weinmeister P. 71, 130, 131, 132
Weirich J. 267
Weiser D. 210
Weiske J. 105
Weiss C. 424, 433, 434
Weiß S. 252
Weissenberg A. 425
Weißgerber P. 146
Weissmann N. 142
Welge P. 468
Welling A. 131, 179
Wendorff S. 204
Wenge B. 5
Wensing J. 170
Wenzel F. 503
Wenzel J. 37
Wenzel P. 76
Werle-Schneider G. 488
Werner F. 261
Werner H. 11
Werthmann R.C. 117, 128
Wettwer E. 164, 165
Wex T. 483
Weyerbrock A. 220
Wichert Grande S. 349
Wichter T. 244
Widder J. 292
Wiechmann N. 195, 196, 197, 198, 199, 209
Wiegand C. 334
Wieland T. 62, 80, 103, 167
Wiench K. 354
Wiencierz A.M. 466
Wiertz M. 371, 372
Wiese J. 359
Wiesner B. 16, 23, 172
Wiesner R. 257
Wiesner S. 484
Wiewrodt D. 423
Wilgenbus P. 77, 183
Wilhelm B. 90, 306
Will J. 193, 206
Willenborg M. 177
Willgeroth S. 35
Wind S. 282
Wingler K. 282
Winkler M. 166
Winter M. 473
Wirkner K. 50, 56a
Wirth A. 163
Wirth M.P. 287
Wissing H. 188
Witt H. 244
Witte I. 77, 183
Wittköpper K. 240
Wittmann H.-J. 15
Woelbing F. 182
Wölfle D. 412
Woermann A. 505
Wohlfart P. 67
Wojnowski L. 68, 241, 299, 485, 486
Wolf A. 139
Wolf T. 464
Wolff S. 319
Wolfrum K. 403
Wolfrum S. 262
Wollin K.-M. 469
Wollny C. 108
Wolpers D. 162
Woltersdorf R. 56
Wong R.J. 276, 285
Wood W.G. 229
Worch S. 259
Wowtscherk M. 287
Wu D.F. 11
Wuest M. 287, 288
Wulfsen I. 167
Wunder F. 124
Wunsch J. 39

Xia N. 66
Xiong G. 336, 337
Xu H. 66, 67

Yadav R. 51
Yan T. 68, 241, 299
Yang L. 434
Yard B. 279
Yassouridis A. 496
Yeh Y. 242

Zahn C. 105, 106
Zahner D.J. 8
Zaichuk T. 6
Zanger K. 136
Zdzienicka M.Z. 401
Zeller F. 289
Zemtsova I. 182
Zenn H.-M. 245
Zenn M. 129
Zettner M. 417
Zhang Y. 257
Zhao H. 163
Zhou X.-B. 163, 167

Zidek N. 366
Ziemann C. 355
Ziesché E. 188
Zietz B.P. 467
Zimmermann B. 129, 245
Zimmermann N. 270
Zimmermann W.H. 251
Zitranski N. 306
Zoidl G. 121
Zolk O. 492
Zolothukina V. 370
Zong X. 82, 149
Zschauer T. 471
Zurawski R. 380, 382
Zurek M. 370
Zwissler B. 188, 189

S 2

Role of the blood-brain barrier in pharmacoresistance of CNS diseases

Löscher W. (1)

Resistance to drug treatment is an important hurdle in the therapy of many brain disorders, including brain cancer, epilepsy, schizophrenia, depression, and infection of the brain with HIV. Consequently, there is a pressing need to develop new and more effective treatment strategies. There is increasing evidence that the blood-brain barrier (BBB) and particularly drug efflux transporters at the BBB can contribute to drug resistance in brain diseases in two ways [1]. First, by their constitutive expression at the BBB, efflux transporters such as P-glycoprotein (P-gp) restrict the brain access of many lipophilic drugs and enhance drug extrusion from the brain, so that the levels of drugs in the brain cannot become sufficiently high for therapeutic efficacy. This is relevant for the treatment of brain cancer and brain HIV infection, among others. Second, in some brain diseases, intrinsic or acquired over-expression of P-gp at the BBB or brain target tissue limits drug penetration into that tissue. This is relevant for brain tumors, HIV, epilepsy and cerebral ischemia and can result from alterations (such as polymorphisms) in *MDR1* or from the effects of disease or drug treatment on expression of P-gp. The consequences of intrinsic or acquired over-expression of P-gp depend on the extent of the over-expression and the affinity of respective substrates for the efflux transporter. In epilepsy, for instance, over-expression of P-gp and multidrug resistance proteins (MRPs) have been determined in epileptogenic brain tissue of pharmacoresistant patients and in rat models of drug resistant epilepsy, and have been associated with subtherapeutic brain levels of antiepileptic drugs. In epilepsy models, pharmacoresistance could be counteracted by P-gp inhibition. Modulation of P-gp at the BBB forms a novel strategy to enhance the penetration of drugs into the brain and may yield new therapeutic options for drug-resistant CNS diseases [1,2].

[1] Löscher, W. and H. Potschka: Drug resistance in brain diseases and the role of drug efflux transporters. Nature Rev.Neurosci., 6, 591-602, 2005.

[2] Deeken, J. F. and W. Löscher: The blood-brain barrier and cancer: transporters, treatment, and Trojan horses. Clin.Cancer Res., 13, 1663-1674, 2007.

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S 3

Impact of genetic factors and comedication on blood-brain barrier function

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Uptake and efflux transporters have been localized to endothelial cells forming the blood-brain barrier. For example, the ATP-dependant efflux pump P-glycoprotein, which transports a wide range of structurally diverse drugs is located in the luminal membrane of endothelial cells forming the blood-brain barrier. Studies with P-glycoprotein deficient mice indicate that the CNS accumulation of P-glycoprotein substrates is considerably increased compared to P-glycoprotein expressing animals. Similarly, if P-glycoprotein inhibitors such as quinidine or PSC-833 (valspodar) are administered to P-glycoprotein expressing animals, there is a considerably accumulation of the drugs in the CNS, which is generally greater than the accumulation of the drug in plasma. Other ABC transporters (e.g. members of the MRP family) have also been localized to the blood-brain barrier and contribute to CNS disposition of drugs. Similar to other organs (e.g. liver), endothelial cells forming the blood-brain barrier also express uptake transporters, for example members of the organic anion transporting polypeptide (OATP) family. It was reported that OATP1A2 and OATP2B1 are expressed in the luminal membrane of endothelial cells forming the blood-brain barrier. Studies will be discussed whether and to which extent genetic polymorphisms in genes encoding for drug transporters located in the blood-brain barrier might affect CNS disposition and effects of drugs.

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S 4

Dynamic regulation of blood-brain barrier function

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The blood-brain barrier is the vascular network of capillaries in the brain. It was originally thought to be merely a simple anatomical barrier separating brain from blood. However, research over the last years by us and other groups demonstrates that the blood-brain barrier is a tightly regulated, highly active and selective, dynamic capillary endothelium with important functions. This includes mediating communication between brain and periphery, and maintaining brain homeostasis by controlling blood-brain barrier function. Proper barrier function is primarily based on 3 distinct molecular components of the capillary endothelium. First, tight junctions sealing adjacent endothelial cells constitute a strong and effective physical barrier. Second, metabolizing enzymes expressed in the endothelium provide a metabolic barrier. And third, a group of ATP-driven efflux transporters represent the active, selective barrier. All these components combined make the brain capillary endothelium a tight and exclusive barrier for a wide range of xenobiotics, including a large number of CNS drugs. Thus, the blood-brain barrier is a tremendous obstacle for the treatment of brain disorders. We will provide an overview of current knowledge on the dynamic regulation of blood-brain barrier function. This will include regulation of tight junctions, metabolizing enzymes and efflux transporters at the blood-brain barrier in health and disease. We will highlight new regulatory mechanisms of blood-brain barrier function and demonstrate how such knowledge can be used to circumvent the barrier for successful pharmacotherapy of brain disorders.

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S 5

Therapeutic strategies to overcome blood-brain barrier function

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Neurological disorders are becoming an increasing public health issue and therapeutics for such diseases represent a considerable market opportunity for the pharmaceutical/ biotechnology industry. However the blood-brain barrier represents a major obstacle for drug entry into the CNS. Efforts are ongoing to overcome this barrier without causing permanent damage, lasting from retrometabolic approaches to modulation of export proteins or targeted drug delivery systems based on targeted polymeric or liposomal systems.

The retrometabolic drug design includes the concept of a chemical delivery system, which is defined as a biologically inert molecule containing the drug in a modified form, that requires enzymatic steps during its conversion to the active drug and that enhances drug targeting to a particular organ. Among these systems the interconvertible dihydropiridine <--> pyridinium salt targeting system has found wide applicability. It has been explored with various classes of drugs including anticancer agents, steroids, anesthetics, anticonvulsants, antivirals, anti-infectives, neurotransmitters, peptides and others. E.g., Estredox™, a novel brain-targeted delivery system for estradiol, bases on this approach and has successfully been tested in clinical trials. Deactivating the function of ABC-proteins remains to be a major challenge in overcoming the barrier. Whereas a therapeutic use of pgp-inhibitors mostly disappoints in various cancer indications, there seems to remain a promising application potential in enhancing drug delivery across the blood brain barrier. E.g., the co-administration of Valspodar (Amdray), a very potent pgp-blocker, with Taxol led to a significant reduction in size of human glioblastoma transplanted into brains of nude mice, whereas administration of Taxol alone was without any effect. Enhanced transcytosis across the endothelial cells can be achieved by using drug loaded liposomes or nanoparticles. Uptake can be further enhanced by specifically targeting the delivery system to receptors on the brain endothelium surface being capable of receptor mediated endocytosis. It requires the discovery and development of receptor specific ligands, which can be attached the drug delivery system itself. Systems under investigation include liposomes linked to transferrin receptor-antibodies, polyether-copolyester dendrimers linked to sugar moieties or surface modified polybutylcyanacrylate nanoparticles, which show an enhanced permeation and delivery of their content to the CNS.

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S 6

The life stories of persecuted scientists in the Third Reich as shown by the doom of pharmacologists

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A year ago Ullrich Trendelenburg published the life stories of 69 ‘persecuted German-speaking pharmacologists 1933-1945’ (Dr. Schrör Verlag). An intensive exchange of ideas led us to the decision of publishing a 2nd edition of the book. Biographical gaps had to be closed and, more importantly, the life stories were regarded within the general context of racist and political persecution. Half of the persecuted pharmacologists emigrated in 1933, while the majority of the German ‘non-aryans’ refrained from emigrating until 1938. In this year the ‘Reichskristallnacht’ (9./10. November) made clear that the persecution would become more and more threatening. For many ‘non-aryans’, emigration was prevented by numerical immigration limits (visa, affidavits of support etc.). The reasons for the early emigration of most of the persecuted pharmacologists were the instantaneous dismissals in 1933 of almost all ‘non-aryan’ scientists employed in research institutions and the presence of a fair chance to continue scientific careers in exile. In 1945, the German pharmacologists emigrated to 12 different countries; almost half settled in the USA and one third in Great Britain. Turkey, Brazil and the Jewish research institutions in Palestine hoped that the immigration of these scientists would raise the academic level towards that of the Western industrial nations. – The individual life stories of the persecuted pharmacologists exposed the whole range of persecution in Germany: the humiliating dismissals, the desperate ‘odyssey’ of emigration, the dramatic escape from the concentration camps, and the holocaust experience; on the other hand, there were cases of bright scientific career in exile (especially in Great Britain). – The behaviour of the ‘aryan’ colleagues in Germany also exhibited a wide range from fanatic following of the national socialist policy, opportunism and Prussian obedience on the one hand to opposition and resistance on the other. The present study reveals similarities between the fate of pharmacologists and biochemists, but there were drastic differences between the life stories of the former and those of the medical doctors.

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1

Functional regulation of OATP2B1 (SLCO2B1) by protein kinases

Köck K. (1), Grube M. (1), Jedlitschky G. (1), Kroemer H.K. (1)

OATP2B1 belongs to the solute carrier family, mediates the uptake of endogenous compounds like estrone-3-sulfate (E1S), dehydroepiandrosteronesulfate (DHEAS) and drugs like atorvastatin or glibenclamide and is expressed in tissues like liver, intestine and heart. Besides regulation on a transcriptional level, posttranslational modifications like phosphorylation by protein kinases might also affect the functionality of this protein. However, these short-term regulatory processes have rarely been addressed so far. We therefore investigated the effect of PKA and PKC modulators on OATP2B1 transport activity.Initially, using in silico analyses based on phosphorylation site prediction programs we identified several potential protein kinase A (PKA) and C (PKC) phosphorylation sites within the amino acid sequence of OATP2B1. Using OATP2B1-overexpressing MDCKII cells we measured the transport function of OATP2B1 in the presence of PKA or PKC activating compounds. While PKA induction by Forskolin, 8-Br-cAMP and DB-cAMP had no effect on OATP2B1-mediated [3H]DHEAS and [3H]E1S uptake, preincubation with the PKC activator phorbol-12-myristate-13-acetate (PMA) significantly inhibited the [3H]DHEAS and [3H]E3S uptake in a time and concentration dependent manner, with a maximum inhibition to around 40% for 10 µM PMA at 3.5 hours of incubation. This effect could be reversed by coincubation with the PKC inhibitor BIM I (1 µM). Kinetic analyses of the E1S uptake revealed a decrease of the maximal transport velocity from 288 (DMSO) to 165 pmol/min/mg protein (100 nM PMA), while the substrate-affinity remained unchanged (9.5 vs. 13.2 µM). A possible explanation for this finding is the internalization of OATP2B1 in response to PKC activation. Using immunofluorescence or live cell imaging of MDCKII cells transfected with GFP-tagged OATP2B1 we could demonstrate rapid redistribution into vesicle-like intracellular compartments. In conclusion, our data suggest a rapid regulation of OATP2B1-mediated transport by PKC, which may be relevant for uptake and transepithelial transport of endogenous or exogenous OATP2B1 substrates, especially in organs like placenta, intestinum or liver.

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2

Cysteine protease activity is responsible for autocatalytic cleavage of *Clostridium difficile* toxin A and B

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Clostridium difficile toxins A and B, which are the causative agents of antibiotic-associated diarrhoea and pseudomembranous colitis, inactivate Rho GTPases by glucosylation. To act in the cytosol, translocation and processing of the catalytic glucosyltransferase is required. Recently it was reported that myo-inositol hexakisphosphate facilitate processing of the toxins (Reineke et al., Nature 2007). We observed that dithiothreitol causes autocatalytic cleavage of toxin A and B into the 63 kDa glucosyltransferase and the C-terminal toxin fragment of 250/210 kDa. The effect of myo-inositol hexakisphosphate was synergistically increased by dithiothreitol. The cleavage was blocked by N-ethylmaleimide, suggesting that cysteine residues are essential for the processing of clostridial glucosylating toxins. Exchange of cysteine 698, histidine 653, or aspartate 587 of toxin B prevented cleavage of full-length recombinant toxin and of an N-terminal fragment covering residues 1-955 and inhibited cytotoxicity of full-length toxin B. Also the cleavage of the N-terminal fragment can be induced by myo-inositol hexakisphosphate, which indicates that the protease, which is involved in the processing of the toxins is located in the N-terminal region of the protein.

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3

Several major antiepileptic drugs are substrates for human P-glycoprotein

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Introduction: The over-expression of multidrug transporters (MDTs), such as P-glycoprotein (P-gp), at the luminal side of the blood brain barrier may lead to lower concentrations of antiepileptic drugs (AEDs) in the brain target tissue, as proposed by the MDT-hypothesis of drug resistance in epilepsy. However, recent reports from in vitro assays seem to indicate that, in contrast to rodent P-gp, human P-gp may not transport AEDs. In these reports kidney cell lines (MDCKII, LLC) over-expressing the human multidrug resistance-1 (MDR1) gene that encodes P-gp were used, and the transport assays were performed in a conventional manner with the Transwell® system, applying the AED to either the apical or basolateral chamber. Since AEDs are lipophilic drugs, passive diffusion could form a bias in such assays by concealing active transport. Our aim was to investigate the transport of AEDs using a method that allows evaluating active transport independently of the passive permeability component. Methods: LLC cells that were transfected with human MDR1 gene were used. Wildtype cell lines were used for comparison. Transport assays with AEDs were either used in a conventional fashion by adding the AED to either the apical or basolateral chamber (concentration gradient condition) or in a modified fashion by adding the AED to both chambers (concentration equilibrium condition); the later allows determining the active efflux component. The P-gp substrate digoxin was used as positive standard. In case of active transport, the P-gp inhibitor tariquidar was used to substantiate the involvement of P-gp in the transport. Results: By using transport assays with human MDR1-transfected cells under concentration equilibrium conditions, a clear basolateral to apical transport was observed for several AEDs, including phenytoin, phenobarbital and levetiracetam. This transport was inhibited by tariquidar, substantiating that these AEDs were transported by human P-gp. Conclusion: Our data demonstrate that human P-gp transports several AEDs, which supports the transporter hypothesis of drug resistance in epilepsy.

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4

The effect of antiepileptic drugs on the expression of ABC drug transporters in different cell types

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Epilepsy is one of the most common chronic neurological disorders mainly treated at present by blocking of the seizures with antiepileptic drugs (AEDs). However up to one-third of all individuals with epilepsy are refractory to AEDs with a wide range of mechanistic actions. The mechanism of pharmacoresistance in epilepsy is poorly understood. The multidrug transporter hypothesis proposes that ABC drug efflux transporter proteins, such as P-glycoprotein (Pgp) and members of the multidrug resistance-associated protein (MRP) family play a major role in pharmacoresistance by extrusion of AEDs from the brain back to the blood. Such multidrug transporters are highly expressed in capillary endothelial cells of the brain-blood barrier and overexpressed in brain tissue from patients with intractable epilepsy. The mechanism of this overexpression is unclear, but induction of transporters by AEDs may be involved.The goal of this study is to evaluate whether AEDs increase the expression of Pgp or MRPs and which mechanisms are involved in this effect. Drugs known to increase Pgp expression were included as reference standard: vincristine, doxorubicine, and mitomycin C.As starting point we used the dog kidney MDCKII cell line, consisting of polarized cells that form tight junctions and express Pgp and MRPs, thus allowing transport studies with AEDs. Furthermore, the immortalized rat brain capillary endothelial cell lines GPNT, GP8 and RBE4 were included in our experiments. The cell cultures were treated with AEDs (phenobarbital, carbamazepine, topiramate, phenytoin) or with known Pgp inducers. The functionality of Pgp was examined by an accumulation test, using either rhodamine or digoxin as Pgp substrates and tariquidar as Pgp inhibitor. Expression of Pgp was quantified by Western blot, and expression of mRNA of ABC transporters (ABCB1, ABCC1, ABCC2, ABCC5) by qRT-PCR. Since the transcription of ABC drug transporters might be regulated by ligand-activated nuclear receptors, NR1I2 (PXR) and NR1I3 (CAR), all cell cultures were tested for expression of PXR and CAR. First data from these experiments show that some AEDs increase Pgp expression, but that this effect is cell-specific.

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5

Interactions of the anticholinergic drugs oxybutynin and trospium chloride with human organic cation transporters

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The muscarinic antagonists oxybutynin (OXY) and trospium chloride (TLC) are used as spasmolytic agents for the treatment of overactive urinary bladder disease. TCL has been shown to be as effective as oxybutynin with better tolerability. The quaternary ammonium base TCL has been proposed to be a substrate of organic cation transporters (OCTs), but hitherto it has only been shown to have affinity to human OCTs (Lips et al. (2007) Eur Urology 51:1042). The aim of the present study was to examine whether the TCL and OXY are substrates, i.e. are transported by the human OCTs (hOCT1, hOCT2 and hOCT3). Therefore, we measured total and specific (decynium22(10 µM)-sensitive) uptake of [3H]OXY and [3H]TCL in human embryonic kidney (HEK293) cells transiently transfected with the cDNA of hOCT1, hOCT2 or hOCT3. In some experiments, non-specific uptake was also measured in mock-transfected cells. Total uptake of [3H]OXY was very high in all transfected HEK293 cells and only a very small portion was due to specific uptake. The high non-specific uptake is obviously a consequence of the high lipophilicity of this drugs, and hOCTs may not play an important role for the inactivation and pharmacokinetics of OXY. However, and in contrast to OXY, uptake of [3H]TCL was mainly due to specific uptake by the three hOCTs. The rank for the efficiency of specific uptake of [3H]TCL by the hOCTs was: hOCT2 > hOCT1 >> hOCT3. Thus, the pronounced transport of TCL by hOCT2 and hOCT1 may lead to interactions with other drugs transported by these transporters.

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6

Expression of steroid sulfatase (STS) and sodium-dependent organic anion transporter (SOAT) in breast cancer

Meerkamp K. (1), Zaichuk T. (2), Ugele B. (3), Petzinger E. (1), Geyer J. (1)

The sodium-dependent organic anion transporter SOAT (SLC10A6) is a recently discovered carrier for sulfoconjugated steroid hormones such as estrone-3-sulfate, dehydroepiandrosterone sulfate, and pregnenolone sulfate, and is present in hormone regulated tissues. Steroid sulfatase (STS) is highly expressed in breast carcinomas and was recognized as an important factor for breast tumor proliferation. Currently, STS inhibitors undergo clinical trials for breast cancer treatment. Sulfoconjugated steroid hormones though not able to penetrate the plasma membrane by simple diffusion require a carrier-mediated import to get access to the intracellular compartment. We assume that SOAT imports sulfoconjugated steroids into breast carcinoma cells. Therefore we analyzed SOAT gene expression in mammary gland and breast carcinoma tissues in comparison with STS. We examined RNA samples from 10 estrogen receptor positive and 10 estrogen receptor negative breast carcinomas and also included normal mammary gland pooled RNA for comparison. Gene expression of SOAT and STS was analyzed by real-time quantitative PCR. For normalization, 2 out of 11 different so called housekeeper genes were selected. Our results demonstrate that in addition to STS, SOAT is expressed in mammary gland and breast carcinoma tissues, and, therefore SOAT might contribute to the hormone dependent proliferation of breast tumors. As gene expression patterns in breast carcinomas highly differ depending on the kind and stage of tumor, examination of a broader range of breast tumor samples will be necessary to identify regulatory mechanisms of SOAT expression due to single tumor characteristics.

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7

Cloning and functional characterization of the mouse sodium-dependent organic anion transporter (SLC10A6)

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The solute carrier family SLC10 is formerly known as the family of "sodium bile acid cotransporters". Recently we identified a novel member of this carrier family, the sodium-dependent organic anion transporter (SOAT) which does not transport bile acids. Instead, SOAT showed specific transport for sulfoconjugated steroid hormones and high expression in human testis and placenta. Therefore, SOAT might be involved in the uptake of sulfated steroids in these organs. To clarify the in vivo importance of SOAT transport, a knockout mouse model is intended. For this reason we cloned the mouse Soat transcript and analyzed sequence characteristics, gene expression, and functional properties in comparison to the human SOAT carrier. The mouse Soat (mSoat) mRNA transcript (GenBank Accession No AJ583504) is coding for a 373 amino acid protein with 71% sequence identity to the human SOAT protein. Soat expression was analyzed in 15 different mouse tissues by real-time quantitative PCR and revealed highest expression in heart and lung. Moderate expression was found in placenta and testis. In contrast, human SOAT showed dominant expression in testis and low expression in heart and lung. For the functional characterization of mSoat we established a stably transfected HEK293 cell line and performed transport experiments with different radiolabeled compounds. We found sodium-dependent transport activity for dehydroepiandrosterone-sulfate, estrone-3-sulfate, pregnenolone-sulfate and taurolithocholic acid-3-sulfate. This data agree with the transport characteristics of the human SOAT carrier. In conclusion: Gene expression and functional properties are in general comparable between the human and the mouse SOAT/Soat carrier. However, differences exist in quantitative gene expression in endocrine and non-endocrine tissues.

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8

Characterisation of the Pgps of sheep and parasite in multiresistant haemonchosis

Zahner D.J. (1), Alber J. (1), Petzinger E. (1)

Infections with parasitic trichostrongylid nematodes endanger the gainful husbandry of small ruminants all over the world. The control of such parasites by modern anthelmintics is essential for the success in keeping these animals. This control is more and more endangered by an increasing incidence of multiresistance against common anthelmintics. Due to this evolution a profitable husbandry has yet become impossible in some areas of South America, southern Africa, Malaysia or southeast USA. There is evidence that nematode resistance against macrocyclic lactones, e.g. ivermectin, is caused by overexpression of an analogue to the Pgp transport system of mammalians conveying multidrug resistance. Overcoming of nematode resistance in vitro can be achieved by Pgp blockers. On the other hand host-Pgp is a major protection system at the blood-brain-barrier (BBB) of ruminants which prevents the entrance of the neurotoxic ivermectin into the brain. Thus non-selective blocking of Pgp-activity for overcoming the ivermectin resistance is not useful. Instead, specific inhibition of H. contortus-Pgp is aquired and seems feasible, due to obvious differences in the amino acid sequence between host and parasite Pgp. To create an assay which allows to compare the kinetics of the two Pgps we cloned the full length cDNA of O. aries-Pgp from gut. This sequence and the cDNA-sequence of H. contortus-Pgp (a kind gift from Prof. R. Prichard, McGill-University, Montreal, Quebec, Canada) have been integrated into an expression vector. By generating stable transfected Madin Darby Kidney Cell (MDCK) lines, overexpressing the Pgps of the parasite and host we have begun to prove the transport activity of H. contortus Pgp and to identify selective blocking agents. Further characterisation of the cell lines by daunorubicin cytotoxicity assays and transepithelial transport activity measurements are now performed in order to identify specific and applicable H. contortus-Pgp-inhibiting substances.

1. Inst. of Pharmacology and Toxicology FB10

9

Function and expression of ABC transporters in choroid plexus and blood brain barrier in diabetic rats

Burghard S. (1), Fricker G. (1), Reichel V. (1)

The tissues separating brain from blood are the blood brain barrier (BBB) and the choroid plexus (CP). Both barriers express transport proteins which regulate entrance and accumulation of drugs, nutrients and other molecules to the brain. Overexpression or function of transporters might lead to an undersupply of the brain with e.g. nutrients, while low expression or function might allow entrance of toxic or harmful molecules into the brain. An alteration in expression or function of transport proteins at the BBB and the CP, respectively, might be a reason for brain damages after long term diabetes.P-glycoprotein (Pgp) is one of the most important ATP-dependent transporters at the BBB. It is the 170 kDa gene product of multidrug-resistance gene. Endothelin-1 (ET1) is known to stimulate Pgp expression. Enhanced ET1 levels in diabetic rats indicate, that Pgp expression might be altered in diabetes. Beside Pgp, the Breast Cancer Resistance Protein (Bcrp) is an important transport protein expressed at the BBB. We also could show expression at the apical membrane of CP epithelial cells. Bcrp, also known as Abcg2, is a 655 amino acids polypeptide which functions as a homodimer.We compared Pgp and Bcrp mRNA levels in brain capillaries and CP of wild-type (Sprague Dawley) and diabetic rats using RT-PCR. The diabetic rats suffered from diabetes induced by streptozotocin (STZ) for at least 14 days. Diabetes was confirmed by blood glucose level. The comparison showed, that the Pgp expression levels in both tissues were slightly enhanced in diabetic rats. However, for Bcrp we found different expression levels and couldn't draw a conclusion so far.A functional comparison of isolated brain capillaries was carried out using fluorescent substances and confocal microscopy. First results showed enhanced transport function of Pgp.Protein expression studies remain to be done.

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10

The role of ARF6 protein in regulation of µ-opioid receptor trafficking and signaling

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Endocytosis of µ-opioid receptor (MOPr) plays an important role in counteracting the development of tolerance to opioid drugs. Activation of phospholipase D2 (PLD2) by opioids has been shown to be a prerequisite for MOPr endocytosis and to be dependent on ADP-ribosylation factor (ARF). However, precise identity of ARF protein (ARF1 or ARF6) as well as the mechanisms involved in opioid-mediated PLD2 activation by ARF proteins are still not clear. Here we tested the effects of overexpression of ARF1 and ARF6 dominant negative mutants (ARF1/T31N and ARF6/T44N) on MOPr endocytosis in HEK293 cells stable expressing MOPr. Data show that blocking ARF6 function significantly decreased MOPr endocytosis after treatment with potent receptor internalizing agonist DAMGO, while overexpression of dominant negative ARF1 mutant had no effect. These results were confirmed in primary cultured cortical neurons cotransfected with MOPr and ARF6/T44N. Moreover, coexpression of constitutively active mutant ARF6/T157N together with MOPr in HEK293 cells induced internalization of receptor after treatment with morphine, an agonist that can not promote endocytosis in HEK293 cells expressing MOPr alone. These data demonstrate that ARF6 and not ARF1 protein is involved in regulation of MOPr endocytosis. Furthermore, we suggest here that ARF6 function is PLD2 mediated since a mutant which is incapable of activating PLD (ARF6/N48I) significantly decreased DAMGO-induced MOPr endocytosis. All together, these results suggest that ARF6 protein acts via PLD2 in regulation of MOPr trafficking and signaling, processes involved in development of opioid tolerance and dependence.

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11

Membrane glycoprotein M6A reduces the development of opioid tolerance

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Opioid drugs, such as morphine, are the most commonly used analgesics but their clinical use is seriously limited by the development of opioid tolerance. It has been demonstrated in vitro and in vivo that, on the cellular level, chronic opioid treatment leads to receptor phosphorylation and rapid reduction of agonist response accompanied by endocytosis of receptors. Several in vitro studies implied a protective role of agonist-induced µ-opioid receptor (MOPr) endocytosis in the development of tolerance via fast reactivation of inactivated receptors. Recently, a neuronal membrane glycoprotein, termed M6A, was shown to interact with the MOPr in cultured cells, and to be essential for the agonist-induced opioid receptor endocytosis and recycling. However, the precise role of M6A in the regulation of receptor endocytosis/recycling and development of opioid tolerance, especially in the intact nervous system, remains to be established. Via generating M6a-deficient mice we show here that M6A plays an essential role in the regulation of agonist-induced MOPr endocytosis and in the reduction of opioid tolerance development in vivo. We found that agonist-induced receptor endocytosis was significantly impaired in thalamic neurons of M6anull mice compared to wildtype mice. Furthermore we demonstrated that M6anull mice displayed a shorter analgesic effect and a higher degree of analgesic tolerance than wildtype mice only to receptor-internalizing agonists such as methadone or etonitazene, but not to non-internalizing drugs like morphine. These findings indicate that proteolipid proteins, such as M6A, are involved in the regulation of G-protein coupled receptor trafficking and signaling in neurons. Our results provide new insights in the mechanistic basis of receptor endocytosis and directly indicates that the agonist-induced receptor endocytosis counteracts the opioid tolerance development in vivo.

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12

Dissecting the functional contribution of GABA-B receptors in peripheral nociceptive neurons towards the endogenous and therapeutic modulation of chronic pain

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GABAB receptors (GABABR), the G-protein-coupled receptors for the neurotransmitter GABA are broadly expressed in the nervous system and mediate slow synaptic inhibition. Numerous studies have implicated GABABR in the endogenous suppression of pain at different peripheral and central levels in the somatosensory pain pathway. Furthermore, Baclofen, a non-selevtive agonist at GABABR has been reported to be antinociceptive in several animals models of chonic pain. Baclofen also produces profound muscle relaxation and motor defects, which confound the analysis of pain behaviours in experimental animals. Indeed, studies in human patients have not fully supported Baclofen-induced antinociception which was predicted based upon animal studies. The GABAB1 subunit is essential for generating functional GABABR in vivo and global. Constitutive absence of GABAB1 in mice frequently causes premature lethality or hyperalgesia besides several other deficits in viable mice. Therefore, to functionally delineate peripheral versus central mechanisms in pain modulation via GABABR, we generated mice with a conditional specific deletion of GABAB1 in peripheral nociceptive neurons of the dorsal root ganglia (SNS-GABAB1-/-). SNS-GABAB1-/- demonstrate reduced response latencies to noxious heat, suggesting that GABABR exert an inhibitory tone over heat-induced acute pain. Furthermore, using the Spared-nerve-injury (SNI) model for neuropathic pain, we observed that SNS-GABAB1-/- demonstrate exaggerated heat hyperalgesia, but not exaggerated mechanical allodynia, following neuropathy. Analysis in the Rotarod test showed no evidence of motor defects in SNS-GABAB1-/- mice. These results suggest that GABABR contribute to endogenous defence mechanisms in neuropathic pain states. We are currently addressing the contribution of GABAB1 expressed in peripheral nociceptors to Baclofen-induced antinociception in pain models. These integrative approaches will help understand the net contribution of GABABR in the peripheral nervous system to inhibitory modulation of

pain and will reveal whether central side effects, such as muscle relaxation, can be overcome upon selectively targeting peripheral GABABR.

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13

Very high constitutive activity of the human histamine H4 receptor

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The human histamine H4 receptor (hH4R) is a GPCR, which stimulates mainly Gαi2 and is activated by histamine (HA). The hH4R is constitutively active and thioperamide (THIO) acts an inverse agonist. To investigate G-protein coupling and constitutive activity of the hH4R in more detail, we co-expressed it with Gαi2 and Gβ1γ2 in Sf9 insect cells (= co-expression system). Moreover, we expressed the hH4R-Gαi2 fusion protein in combination with Gβ1γ2. Ternary complex formation was studied by [3H]HA saturation binding. Interestingly, after guanosine 5'-[γ-thio]triphosphate (GTPγS) addition, the KD and the Bmax values were not significantly altered in both fusion protein and co-expression system, indicating that GTPγS does not disrupt the ternary complex. This behaviour is in contrast to most other Gi-coupled GPCRs. Most surprisingly, we found a high-affinity state of the hH4R, but no [γ-35S]GTPγS binding signal in membranes expressing the hH4R without mammalian Gi-proteins. Thus, we suppose a G-protein-independent form of an active receptor state. In both the co-expression and the fusion protein system about 70 % of the total receptor-regulated GTPγS binding was due to constitutive activity. In the presence of HA and of THIO, the affinity of GTPγS to Gαi2 was higher in the fusion protein system than in the co-expression system. This indicates a more effective stimulation of Gαi2 in the fusion protein while THIO is less able to reduce the GTPγS affinity of Gαi2. To study the absolute effect of THIO in the co-expression system, we compared the THIO-blocked co-expression system with a membrane lacking the hH4R (only Gαi2 and Gβ1γ2) in [γ-35S]GTPγS binding. In the absence of the hH4R, GTPγS binding was significantly lower. Thus, although THIO is one of the most effective inverse agonists at the hH4R, it does not exert the maximally possible effect. Finally, constitutive activity of hH4R, in contrast to all other Gi-coupled GPCRs studied so far, exhibited Na+-insensitivity. Collectively, our data show that the hH4R belongs to the wild-type GPCRs with the highest constitutive activity, suggesting that in vivo, hH4R may support maintenance of a tonic cell function.

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14

Analysis of putative ligand-specific conformations of histamine H3 receptor species isoforms

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Rat and human histamine H3-receptors (H3Rs) [1, 2] were expressed with four different mammalian G proteins in Sf9 cells using the baculovirus expression system. When co-expressed with Gi/o proteins (Giα1, Giα2, Giα3 or Goα1 and β1γ2 dimer), the receptors displayed a high-affinity binding site for the agonist radioligand [3H]N-a-methylhistamine ([3H]NAMH), which was sensitive to GTPγS (10 μM), demonstrating an interaction between the receptor and the different G proteins, whereas binding to H3Rs in the absence of mammalian G protein was GTPγS-insensitive and still present without a change in apparent Bmax. The receptor to G protein ratio was evaluated using quantitative immunoblot to show a large relative excess of G protein over H3Rs. Several ligands were tested for their ability to stimulate hydrolysis of [γ-32P]GTP in membranes of Sf9 cells co-expressing the receptors and various G proteins. All compounds showed a similar pharmacology independent of the type of G protein co-expressed, including partial agonists, such as proxyfan, previously described as protean agonist at H3Rs [3]. However, large species-differences were found when imoproxyfan [4], originally designed as a H3R antagonist, were functionally characterized at rat and human H3Rs. At rat H3R, improxyfan was an inverse agonist. In contrast, at human H3R, imoproxyfan not only showed a lower affinity and potency compared to the rodent receptor, but also was found to act as a very efficacious partial agonist. Collectively, our data show the following: (i) As shown by agonist radioligand binding and steady-state GTPase assay, the rat and human H3R equally couple to all co-expressed G proteins, but can exist in a high-affinity state independent of G-proteins. (ii) Ligand-specific receptor conformations, resulting in coupling preferences, do not exist for the compounds investigated in this study. Therefore, we suggest that other effects may be responsible for the effects of proxyfan reported in the literature [3, 5]. (iii) Pharmacology of H3R ligands is highly species-dependent, and can even span from inverse agonism to agonism. [1] Lovenberg TW et al., Mol Pharmacol, 1999, 55, 1101-7.[2] Lovenberg TW et al., J Pharmacol Exp Ther, 2000, 293, 771-8.[3] Gbahou F et al., Proc Natl Acad Sci, 2003, 100, 11086-91.[4] Sasse A et al., J Med Chem, 2000, 24, 3335-43.[5] Krueger KM et al., J Pharmacol Exp Ther, 2005, 314, 271-81. (Supported by Deutsche Forschungsgemeinschaft - GRK 760)

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15

Analysis of a series of phenylhistamines at four H1-receptor species isoforms

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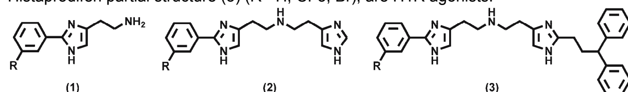
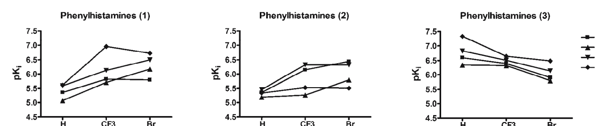
Histamine H1-receptor (H1R) agonists are important tools to analyse differences in species isoforms of the H1R at a molecular level. Phenylhistamines (1), Phenylhistamines with an additional histamine moiety (2) and Phenylhistamines with a Histaprodifen partial structure (3) (R =H, CF3, Br), are H1R agonists.



The aim of this study was to dissect pharmacological differences between the human (h), bovine (b), rat (r) and guinea-pig (gp) H1R. Therefore the compounds were characterized with the GTPase assay as well as with the mepyramine competition binding assay. The figures below show trends in pKi values for the three groups of ligands with respect to R for all four species isoforms. The highest pKi values were obtained for the Phenylhistamines with an additional histaprodifen moiety (3). In the series R = H → CF3 → Br an increase in pKi values is found for ligand groups (1) and (2), in most cases, but a decrease for (3).



Molecular modelling studies based on active H1R models show, that members of the groups (2) and (3) can bind in two different orientations into the binding-pocket. 3D-QSAR studies indicate that the orientation is species dependent.

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16

Identification of new pharmacological chaperones by high throughput screening and automated microscopy

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Mutations in the genes of membrane proteins frequently lead to misfolded protein variants thereby causing disease. Examples are mutants of the G protein-coupled vasopressin V2 receptor (V2R) leading to nephrogenic diabetes insipidus or mutants of the cystic fibrosis transmembrane conductance regulator (CFTR) causing mucoviscidosis. Misfolded proteins are retained in the early secretory pathway by a quality control system and subsequently subjected to degradation. Recently, it was shown that misfolding of membrane proteins can be corrected in vitro by “pharmacological chaperones”, specific small molecule ligands that appear to facilitate correct protein folding thereby helping to overcome transport and functional defects. Taking the V2R as a model, we have developed a strategy to identify new pharmacological chaperones by high throughput screening and automated microscopy in live cells: GFP-tagged V2R variants are stably expressed in HEK 293 cells, spread in 386 well titer plates and incubated with substance libraries. To analyze receptor transport automatically, we have developed algorithms to define three subcellular regions: the nuclear region is defined by a Hoechst 33258 stain, the plasma membrane region by a Trypan blue stain and the intermediary region by subtraction. A rescue of the receptor transport is assessed by calculating the ratio of the receptor fluorescence at the plasma membrane and in the intermediary region. This methodology will not only help to identify new pharmacological chaperones. Using switchable fluorescence tags instead of GFP, substances affecting trafficking of membrane proteins between the individual subcelluar compartments (such as the endoplasmic reticulum and the Golgi apparatus) may be detected as well.

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17

Parathyroid hormone acts as a pharmacological chaperone on the parathyroid hormone receptor

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Parathyroid hormone receptor (PTH1R) belongs to class B G-protein-coupled receptors and is the major regulator of extracellular calcium homeostasis. Binding of the native ligand parathyroid hormone (PTH) or biologically active peptide fragments of PTH to the receptor lead to activation of Gs and Gq/G11 pathways with subsequent stimulation of adenylyl cyclase and phospholipase C. Activation of the PTH1R leads to the recruitment of beta-arrestin and subsequently to an efficient internalization of the receptor from the cell surface within several minutes.Here we present that long-term stimulation of the PTH1R for several hours with PTH resulted in increased cellular protein levels and enhanced membrane distribution of the receptor. Metabolic labeling of the PTH1R revealed a prolonged half-live of the PTH1R after long-term stimulation. This apparent increase of receptor stability was accompanied by an accumulation of high molecular mass moieties of the PTH1R suggesting additional posttranslational modification of the protein.We are currently searching for possible molecular explanations regarding the nature of the increased half live and molecular weight after long-term stimulation of the PTH1R, and we will present the actual status of our approaches.

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18

Persistent signal transduction of FTY720 via internalized sphingosine-1-phosphate type 1 receptors

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The novel immunomodulator FTY720 has demonstrated efficacy in the treatment of Multiple Sclerosis. Following phosphorylation in vivo it acts as an agonist on S1P receptor types 1, 3, 4 and 5. Most of the clinical effects of FTY720-phosphate (FTY720P) are thought to be mediated via S1P1 receptors on lymphocytes and endothelial cells, leading to sequestration of lymphocytes in secondary lymphoid organs. FTY720P was described to act as a “functional antagonist” by promoting efficient internalization and degradation of S1P1 receptors. Measuring receptor internalization and signaling activity in parallel, we demonstrate here that S1P1-FTY720 complexes undergo quantitative internalization but retain signaling activity for hours, even after agonist washout, and are trafficked to the trans-Golgi network. The persistent signaling translates into function, as demonstrated by an increased chemokinetic migration of

primary human endothelial cells. In contrast to the concept of functional antagonism, our data identify sustained agonism on S1P1 receptors as a crucial parameter in the mechanism of action of FTY720.

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19

Receptor polymorphisms determine the kinetics of beta-adrenergic receptor conformational changes

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β1-and β2-adrenergic receptors (βARs) mediate crucial catecholamine effects in the body and thus play a key role in the sympathetic nervous system. Several frequently occurring polymorphisms have been identified whose clinical relevance is controversially discussed. The most important are Ser49Gly and Gly389Arg for the β1AR, Arg16Gly and Gln27Glu for the β2AR. In order to investigate the impact of these single amino acid changes on the receptor level we generated mutant βAR variants which contain the cyan- and yellow-emitting variant of the green fluorescent protein (CFP/Cer and YFP). By using a fluorescence resonance energy (FRET) approach we could determine the activation characteristics of the polymorphic receptors in real time and in living cells. The sensors retained the pharmacological properties (i.e. ligand affinities) of the native receptors. Furthermore they reflected the functionality of the wild-type receptors since they were able to produce cAMP to the same extent. Comparing the activation and deactivation kinetics of the different receptor variants upon a single agonist stimulation did not reveal any significant differences. In contrast, we found that receptor polymorphisms critically determine the kinetics of receptor activation and inactivation upon repetitive stimulation by agonists. Whereas an increase in the activation speed could be observed for the Gly16/Gln27 and Gly16/Glu27 variants of the β2AR, we found the activation constant of the Arg16/Gln27 β2AR to become slower after repetitive agonist exposure. Thus, carriers of the Gly16/Gln27 and Gly16/Glu27 variants of the β2-adrenergic receptor may respond with a relatively longer active state of the receptor protein itself compared to Arg16/Gln27 carriers. These data suggest that naturally occurring receptor polymorphisms critically determine the kinetics of activation and deactivation of a G protein-coupled receptor with likely consequences for its signalling properties.

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20

EP4 receptor stimulation reduces endothelial cell permeability

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Vascular inflammation is associated with an increased generation of endothelial derived mediators and endothelial barrier dysfunction, leading to perivascular accumulation of macromolecules, inflammatory cells and edema. There is evidence that the adherens junction protein VE-cadherin, which decreases endothelial paracellular permeability, is regulated in a cAMP-dependent manner. The present study evaluates the role of E- and I-type prostaglandin receptors on endothelial permeability and VE-cadherin expression in adult human saphenous vein endothelial cells (AHVEC). RT-PCR demonstrated the expression of E-type (EP1-EP4) and I-Type (IP) prostaglandin receptor subtypes, with predominant expression of EP4 and IP. Confluent AHVEC grown on 0.4 µm porous membranes were stimulated with selected EP and IP receptor agonists for 2h. Endothelial permeability was determined by the passage of FITC-labelled BSA (1 mg/ml), which was added 1h after the prostaglandin receptor agonists. Both PGE2 (100 nM) and the selective IP agonist cicaprost (10 nM) significantly reduced vascular permeability in a concentration-dependent manner, with a maximum inhibition by 56 ± 2 and 66 ± 7 %, respectively (each p<0.05, n=3). Similar to PGE2, EP4 receptor specific activation with a synthetic agonist reduced basal endothelial permeability. The agonist also abolished the thrombin-induced (3 U / ml) fenestration of endothelial cells. In AHVECs cultured on coverslips and immunostained with CY3-labeld VE-cadherin, acute (15 min) E- and I-type prostaglandin receptor stimulation markedly increased VE-staining compared with paired controls. In summary, prostaglandin receptors (EP4 and IP subtype) regulate basal and thrombin-stimulated endothelial barrier function, at least in part via increased expression of VE-cadherin.

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21

Anti-proliferative effects of prostanoid EP2 receptors and cAMP in human lung fibroblasts are mediated via Epac1

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In lung fibroblasts activation of adenylyl cyclase, for example via EP2 receptors exerts inhibitory effects on various functions including proliferation. Beside the classic effector for cAMP, protein kinase A (PKA), alternative effectors have been identified, among them Epac1 and -2 (exchange proteins activated by cAMP). The present study aimed to illuminate transduction pathways mediating the anti-proliferative effects of EP2 receptor activation in lung fibroblasts. Human lung fibroblasts (MRC-5 cells) were cultured and cell proliferation was quantified by measuring incorporation of [3H]-thymidine.The EP2 receptor agonist butaprost (1 nM-1 µM) inhibited [3H]-thymdine incorporation maximally by 72±2% with an IC50 of 2.3 nM. The selective Epac-agonists 8-CPT-2 -O-Me-cAMP and Sp-8-sCPT-2`-O-Me-cAMPS (1-100µM) inhibited [3H]-thymidine incorporation maximally by 69±3% and 70±1%, resp., whereas the selective PKA-agonist 6-Bnz-cAMP (up to 300µM) showed no effect. Pretreatment of cells with siRNA directed against Epac1 resulted in 75% reduction of Epac1 mRNA within 24 h (with no effects on Epac2 mRNA), but the downregulation of Epac1 protein occurred delayed. When cells were treated twice with Epac1 siRNA at an interval of 24 h, a consistent reduction in Epac1 protein was observed 72 h after the last treatment. Under these conditions of partial knock-down of Epac1 the concentration response curve of butaprost was markedly shifted to the right, whereas in parallel experiments in which cells were exposed to non-silencing siRNA or siRNA directed against Epac2, the inhibitory effect of butaprost remained unaffected.In conclusion, EP2 receptor-mediated anti-proliferative effects in human lung fibroblasts involve Epac1 indicating that this pathway rather then the classical PKA pathway mediates anti-proliferative effects of cAMP-elevating signals in human lung fibroblasts.

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22

S100A10 and the plasmin cleavage-derived annexin A2 N-terminal peptide form a complex inducing monocyte chemotaxis

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Plasmin is a potent proinflammatory activator of monocytes and macrophages. Recently we have identified the annexin A2 heterotetramer composed of annexin A2 and S100A10 as plasmin receptor on human monocytes and macrophages. Plasmin initiates signaling in monocytes by proteolytic cleavage of the annexin A2 subunit of the receptor, followed by the dissociation of the heterotetrameric complex. The exact mechanism linking the dissociation of the receptor and activation of the intracellular signaling remains elusive. Plasmin cleaves annexin A2 at a single position at the lysine 27 releasing an annexin A2 N-terminal peptide (A2NTP). After treatment of human monocytes with plasmin, S100A10 can be detected in the cell supernatant suggesting that S100A10 is released from the complex at the cell surface. In order to analyze binding of S100A10 to the cleaved 27 amino acids long annexin A2-derived peptide A2NTP, we performed surface plasmon resonance analysis, which showed that in contrast to scrambled control peptide, A2NTP indeed binds S100A10. These data were validated by an in vitro pull down assay using biotinylated A2NTP and recombinant S100A10. In order to investigate the functionality of the A2NTP/S100A10 complex, we performed analysis of the monocyte chemotaxis. Neither the A2NTP peptide, nor S100A10 alone were able to induce cell migration. On the contrary, a reconstituted complex containing both, A2NTP peptide and S100A10, induced chemotaxis to the similar extend as plasmin or fMLP used as positive controls. These data suggest that the complex of S100A10 and the N-terminal part of the annexin A2 molecule acts as a new activating protein/peptide complex and may mediate the plasmin-induced signaling in monocytes. Supported by the DFG, SFB 451.

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23

Use of Kaede fusions to visualize recycling of the corticotropin-releasing factor receptor

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The corticotropin-releasing factor receptor type 1 (CRF1R) is involved in the regulation of the hypothalamic-pituitary-adrenal stress axis. Following agonist treatment, the receptor is rapidly desensitized and internalized. It was unknown whether the receptor is then targeted to the lysosomal pathway or recycles back to the plasma membrane. Here, we have addressed this question and developed a new methodology using CRF1R fusions with the recently cloned Kaede protein. In contrast to the widely used green fluorescent protein (GFP), the fluorescence of Kaede can be converted from green to red using UV irradiation. Our novel assay allows to study the fate of G protein-coupled receptors (GPCRs) microscopically in real time: Initially, receptors at the plasma membrane are internalized using an agonist. The fluorescence in endosomal compartments is then switched from green to red. Thereafter, trafficking of the receptors to target compartments, such as the plasma membrane or lysosomes, can be easily visualized by monitoring their new fluorescence. Repeated internalization is blocked by the addition of an antagonist. Using this methodology, we show that the CRF1R is rapidly recycled from endosomal compartments. The Kaede technology may be a powerful tool to study trafficking of GPCRs and unrelated membrane proteins in general.

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24

Probing the zone sensitive for allosteric modulation of muscarinic receptor activation

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Applying small allosteric molecules as probes we have shown that a junction between the second extracellular loop (E2) and the beginning of transmembrane helix seven (TM7) is opened during activation of the muscarinic M2 receptor [1]. M2422Trp at the beginning of TM7 was identified as critical for agonist-induced receptor activation. In the inactive receptor state M2422Trp forms an aromatic interaction with E2-M2177Tyr that is stabilized by allosteric molecules which intercalate into this contact [1]. We aimed at checking whether the zone sensitive for allosteric modulation of receptor activation goes beyond the M2177Tyr/M2422Trp-junction. In a 3-dimensional receptor model, the atypical allosteric modulator Duo3 docked to the allosteric site without reaching into the allosteric core region where the M2177Tyr/M2422Trp-junction is located [2]. In order to check this prediction we used a COS7-hM2422Trp→Ala receptor mutant. Radioligand binding experiments revealed that, in contrast to common allosteric agents, the binding affinity of Duo3 is not sensitive to this mutation. This finding verifies that Duo3 does not interfere with the M2177Tyr/M2422Trp-junction. We measured CHO-hM2-induced signalling applying a [35S]GTPγS assay. Duo3 almost switched off partial agonist (pilocarpine)-induced receptor activation and strongly reduced agonist potency. Also the full agonist acetylcholine greatly lost affinity under the influence of Duo3 but maximum receptor activation was maintained. This pattern of findings fully corresponds with our findings made previously with typical allosteric modulators that intercalate into the M2177Tyr/M2422Trp-contact. As the atypical probe Duo3 docks into the allosteric binding crevice distant from the M2177Tyr/M2422Trp-junction we conclude that the receptor's off-on transition goes along with a far reaching rearrangement of the

extracellular loop region. [1] Jäger D, et al. (2007) J Biol Chem 282:34968-34976[2] Tränkle C, et al. (2005) Mol Pharmacol 68:1597-1610

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25

A FRET-based M2 muscarinic receptor sensor to study the mechanisms of allosteric modulation

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In recent times allosteric modulators have been proposed as promising new compounds to modify protein function. In contrast to orthosteric agonists, which occupy the “classical” binding site for endogenous agonists, these agents bind to an additional site of a protein and alter its activity. Since these allosteric agonists can only enhance or decrease protein function in the presence of an orthosteric ligand, adverse effects and side effects may differ from conventional drugs. Allosteric binding sites have been discovered for several G-protein-coupled receptors, including M1-5 muscarinic receptors. Since these receptors play a pivotal role in the regulation of a plethora of organ functions it is particularly important to investigate the mechanisms of allosteric modulation of these receptors. To study the mechanisms of several known allosteric modulators of the M2 muscarinic receptor, we designed a new FRET-based sensor. We used CFP fused to the C-terminus of the receptor and the small fluorescent compound FlAsH, which can be attached to the six-amino acid motif CCPGCC inserted into the third intracellular loop, as donor and acceptor fluorophores, respectively. Such a sensor was expressed in intact cells to perform real-time monitoring of the M2-receptor activation and deactivation by various agonists and antagonists. This approach should further allow to distinguish between conformational changes in receptors that are brought about by orthosteric and allosteric ligands in order to delineate the mechanisms of allosteric modulation.

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26

Critical role of an extracellular E2/transmembrane helix 7 junction for the constitutive activity of a G protein-coupled receptor

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The human muscarinic M2 acetylcholine receptor belongs to the superfamily of G protein-coupled receptors and is prototypal for the study of allosteric effects. A key epitope of the allosteric binding site is M2422Trp in position 7.35 that is located at the top of transmembrane helix 7 and that contacts, in the inactive receptor, the extracellular loop E2 via an aromatic interaction with M2177Tyr. At the pointmutated receptor hM2422Trp→Ala the full agonist acetylcholine keeps its intrinsic efficacy whereas the partial agonist pilocarpine looses most of its efficacy. hM2422Trp appears to be essential for stabilizing the active conformation in pilocarpine bound receptors, but not in acetylcholine bound receptors [1].The question arises whether the spontaneous off-on switch of the receptor protein in the absence of agonist is impeded in the hM2422Trp→Ala mutant.Receptor mediated G protein activation was measured using a [35S]GTPγS-binding assay carried out with membranes of CHO cells stably transfected with the human M2 wild-type receptor gene or the point-mutated receptor gene, respectively.Receptor dependent G protein activation was defined as the difference between [35S]GTPgS-binding in the presence of the inverse agonist atropine (1µM) set as 0% and the full agonist acetylcholine (100µM) set as 100%. The wild type hM2 receptor shows a constitutive, agonist-independent activity amounting to 41% ± 2%. In the hM2422Trp→Ala mutant spontaneous receptor activity is strongly reduced to a level of 11% ± 1% (n=3, mean values ± S.E.), whereas the acetylcholine induced maximum was unchanged.In conclusion the allosteric epitope M2422Trp is essential for stabilizing the active receptor conformation in the absence of agonist. [1] Jäger et al. (2007) J Biol Chem. 282(48):34968-76 Supported by the DFG (GRK 677)

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27

Dynamics of agonist-induced interaction of M3 acetylcholine receptor and Gq proteins

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Traditional methods to analyze G protein-coupled receptor signaling mostly are done using biochemical methods requiring cell homogenization. Therefore, we set out to investigate influences of different agonists to stimulate receptor / G protein interaction in a more physiological setting. We established a fluorescence resonance energy transfer (FRET) -based assay to monitor the interaction of M3 receptors with Gq proteins in intact cells. In HEK293 cells, an M3 receptor-YFP fusion protein was coexpressed with Gαq β1 CFP-γ2, and single-cell fluorescence recorded while the cell was perfused with the agonists carbachol, acetylcholine or buffer. Upon agonist stimulation, an increase in YFP and a decrease in CFP fluorescence was observed, leading to an increase in ratiometric FRET. The FRET signal change depended in amplitude and onset kinetics on the agonist concentration. Both carbachol and acetylcholine stimulated M3 / Gq interaction with fast kinetics (time constants <100ms), which were in the range of M3 receptor activation kinetics measured by FRET. While receptor deactivation kinetics had time constants of ~800ms for acetylcholine and ~600ms of carbachol, the FRET signal between the receptor and the G protein had a much slower deactivation time constant (9.4s and 3.8s for washout of acetylcholine and carbachol, respectively). Taken together, by direct measurements in living cells, we analyzed kinetics of M3 receptor / Gq interaction. The interaction termination kinetics were slower than M3 receptor deactivation and depended on the agonist applied before.

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28

Constitutively active human histamine H2 receptor lacks constitutive internalization in HEK293 cells

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The aim of this study was to investigate the membrane trafficking of the human histamine H2 receptor (hH2R) in vitro with respect to its pharmacological profile. HEK293 cells were stably transfected with human histamine H2 receptor DNA, encoding N-terminal FLAG-tag and C-terminal hexahistidine-tag epitope sequences. In vitro expression of tagged hH2R was demonstrated by immunofluorescence, whereas wild type HEK293 cells served as a negative control. Strong constitutive receptor activity was detected in membrane preparations of hH2R expressing cells using the adenylyl cyclase (AC) activity assay. The AC activity was increased by the agonists histamine, arpromidine and impromidine, and was decreased by the inverse agonists famotidine and ranitidine, respectively. Incubation of hH2R-positive HEK293 cells with the agonists for 1h resulted in noticeable internalization of immunolabeled receptors, which was reversible by the consecutive incubation with the inverse agonists. No internalization was observed with inverse agonist incubation only. Surprisingly, incubation without any exogenously added agonist did not lead to internalization of hH2R. In conclusion we suggest that the constitutively active hH2R undergoes a conformational change upon agonist binding, which enables the interaction with mediators of internalization and initial trafficking steps.

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29

Interaction of histamine receptors with antipsychotic drugs

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The local mediator and neurotransmitter histamine plays a central (patho)physiological role in a number of processes. The histamine H4-receptor (H4R) was discovered not until the year 2000 and, therefore, its precise function is still poorly defined. It is primarily expressed in haematopoietic cells, specifically T-lymphocytes, mast cells and eosinophils. This suggests an involvement of the H4R in immune reactions and inflammatory processes. Since clozapine is known to show partial agonism at the H4R it is also discussed that there is a connection between the H4R and the development of agranulocytosis, a severe and potentially lethal side effect of atypical antipsychotic drugs. For a better understanding of the relationship between antipsychotic drug-induced agranulocytosis and the H4R, we expressed various HxRs in Sf9 insect cells. We determined the affinities (Ki-values) of antipsychotic drugs such as phenothiazines, butyrophenones, thioxanthenes and atypical antipsychotics by performing radioligand binding studies using [³H]histamine (H4R), [³H]N-a-methylhistamine (H3R) and [³H]mepyramine (H1R) as radioligands. Most of the antipsychotic drugs bound to the H4R and H3R only with moderate affinities (Ki-values from 500 nM to 100 µM), whereas some of them showed a high affinity to the H1R (Ki-values from 100 pM to 3 µM). Among the tested substances the highest affinity at the H4R could be determined for clozapine (Ki-value 500 nM). As these concentrations are easily reached in the plasma during treatment a correlation between the H4R and agranulocytosis is possible.

Table 1: Ki-values [nM] of selected antipsychotic drugs at HxRs

	H1R	H3R	H4R
clozapine	3	3,100	500
N-desmethyl-clozapine	4	30,000	530
clozapine N-oxide	2,700	50,000	70,000
chlorpromazine	2	19,000	3,000
chlorprothixene	0.1	5,800	2,900
fluphenazine	6	3,300	70,000
thioridazine	3	2,400	8,800

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30

Role of individual disulfide bonds on the assembly and cell surface expression of P2X1 receptors

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During synthesis, membrane proteins are translocated into the ER lumen, where they undergo folding, assembly, and other co- and post-translational modifications. A critical step in the proper folding of the ectodomain of many membrane proteins is the formation of native disulfide bonds, which typically serve to stabilize the correctly folded three-dimensional conformation of the protein. Here, we analyzed the contribution of individual disulfide bonds on the assembly and cell surface expression of the P2X1 receptor, which is a member of the P2X family of ATP-gated cation channels. P2X receptors contain ten perfectly conserved cysteine residues within the extracellular loop, which are contained in two cysteine-rich domains, CRD1 (C1-C6) and CRD2 (C7-C10). We have previously reported that rP2X1 mutants having one or several cysteine residues of CRD1 replaced by serine were capable of forming homotrimers that were exported to the plasma membrane, albeit often less efficiently than the wild type rP2X1 receptor. In contrast, mutants lacking one of the CRD2 cysteines had a strong propensity to aggregate, were unable to form defined oligomers, and did not appear at the cell surface.To determine the role of individual disulfide bonds in CRD1, we simultaneously replaced four distinct cysteine residues by serine, and examined the cell surface expression and assembly by protein labelling with [35S]methionine and fluorescent dyes combined with blue native PAGE. *Xenopus laevis* oocytes were used for heterologous expression of the P2X1 mutants. Provided that a native disulfide bond could be formed, the corresponding rP2X1 mutant formed defined homotrimers and was exported as a functional receptor to the plasma membrane. The following disulfide connectivities supported functional receptor formation: C1-C6, C2-C4, and C3-C5. Analysis by non-denaturing and denaturing SDS-PAGE revealed that a single disulfide bond in CRD1 was sufficient to establish a wild type-like compact structure of the receptor mutant. We conclude that one single disulfide bond out of the three naturally occurring disulfide bonds in CRD1 is sufficient to drive the formation of functional rP2X1 receptors.

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31

Quantitative investigation of receptor-receptor interactions by FRAP microscopy

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Many G protein coupled receptors have been described to assemble to dimers or higher order oligomers. Based on current techniques extent, stability and size of oligomerization of these receptors is difficult to determine. Therefore, we utilized a generally applicable approach based on dual-color fluorescence recovery after photobleaching (FRAP) to study the stability and extent of oligomerization between membrane proteins on the basis of the mobility of these proteins. It was important to exactly determine the relative expression of YFP and CFP tagged receptors in each individual experiment. To do so YFP and CFP fluorescence of individual cells were compared to reference constructs containing both an extracellular YFP and an intracellular CFP. The lateral mobility of an extracellularly YFP-tagged receptor was restricted using an antibody against YFP whereas the other coexpressed protein of interest labelled intracellularly with CFP was not affected by this antibody treatment. The effect of the mobility restriction of the former protein on the mobility of the latter protein is determined and used as readout for protein-protein interactions. Thus, the mobility at different expression ratios could be compared. We applied this approach to analyze homo-interactions between β1- and β2-adrenergic receptors (AR). For β1-AR we found that the majority of receptors were mobile. Expression of a majority of β1-AR immobilized by antibody treatment showed only little effect on the size of the immobile fraction of the non-crosslinked minority, however the fluorescence recovery was substantially slowed demonstrating that β1-AR di-/oligomerization is not stable but rather dynamic. In contrast even at a four fold excess of non-crosslinked over crosslinked β2-AR no fluorescence recovery was measurable. These results indicate that β2-AR indeed oligomerize to stable higher order complexes.

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32

PKA anchoring via AKAP150 is essential for TRPV1 modulation by forskolin and PGE2 in mouse DRG neurons

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Phosphorylation-dependent modulation of the vanilloid receptor TRPV1 is one of the key mechanisms mediating the hyperalgesic effects of inflammatory mediators, such as prostaglandin E2 (PGE2). Little is known about the molecular organization of the TRPV1 phosphorylation complex and specifically about scaffolding proteins that position the protein kinase A (PKA) holoenzyme proximal to TRPV1 for effective and selective regulation of the receptor. Here, we demonstrate the critical role of the A-kinase anchoring protein AKAP150 in PKA-dependent reduction of TRPV1 desensitization in adult mouse dorsal root ganglion (DRG) neurons. We found that AKAP150 is expressed in ~80% of TRPV1-positive DRG neurons and is co-immunoprecipitated with the capsaicin receptor. In addition, the regulatory subunit RIIa of PKA (PKA RIIa) also coimmunoprecipitates with TRPV1. In functional studies, PKA stimulation with forskolin markedly reduced desensitization of TRPV1. This effect was blocked by the PKA selective inhibitors KT5720 and H89, as well as by the AKAP inhibitory peptide Ht31. Similarly, PGE2 decreased TRPV1 desensitization in a manner sensitive to the PKA inhibitor KT5720. Both forskolin and PGE2 effects were strongly impaired in DRG neurons from knock-in mice that express a mutant AKAP150 lacking the PKA-binding domain (Δ36 mice). Importantly, the D36 mutation also disrupted co-immunoprecipitation of PKA RIIa with TRPV1. The PGE2/PKA signaling defect in DRG neurons from D36 mice was rescued by overexpressing the full-length human ortholog of AKAP150 (AKAP79), but not other AKAPs, such as D-AKAP1, D-AKAP2, AKAP18 or AKAP250. TRPV1 sensitization induced by stimulating protein kinase C (PKC) with phorbol-12,13-dibutyrate remained intact in Δ36 mice. Taken together, these data suggest that PKA anchoring by AKAP150 is essential for the enhancement of TRPV1 function by activation of the PGE2/PKA signaling pathway.

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33

Epac-mediated inhibtion of TNF-α-induced apoptosis

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Tumor necrosis factor-α (TNF-α) is a cytokine with various functions in signal transduction cascades involved in cell survival, differentiation, and death. Cyclic adenosine monophosphate (cAMP), is known to be involved in the regulation of apoptosis. Both, pro- and anti-apoptotic effects of cAMP have been described. These cAMP-dependent effects have so far been attributed to activation of protein kinase A. Recently, however, Epac proteins, direct cAMP targets and guanine nucleotide exchange factors for Ras-like GTPases, have been implicated to contribute to cAMP-dependent regulation of apoptosis. Therefore, we investigated the effects of 8-(4-chlorophenylthio)-2'-O-methyl cyclic AMP (8-pCPT-2Me-cAMP), a selective Epac activator, on TNF-α-induced apoptosis in U937 cells, a monocytic cell line. For this purpose, cells were incubated for 24 hours with TNF-α without and with 8-pCPT-2Me-cAMP. Cells undergoing apoptosis were analyzed by detection of caspase-3 processing, poly(ADP-ribose)polymerase (PARP) cleavage, DNA fragmentation (propidium iodide staining), as well as annexin-V binding (flow cytometry). We report here that 8-pCPT-2Me-cAMP significantly inhibited TNF-α-induced apoptosis. These data indicate that Epac proteins modulate the signalling cascades leading to apoptosis in U937 cells after stimulation of the TNF-α receptor.

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34

Determination of subunit stoichiometry of heterotrimeric P2X receptors by quantitative fluorescent cell surface labelling

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Most ion channels are multimeric proteins consisting of identical or homologous subunits that arrange in such a way as to form an ion permeation pathway channel in the centre of the subunit complex. P2X receptors assemble from a repertoire of seven subunit isoforms to form homotrimeric and also heterotrimeric receptor channels, which serve to mediate the ATP-gated permeation of Na+, K+ and Ca2+ across cell membranes. Heteromeric P2X receptors that have been identified include P2X1+4, P2X1+5, and P2X2+3. Among these, P2X2+3 receptors have been particularly well studied, because sympathetic ganglion cells involved in pain propagation express an αß-methylene-ATP-sensitive non-desensitizing P2X receptor phenotype that is mimicked by co-expression of recombinant P2X2 and P2X3 receptors. Coimmunoprecipitation experiments confirmed that P2X2 and P2X3 subunits are physically linked to each other. From functional analysis, it has been inferred that the P2X2+3 receptor is composed of one P2X2 and two P2X3 subunits.Most likely that the stoichiometry of heteromeric P2X receptors is precisely regulated and is fundamental to its functional properties. In this study, we exploited quantitative fluorescent cell surface detection to assess biochemically the stoichiometry of heteromeric P2X receptors. The principle of this method consists of co-expressing various ratios of a StrepII-tagged P2X subunit and a His-tagged P2X subunit as bait and prey proteins, respectively. Plasma membrane expressed receptors are covalently labelled by a fluorescent dye, affinity purified under non-denaturing conditions, and resolved by SDS-PAGE. The fluorescence intensity of the co-purified subunits is quantified and correlated with the theoretical probability distribution of having one of the possible stoichiometries. Co-expression of P2X2 and P2X3 subunits yielded a clear correlation between the experimentally determined prey subunit at the plasma membrane and a 1:2 (P2X2:P2X3) subunit, which is consistent with the functional data. Having demonstrated the feasibility of this approach, the subunit stoichiometry of other heteromeric P2X receptors is amenable to analysis.

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35

Regulation of prostacyclin receptor-mediated signaling in human pulmonary fibroblasts

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Iloprost (ilo), a synthetic prostacyclin mimetic is in clinical use for treatment of pulmonary hypertension. Continuous administration of the natural ligand prostacyclin over months is associated with the development of tolerance which requires marked increase in dosing and is probably due to agonist-induced down-regulation of the Gs-coupled prostacyclin receptor. This study investigates whether ilo has a similar effect and whether this effect is seen at therapeutically relevant doses. Human pulmonary artery fibroblasts were incubated in vitro with ilo. Changes in cellular cyclic AMP (cAMP) were measured as a surrogate parameter for Gs-mediated stimulation of adenylate cyclase. Stimulation with ilo at nanomolar concentrations (0.1 – 10 nM) increased cAMP levels from 12 ± 1 to 19 ± 2; 30 ± 3; 115 ± 15; 216 ± 12 and 847 ± 116 at 0.1, 0.3, 1.0, 3 and 10 nM ilo, respectively. Incubation with ilo (10 nM; 2 - 24 h) caused a time dependent decrease of stimulated cAMP levels from 958 ± 148 at first stimulation (control) to 312 ± 76 pmol cAMP/mg protein (n=3). Interruption of incubation with the agonist for 2h caused a comparable receptor desensitization after 10h. Resensitization experiments revealed that more than 5h of agonist free intervals were required for significant receptor resensitization. This suggests de novo synthesis of receptor protein. Incubation with ilo at therapeutic concentrations (0.3 nM) for up to 24h did not result in substantial loss of receptor activity: 735 ± 27 vs 722 ± 95 pmol cAMP/mg protein. There was also no significant change at 1 nM ilo while cAMP was decreased from 913±190 to 440 ± 64 pmol cAMP/mg protein at 3 nM ilo. These data show that ilo has the capacity to downregulate prostacyclin receptors at higher concentrations if present for sufficient periods of time. However these effects are not seen at lower concentrations (< 1 nM) even after 24h of continuous incubation and, therefore, might not be relevant in vivo.

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36

Influence of S-adenosyl-L-homocysteine hydrolase overexpression on cell viability and DNA methylation

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S-adenosyl-L-homocysteine (AdoHcy) hydrolase catalyzes the reversible hydrolysis of AdoHcy to adenosine (Ado) and homocysteine. This enzyme regulates intracellular AdoHcy concentrations and controls thereby the transmethylation activity since AdoHcy is a product inhibitor of S-adenosyl-L-methionine (AdoMet)-dependent methyltransferases. In the present study we assessed the effect of enhanced AdoHcy hydrolase activity on AdoMet/AdoHcy metabolism, cell viability, and methylation reactions. We generated HEK-293 cell lines stably overexpressing AdoHcy hydrolase using the mammalian expression vector pcDNAcd20 encoding human placental AdoHcy hydrolase. Cells were cultured in MEM + 10% newborn calf serum at 37°C with 5% CO2. Cell viability was monitored using a cell analyzer system. The activity of caspase 2,3,6, and 8 was determined fluorometrically. Ado, AdoMet, and AdoHcy were measured by HPLC. AdoHcy hydrolase activity was determined photometrically at 292 nm. The global DNA methylation was determined using cytosine extension assay.A 2-10-fold AdoHcy hydrolase overexpression decreased the intracellular AdoHcy levels from 0.18±0.01 to 0.12±0.01 nmol/107 cells and increased the Ado levels from 0.05±0.006 to 0.19±0.01 nmol/107 cells. In contrast, a 16-fold AdoHcy hydrolase overexpression increased AdoHcy and Ado levels to 0.51±0.1 and 0.81±0.07 nmol/107 cells, respectively, lowered energy charge, and altered cell morphology. Furthermore, we found a correlation between AdoHcyase activity, adenosine levels, and cell viability. Caspase-activity assays and DNA fragmentation analysis revealed that the cell death in AdoHcy hydrolase overexpressing cells was due to apoptosis. Global DNA methylation was not altered in the different AdoHcy hydrolase overexpressing cell lines. Our data show that 2-5-fold enhanced AdoHcy hydrolase activity is well tolerated by the cell, while greatly enhanced AdoHcy hydrolase activity results in adenosine-induced apoptosis.

The fact that enhanced AdoHcy hydrolase activity does not increase transmethylation activity suggests that AdoHcy hydrolase activity under physiological conditions is sufficient for efficient transmethylation.

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37

Isolation and characterization of a novel dominant-negative splice variant of the human orexin type-2 receptor

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Orexin A and orexin B are hypothalamic peptides that are involved in the regulation of feeding behavior, neuroendocrine and autonomic functions and sleep wakefulness. There are two orexin receptor subtypes, OX1 and OX2 receptor, which are members of the G-protein coupled receptor family and act via Ca2+ mobilizing signals.Using RACE-cloning we isolated a novel splice variant of the human OX2 receptor. This new isoform lacks the seventh transmembrane domain and contains an alternative non-homologous sixth transmembrane domain. The expression ratios of the OX2 receptor wild type to splice variant vary in different human tissues such as brain, lung and adrenal gland as shown by real time PCR. To identify potential functional effects of the OX2 splice variant on the wild-type receptor, both isoforms were expressed in *Xenopus laevis* oocytes via mRNA microinjection. Under voltage clamp conditions application of orexin A induced a Ca2+ dependent chloride current in oocytes expressing the wild-type OX2 receptor while no effects of orexins were found in oocytes solely expressing the splice variant. Coexpression of the splice variant with the wild-type OX2 receptor resulted in significantly lower amounts of the evolved currents. A similar effect of the splice variant was also observed in a human adrenocortical carcinoma cell line (NCI-H295R) and in Chinese hamster ovary (CHO) cells. We transfected different concentrations of splice variant receptor DNA into NCI-H295R cells that endogenously express the wild-type as well as the splice variant receptor. CHO cells which do not express any orexin receptors were treated in the same manner, but were additionally transfected with wild-type receptor DNA. Intracellular calcium before and after orexin A application was monitored using the fluorescent Ca2+ indicator Fluo-4 AM and a fluorescence microplate reader. Both cell lines showed up to 50% reduction of the Ca2+ response following orexin application depending on the expression of the splice variant. Our results suggest that the novel OX2 receptor splice variant modulates the wild-type receptor signaling in a dominant-negative manner.

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38

Muscarinic receptors mediate stimulation of collagen synthesis in human lung fibroblasts

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The decline in lung function in patients with COPD is delayed by treatment with the long-acting muscarinic antagonist tiotropium (Spiriva©), suggesting that cholinergic mechanisms may contribute to long-term structural changes. We recently demonstrated that human lung fibroblasts express muscarinic receptors which can mediate proliferative effects. The present experiments aimed to explore whether collagen synthesis by human lung fibroblasts is affected by muscarinic mechanisms. MRC-5 cells and primary human lung fibroblasts were cultured and [3H]-proline incorporation ([3H]-PI) into cellular proteins served as a measure of collagen synthesis. In MRC-5 cells [3H]-PI under control conditions amounted to 10,928±618 d.p.m. (n=22). The muscarinic receptor agonist carbachol concentration-dependently enhanced [3H]-PI, maximally by 60% at 10 µM (EC50: 220 nM). Likewise, 10 µM oxotremorine caused an increase by 69% (n=18). For comparison, TGF-β (5 ng/ml) caused an increase by 105% (n=9). Digestion of labelled proteins with collagenase II for 1 h released 80% of [3H]-proline from the protein fraction, confirming that total [3H]-PI largely reflects collagen synthesis. Furthermore, effects of carbachol on collagenase-sensitive [3H]-proline were similar to those on total [3H]-PI. The stimulatory effect of 10 µM carbachol on [3H]-PI was inhibited by tiotropium in a concentration-dependent manner (IC50: 110 pM). Pretreatment of cells with pertussis toxin (0.1 µg/ml) caused a slight reduction of [3H]-PI and prevented the stimulatory effect of 10 µM carbachol. The stimulatory effect of 10 µM carbachol on [3H]-PI was also prevented by the MAP kinase inhibitor PD 98059 (30 µM). Carbachol enhanced [3H]-PI in a concentration-dependent manner also in primary human lung fibroblasts, maximally by 47% at 10 µM (EC50: 165 nM) and 10 nM tiotropium prevented the effect of 10 µM carbachol. In conclusion, collagen synthesis in human lung fibroblasts is stimulated via Gi coupled muscarinic receptors.Funded By: Boehringer Ingelheim

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39

Expression of the orphan carrier SLC10A4 in cholinergic neurons of the peripheral and enteric nervous system, and lung and bladder epithelium

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The solute carrier family SLC10 was formerly referred to as the “sodium bile acid cotransporter family”. Indeed the founding members are bile acid carriers which belong to the class of sodium-coupled cotransporters at the plasma membrane. They maintain the enterohepatic circulation of bile acids between the liver via Ntcp (Slc10a1) and the ileum via Asbt (Slc10a2) and so participate in the homoeostasis of cholesterol. In 2004 we cloned a new member of this carrier family which is referred to as Slc10a4 (GenBank accession no. AY825923). Phylogenetic analysis revealed that Slc10a4 and Ntcp emerged from a common ancestor gene, but both proteins clearly differ in function. In contrast to Ntcp, Slc10a4 showed no transport activity for bile acids and sulfoconjugated steroid hormones when expressed in *Xenopus laevis* oocytes and HEK293 cells. Gene expression analysis by real-time quantitative PCR revealed that Slc10a4 expression is highest in the brain. High SLC10A4 expression was also detected in human digestive organs pointing to an additional role of this protein outside the brain. By applying a polyclonal rabbit antibody directed against a C-terminal epitope of the rat P4 protein we localized the Slc10a4 protein in cholinergic neurons of the rat central nervous system. Additionally, we detected Slc10a4 expression in neurons and fibers of the myenteric plexus of the enteric nervous system. In addition Slc10a4 was found in lung and bladder epithelium. Although the functional properties of Slc10a4 are so far unknown, its distribution suggests any kind of carrier or regulatory function in cells of the neuronal and non-neuronal cholinergic system.

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40

α2-adrenoceptor subtypes modulate retinal angiogenesis and intraocular pressure

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α2-Adrenoceptor agonists are clinically used for a variety of therapeutic effects including hypotension, analgesia, sedation, increased sympathetic tone and glaucoma. Three subtypes of α2-adrenoceptors (α2A, α2B, α2C) have been cloned and mice with targeted deletions in the α2-adrenoceptor genes have been successfully applied to identify the subtype(s) involved in the biological and pharmacological functions of these receptors. However, the subtype(s) involved in the α2-mediated reduction in intraocular pressure and the acute and chronic effects on the retinal vasculature have not been identified, previously. Thus, we used mice carrying targeted deletions in the α2-adrenoceptor genes to search for the subtypes involved in these functions. In order to determine intraocular pressure, mice were anesthetized with ketamine and the ocular pressure was determined before and 20 min after topical application of brimonidine using a TonoLab (Colonial Medical Supply Co., USA) ophthalmological tonometer. In wild-type C57BL/6 mice brimonidine significantly lowered intraocular pressure from 11.9 ± 0.2 mm Hg at baseline to 9.9 ± 0.1 mm Hg (p<0.05). Similar effects were observed in α2AC-deficient mice but not in animals lacking α2B-receptors. As α2B-receptors have previously been shown to be essential for vascular development in the placenta, we assessed the postnatal vascularization of the retina in mice. At four time points after birth (1-10 days), mice were perfused with FITC-dextran solution and retinal flat mounts were prepared. In α2B-/- mice, there was a significant delay of vascularisation in the retina at day 4 and day 7 compared to the wild-type group. In both groups, the vascular network reached the edge of the retina on postnatal day 14. On day 7, mRNA expression of sFLT1, VE-Cadherin and PECAM were significantly decreased and VEGFR-2 expression was increased in α2B-/- retina. Thus, α2B-adrenoceptors accelerate the development of the retinal vasculature and lower intraocular pressure in the murine eye.

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41

Diverse functions of α2-adrenergic auto- and heteroceptors

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Previous studies revealed distinct physiological functions of the α2-adrenoceptor subtypes (α2A,B,C-AR). Less is known whether these functions are mediated by autoreceptors, i.e. α2AR on adrenergic neurons, or heteroreceptors, i.e. α2AR on non-adrenergic neurons. To clarify this issue, we generated transgenic mice expressing α2AAR under control of the dopamine-β hydroxylase (DBH) promoter. These mice were backcrossed onto a α2AC-/- background. For our studies we compared the following mouse lines: 1) Mice with endogenous α2AAR (+/+), 2) transgenic mice which express α2AAR only in nor/adrenergic (i.e. DBH positive) cells (-/-TG), and 3) mice with a targeted disruption of α2AAR (-/-). The genetic model was confirmed by PCR genotyping, by quantitative RT-PCR and by autoradiography. A comparison of the results of superfusion experiments with [3H]noradrenaline and HPLC measurements of plasma noradrenaline levels revealed functional feedback inhibition of noradrenaline release in -/-TG like in +/+ mice. As a cardiac consequence of elevated noradrenaline plasma levels, -/- mice developed hypertension and heart hypertrophy. Interestingly α2-AR agonist mediated hypotension and pronounced bradycardia was present only in +/+ mice and this effect was atropine sensitive. The α2-agonist mediated analgesia in the tail flick test as well as hypothermia were observed in +/+ but not in -/- and -/-TG mice. Sedative and anesthetic sparing responses as measured by loss of righting reflex were also absent in -/- and -/-TG mice. In the forced swim test, the most commonly used test to assess antidepressant activity, the immobility time of -/- mice was reduced compared to +/+ and -/-TG mice under basal conditions. The antidepressant drugs reboxetine and mirtazapine reduced the immobility time in +/+ and -/-TG mice but did not elicit an additional effect in -/- mice. In conclusion, α2A autoreceptors are implicated in the antidepressant effect of the α2-antagonist mirtazapine and inhibit noradrenaline release from adrenergic neurons. α2A heteroreceptors mediate analgesia, hypothermia, sedation and anesthetic sparing effects as well as hypotension and bradycardia after application of α2-agonists.

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42

Phenotypic changes in the adrenal medulla of mice subjected to transverse aortic constriction

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Cardiac overstimulation by the sympathetic nervous system, reflected by elevated circulating levels of catecholamines, is a salient characteristic of heart failure (HF). In this study we have sought to characterize the phenotype of the adrenal medulla during

development of hypertrophy and HF in mice subjected to transverse aortic constriction (TAC).Morphometric analysis of histological sections of the adrenal gland, adrenaline (AD) levels in adrenal and plasma, basal hemodynamics and susceptibility to develop HF after 8 weeks of TAC were evaluated in wild type (WT) mice (C57BL/6) and in mice lacking the α2C-adrenoceptor (α2CKO). The aortic banding operation resulted in similar degrees of aortic stenosis and cardiac hypertrophy in WT and α2CKO mice. Total adrenal gland weight was significantly higher in mice subjected to TAC (WT: sham 0.05±0.01, TAC 0.18±0.02*; α2CKO: sham 0.04±0.01, TAC 0.13±0.02* mg/g body weight, *P<0.05 sham vs TAC), leading to an increased cortex volume (WT +40%; α2CKO +45%), but an even greater increase in adrenal medulla volume (WT +120%; α2CKO +149%). TAC increased adrenal AD content (WT: sham 18±1, TAC 26±2*; α2CKO: sham 14±3, TAC 34±8 nmol, *P<0.05 sham vs TAC) and AD plasma levels (WT: sham 4±1, TAC 21±6*; α2CKO: sham 9±2#, TAC 26±8* pmol/ml, *P<0.05 sham vs TAC, #P<0.05 WT vs α2CKO). In the adrenal medulla of α2CKO sham mice, GRK2 (G-protein receptor kinase type 2) mRNA levels (measured by real time PCR) were 4 fold higher compared to WT sham. In WT mice adrenal medulla TAC increased mRNA levels of GRK2 (8 fold), GRK3 (4 fold), tyrosine hydroxylase (4 fold) and β1- (8 fold) and β2- (4 fold) adrenoceptors.Our results indicate that the adrenal medulla demonstrates two distinct phenotypic changes: one in response to increased AD release (α2CKO) and the other to induction of cardiac hypertrophy (TAC). Deletion of α2C-adrenoceptors, which are important for the control of AD release, leads to an up-regulation of GRK2. In contrast, cardiac hypertrophy elicits an adrenal hypertrophic signal characterized by up-regulation of GRK3 and tyrosine hydroxylase and increased adrenal AD content and release.Supported by grant PTDC/SAU-FCF/66502/2006
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43

Lack of urethane to blunt the effect of agonists at three presynaptic receptors under in vitro conditions

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In pithed and atropinized rats anaesthetized with pentobarbitone 300 µmol/kg, the cannabinoid receptor agonists CP-55,940 1 µmol/kg and R-(+)-methanandamide 3 µmol/kg inhibited the electrically induced increase in heart rate by 43 (P<0.001) and 17 % (P<0.01), respectively; either effect was abolished by the CB1 receptor antagonist AM 251 3 µmol/kg. When urethane 14 mmol/kg was used as an anaesthetic instead, the inhibitory effect of CP-55940 was 23 % only (P<0.05) and that of R-(+)-methanandamide was no longer significant. These data are reminiscent of results by Armstrong et al. (J Pharmacol Exp Ther 223, 524, 1982), who found that α2-adrenoceptor-mediated cardiovascular responses in anaesthetized or pithed rats were less pronounced in urethane- when compared to pentobarbitone-treated animals. In an attempt to further elucidate the effect of urethane, we studied its effect on the basal and electrically evoked tritium overflow and its interaction with agonists at CB1, α2 and histamine H3 receptors in mouse tissues preincubated with 3H-noradrenaline. Urethane up to 10 mM did not affect basal and evoked tritium overflow from cortical slices and vas deferens pieces. In the absence of urethane, the evoked tritium overflow from vas deferens pieces was inhibited by the cannabinoid receptor agonist WIN 55,212-2 (maximum effect 65 %, pEC50 5.9) and the evoked overflow from cortical slices was inhibited by the α2-adrenoceptor agonist clonidine (maximum 90 %, pEC50 8.5) and by histamine (maximum 65 %, pEC50 6.9). Urethane 10 mM had virtually no influence on the concentration-response curve of each agonist (both with respect to the maximum effect and the pEC50). In conclusion, the urethane-related attenuation of the effects of cannabinoid and α2-adrenoceptor agonists in vivo cannot be explained by an antagonistic effect of this anaesthetic at either receptor in vitro.
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44

Phenylephrine activates both α1 and α2-adrenoceptors in porcine pulmonary veins

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Phenylephrine (PE) is commonly used as an agonist to characterize α1-adrenoceptor-mediated responses in various tissues. It was previously hypothesized that PE might also activate α2-adrenoceptors [Guimarães et al. (1987) Naunyn-Schmiedeberg's Arch Pharmacol 335:397–402]. In the present study we used a tissue bath protocol and second messenger experiments to substantiate the ability of PE to elicit α2-adrenoceptor-mediated effects. In tissue bath studies, vascular rings of porcine pulmonary veins (bioassay for α2C-adrenoceptors; Görnemann et al. (2007) Br J Pharmacol 151:186–194) were mounted in modified Krebs-Henseleit solution at 37oC for measurement of isometric force changes. Concentration-response curves (CRCs) to PE in the absence of antagonist were apparently monophasic. However, PE curves became biphasic in the presence of the selective α2-adrenoceptor antagonist rauwolscine. Whereas the first phase of contraction to PE remained nearly unaffected in the presence of rauwolscine (1–30 nM), the second phase was concentration-dependently shifted to the right. In the presence of the α1-adrenoceptor antagonist prazosin at a concentration (30 nM) that did not block α2-adrenoceptors, CRCs to PE were concentration-dependently shifted to the right by the selective α2-adrenoceptor antagonist yohimbine (3–100 nM; pA2 8.57 ± 0.05, slope of the Schild plot 0.83 ± 0.11, not significantly different from unity). In second messenger experiments, porcine pulmonary veins were treated with forskolin (5 µM), an activator of adenylyl cyclase, in modified Krebs-Henseleit solution at 37oC. The level of cAMP did not further increase after the addition of 100 µM PE (13.6 ± 2.3 vs. 16.5 ± 2.5 pmol/mg tissue; P>0.05) but was enhanced by 100 µM PE in the presence of 0.1 µM rauwolscine (35.9 ± 6.7 pmol/mg tissue; P<0.05). In is concluded that the widely used α1-adrenoceptor agonist PE is not selective for this type of receptors. PE activates both α1- and α2-adrenoceptors.
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45

Isolation and culture of primary equine tracheal epithelial cells

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Culture of airway epithelial cells is a useful model to investigate physiology of airway epithelia and airway disease mechanisms. In this report, we describe the first time the development of a procedure for the isolation, characterization and culture of primary equine tracheal epithelial cells. Epithelial cells were isolated from the trachea by exposing and stripping the mucosal epithelium from the adjacent connective tissue and smooth muscle. The tissue was minced and dissociated using 0.25% trypsin-EDTA solution for 2 hours at 37°C and 5% CO2 atmosphere. Cells were collected by sieving and centrifugation, and contaminating fibroblasts were removed by differential adhesion. Incubation of freshly isolated cells for 30 minutes in 5% CO2 and collection of unattached epithelial cells was the most effective for removal of contaminating fibroblasts. This procedure resulted in a typical yield of viable (95% by trypan blue exclusion) 107 cytokeratin-positive epithelial cells/g tracheal tissues with approximately 94% cytokeratin-positive cells of epithelial origin and only 9% of fibroblasts. Cells seeded at a density of 6.9 x 104 cells/cm2 in serum-free airway epithelial cell growth medium formed a monolayer near confluency within a week. Confluent monolayers were dissociated using dispase II and primary (P1) and secondary passages (P2) were successfully established in collagen free cell culture flasks and cells upon P2 could be maintained over 30 days. However, trypsin dissociated cells could not attach to culture flasks upon primary passage. Collagen coating of tissue culture flask was not required for cell adhesion. In our study high yield-protocol for isolation and culture of equine tracheal epithelial cells was established that can serve for in vitro / ex-vivo studies on pharmacological and toxicological targets relevant to (equine) airway diseases.
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46

Role of the myosin-light-chain-kinase for the α1-adrenergic positive inotropic effect

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Background: The mechanisms of the α1-adrenergic positive inotropic effect are enigmatic for more than 30 years. Pharmacological evidence recently suggested involvement of myosin-light-chain-kinase (MLCK), a calcium/calmodulin-dependent protein kinase, and increased MLC2 phosphorylation causing Ca2+-sensitization of myofilaments. Given that this conclusion based exclusively on potentially nonspecific components such as wortmannin and ML-7, the present study pursued a molecular approach to test this hypothesis. Methods: Western Blot and quantitative RT-PCR were used to detect the known MLCK isoforms in mouse heart and neonatal rat cardiac myocytes (NRCM). Engineered heart tissue (EHT) were generated from NRCM to study the consequences of MLCK knockdown on cardiac contractility. Knockdown was performed with lentivirus encoding shRNA against the 130 kDa smMLCK isoform or a non-target control. EHTs were infected on day 0 and subjected to isometric force measurements 12-14 days later. Force was measured under basal conditions and under cumulative concentrations of the α1-adrenoceptor agonist phenylephrine in the presence of nadolol and a final high concentration of the β-adrenoceptor agonist isoprenaline. Changes in MLC2-protein level and –phosphorylation were analyzed in shock-frozen EHTs by Western Blot. Results: Western Blots and qRT-PCR analyses revealed the 130 kDa smMLCK to be the most prominent isoform in mouse heart and NRCM/EHTs. In mouse left atrium transcript levels of 130 kDa smMLCK, 220 kDa smMLCK and skeletal MLCK amounted to 77%, 19% and 4%, respectively. Knockdown of 130 kDa smMLCK in NRCM and EHTs amounted to ~50% on mRNA and protein level, the lentivirus encoding the non-target shRNA had no effect. Knockdown of 130 kDa smMLCK was accompanied by a massive decrease of basal force and phenylephrine-mediated positive inotropic response. Unexpectedly, the isoprenaline response was also almost abolished. MLCK 130 knockdown caused a drastic reduction of MLC2-protein level and –phosphorylation. Summary and conclusion: The 130 kDa smMLCK was found to be the major of the known MLCK isoforms in mouse heart. Knockdown of this isoform in EHTs caused a drastic and global effect on force of contraction, suggesting that this enzyme could play a more general role in cardiac myocytes than anticipated. Model-specific effects have to be considered.
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47

COMT inhibition and melanin decrease by tolcapone in SK-mel melanocytes

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Melanin synthesis in melanocytes includes several substrates containing a catechol moiety which can be O-methylated. It has been suggested that catechol-O-methyl transferase (COMT) activity has a protective role in melanocytes resulting from O-methylation of the reactive dihydroxyindolic intermediates of melanogenesis leaking from melanogenic compartments.In the present study, COMT activity and melanin synthesis were measured in cultured human melanoma cells (SK-mel). COMT activity was evaluated in homogenates of SK-mel cells using adrenaline as substrate. Assay of the O-methylated product of adrenaline, metanephrine, was done by HPLC-EC. Melanin content was determined with a spectrophotometer at 420 nm. Experiments were done in the absence and in the presence of tolcapone (10-100 nM), a potent COMT-inhibitor. SK-mel cells were treated for 24 hours with tolcapone (10-100 nM) before melanin quantification. The kinetic parameters, Vmax an Km found for COMT activity in SK-mel cells were 15.36±0.32 nmol mg-1 protein h-1 (arithmetic mean±SEM, n=5) and 1.1(0.94;1.13) µM (geometric mean and 95% confidence limits, n=5), respectively. The IC50 value of tolcapone at inhibiting COMT in melanocytes was 4.3(2.8; 6.4) nM (n=5). Tolcapone caused a concentration-dependent decrease of the melanin content in SK-mel cells. The maximal effect of tolcapone was higher than that of the prototypic inhibitors of melanogenesis hydroquinone and kojic acid. Melanin concentration in SK-mel cells in the absence (control) and in the presence of 100 nM tolcapone, 363 µM hydroquinone or 700 µM kojic acid was, respectively, 36.2±0.7, 20.1±0.8, 24.3±2.1, 28.3±0.9 µg ml-1 (n=5, each). The results show that SK-mel cells

have a high COMT activity, which is highly sensitive to inhibition by tolcapone at low concentrations. At these concentrations tolcapone also causes a marked reduction in the melanin content of melanocytes. Vieira-Coelho MA, Soares-da-Silva P (1996) Ontogenic aspects of liver and kidney catechol-O-methyltransferase sensitivity to tolcapone. Br J Pharmacol 117:516-520

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48

Functional characterization of naturally occuring variants of the human 5-HT3B receptor subunit

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Within the family of serotonin receptors the 5-HT3 receptor, which is composed of five subunits, is the only ligand-gated ion channel. 5-HT3 receptor antagonists are effective in the treatment of chemotherapy-induced emesis and irritable bowel syndrome. Furthermore, 5-HT3 receptors are involved in cognitive processes, the mediation of pain and psychiatric diseases. Characterization of variants in HTR3 genes may help to elucidate the role of 5-HT3 receptors in the mentioned pathological states and to find more effective therapies.To date five 5-HT3 receptor subunit genes have been cloned (A-E) and their proteins have been functionally characterized.The 5-HT3B subunit has been shown to modulate the pharmacological and biophysical characteristics of the receptor when co-expressed with the 5-HT3A subunit. Thus, variants of the HTR3B gene could influence the function of heteromeric 5-HT3A/B receptors and may have and impact on gene-related psychiatric diseases. Two frequent and two rare HTR3B variants were characterized in HEK293 cells transiently co-transfected with 5-HT3B and 5-HT3A cDNAs. For functional characterization a recently established assay, based on aequorin bioluminescence upon Ca2+ influx through the 5-HT3 channel pore, was used (Walstab et al. [2007] Anal Biochem 382:185-192). Radioligand binding studies with the 5-HT3 receptor antagonist [3H]GR65630 were carried out to determine the expression levels of the 5-HT3B subunit containing complexes. 5-HT exhibited different efficacies at nearly all characterized variant receptors that cannot in all cases be explained by altered receptor densities on the cell membrane. Only one of the variant receptors showed a different concentration-response relationship for 5-HT compared to the wildtype 5-HT3A/B receptor.In conclusion, some of the examined 5-HT3B subunit variants significantly altered the characteristics of the heteromeric 5-HT3A/B receptor which is a further step towards the enlightenment of the role for 5-HT3 receptors in gene-related diseases.

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49

Effect of major constitutents of ginger oil on the 5-HT3 receptor induced [14C]guanidinium-influx into N1E-115 cells

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Ginger oil was shown to have antagonist properties on the 5-HT3 receptor channel system. Depending on the origin, extraction and storage conditions of the volatile oil, the content of sesquiterpenes and of citral may differ. The aim of this study was to elucidate the importance of these constituents for the effect of the volatile oil on 5-HT3 receptors.Ginger oil containing 79.5 % sesquiterpenes albeit no citral, was obtained by steam distillation. The sesquiterpenes ar-curcumene [1], β-sesquiphellandrene [2] and β-bisabolene / α-farnesene [3] were isolated by flash-chromatography, FCPC and preparative HPLC. α-zingiberene [4] was isolated by selectively forming a Diels-Alder adduct which was purified by flash-chromatography and hydrolyzed to reconstitute pure α-zingiberene. N1E-115 mouse neuroblastoma cells were preincubated for 20 min with the compounds to be tested. 100 μM serotonin and 5 μM [14C]guanidinium were added and the incubation was terminated after 2.5 min by rapidly washing. Cells were dissolved and the content of [14C]guanidinium as a marker for ion influx was determined by liquid scintillation counting.The sesquiterpenes inhibited the serotonin induced [14C]guanidinium influx in a concentration dependent manner, except [4] which showed agonist properties. The order of inhibitory potency was [1] > [2] ≈ [3]. The examination of a mixture of all sesquiterpenes in concentrations equivalent to the amount in the volatile oil revealed that the effect of the oil is not mainly caused by the pure sesquiterpene fraction. Citral potently inhibited the [14C]guanidinium influx and the addition of 20 % citral to the volatile oil enhanced the effect of the native volatile oil.In conclusion: the main components contribute to, but do not completely cause the effect of ginger oil on 5-HT3 receptors. Also minor constituents and synergistic effects have to be considered.

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50

Potentiation of NMDA- but not AMPA-evoked currents by the excitatory amino acid uptake inhibitor L-trans-2,4-PDC in layer V pyramidal neurons of the rat prefrontal cortex

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Glutamate, the major excitatory transmitter of the CNS is inactivated by neuronal and glial uptake mechanisms. L-trans-pyrrolidine-2,4-dicarboxylic acid (PDC) is a preferential blocker of the glial glutamate uptake transporter in the prefrontal cortex. The whole-cell variant of the patch-clamp technique was used to record current responses from layer V pyramidal neurons of the rat prefrontal cortex. In the absence of external Mg2+, PDC caused a slowly developing inward current response, which was abolished both by the NMDA receptor antagonist D(-)-2-amino-5-phosphonopentanoic acid (AP5) and Mg2+. The AMPA receptor antagonist 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) failed to alter this effect. Tetrodotoxin did not influence the mean current response to PDC although it depressed the frequency of spontaneous excitatory postsynaptic currents which reflect the basal exocytotic release of glutamate. N-methyl-D-aspartate (NMDA)-induced currents were potentiated by PDC both in the presence and absence of external Mg2+. By contrast, PDC did not modulate AMPA-evoked currents. Inclusion of GDP-b-S, that inhibits G protein-coupled intracellular reactions, into the pipette solution, abolished the effect of PDC on NMDA currents, suggesting the involvement of a postsynaptic mechanism via a G protein-coupled receptor. The potentiation of NMDA currents persisted in the presence of the metabotropic glutamate receptor (mGluR) group I antagonist (R,S)-1-aminoindan-1,5-dicarboxylic acid (AIDA), but was prevented by the mGluR group II antagonist (2S)-2-amino-2-[(1S,2S)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY341495). Moreover, the mGluR group II agonist (1R,4R,5S,6R)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268), but not the mGluR group I agonist (S)-3,5-dihydroxyphenylglycine (DHPG) mimicked the effect of PDC on NMDA-induced currents. We propose that PDC increases ambient glutamate concentrations by inhibition of glutamate uptake. This glutamate may then activate postsynaptically localized mGluR group II that, in turn, positively interact with NMDA receptors.

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51

A2B adenosine receptors mediate an inhibition of ERK1/2 phosphorylation in the breast cancer cell line MDA-MB-231

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The estrogen receptor-negative breast cancer cell line MDA-MB-231 expresses A2B adenosine receptors as the sole adenosine receptor subtype at remarkably high levels (1). In addition to a stimulation of adenylyl cyclase it was shown that the A2B receptor mediates a Ca2+ signal in these cells, most likely via activation of Gq. Attempts to detect a potential A2B-mediated MAP-kinase activation failed due to very high basal MAPK activity which seems to be maximal as it can not be stimulated further with FCS or EGF. This high basal activity in MDA-MB-231 cells was dependent on src and her2 as inhibitors for both proteins abolished the high basal ERK1/2 phosphorylation almost completely. Interestingly, we observed that stimulation of A2B adenosine receptors with NECA caused a time-dependent inhibition of ERK1/2 phosphorylation as well. This inhibition was antagonized with DPCPX confirming that its was indeed adenosine receptor-mediated. A similar inhibitory effect on basal ERK1/2 phosphorylation was detected by forskolin-stimulated activation of adenylyl cyclase suggesting the involvement of cAMP in this effect. However, it was independent of the PKA pathway as the inhibiton persisited in the presence of PKA inhibitors. The presence of U73221 abolished the NECA effect suggesting a role for PLC for the A2B receptor-mediated inhibition of ERK1/2 phosphorylation. The modulation of MAP-kinase activity suggests that A2B adenosine receptors may be a target to control breast cancer cell growth and proliferation.(1) M. Panjehpour, M. Castro, K.-N. Klotz (2005) The human breast cell line MDA-MB231 expresses endogenous A2B adenosine receptors mediating a Ca2+ signal. Br. J. Pharmacol. 145:211-218

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52

Characterization of the ligand-binding site of non-nucleotide antagonists at the human platelet P2Y12 receptor

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The P2Y12-receptor plays a crucial role in platelet aggregation. Previously, we have identified a number of amino acid residues of the human P2Y12-receptor including S101 in transmembrane region 3 (TM3), R256 in TM6 and K280 in TM7 which are involved in binding of nucleotide agonists and antagonists to the receptor. In the present study, we determined potencies of non-nucleotide antagonists at recombinant wild type (WT) and mutant receptors. Receptor function was assessed by measuring the cAMP response element (CRE) directed luciferase expression in Chinese Hamster Ovary cells. The cellular cAMP production was accelerated by forskolin. In cells expressing WT-P2Y12-receptors, the agonist 2-methylthio-ADP inhibited the CRE-dependent luciferase expression with an EC50 concentration of about 1 nM. The anthraquinone derivative reactive blue-2 shifted the concentration-response-curve to the right with an apparent pKB-value of 7.4. In cells expressing S101A mutant receptors, reactive blue-2 had a similar potency (pKB 7.6). In contrast, in cells expressing R256A-mutant receptors the potency of reactive blue-2 was markedly decreased (pKB 5.9). The structurally related compound reactive red-2 had similar potencies at WT-receptors (pKB 6.5), R256A mutant receptors (pKB 6.4) and S101A mutant receptors (pKB 6.6). The same was true for the non-nucleotide P2Y-receptor antagonist suramin (WT pKB 5.5, R256A pKB 5.2, S101A pKB 5.5) and its analogue Bay u9421 (sym-bis(benzoyl-1-naphthylamino-3,6-bisulfonate)chlorotriazine; WT pKB 5.9, R256A pKB 5.9, S101A pKB 6.2). These data show that, of the non-nucleotide antagonists tested, reactive blue-2 had the highest potency (pKB 7.4). Moreover, the results indicate that reactive blue-2 interacts with the residue R256 in TM6 which has been shown to be involved in agonist binding. Hence, reactive blue-2 may serve as a chemical lead for the development of new potent non-nucleotide platelet aggregation blockers.

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53

Early effects of ADP and adenosine on gene expresion in human coronary artery vascular smooth muscle cells

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Adenine nucleotides play important roles in the regulation of the vascular tone. Less is known about the induction of gene expression by ATP and ADP as compared with other endogenous agonists acting on vascular smooth muscle cells. For our experiments we used human coronary artery smooth muscle cells (HCASMCs) in primary culture. It turned out that the endogenous agonists ATP and ADP do not act on HCASMCs via P2Y-receptors for nucleotides, but - after dephosphorylation to adenosine or AMP - via adenosine receptors. The effects of ADP and adenosine on gene expression were remarkably strong and appeared completely cAMP-mediated because they were fully mimicked by forskolin. In agreement with this view, adenosine also increased luciferase expression in HCASMCs transiently transfected with the pCRE (cAMP response element)-luc vector. PMA (phorbol 12-myristate 13-acetate) induced a different (only

partially overlapping) pattern of early gene expression. The effect of ADP was decreased by xanthine antagonists, but not by P2Y-receptor antagonists including MRS2179 (2´-deoxy-N6-methyladenosine-3´,5´-bisphosphate), indicating an involvement of adenosine receptors, most likely of the A2b subtype. Interestingly, noradrenaline behaved virtually identical to forskolin and adenosine. Other endogenous agonists which are known to act via the phosphoinositide pathway (angiotensin II, vasopressin and bradykinin) induced only a very weak immediate-early gene expression. Therefore, adenosine, along with noradrenaline, was identified as the strongest endogenous GPCR-mediated inductor of gene expression in HCASMCs. One of the most intensely adenosine-induced genes was NR4A1 (also called TR3), for which an important role in atherogenesis was suggested earlier (Arkenbout et al. 2002, Circulation 106;1530-1535).

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54

Evidence of various functional P2Y as well as P2X7 nucleotide receptors in cultured cerebrocortical astrocytes of the rat

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In recent years, nucleotides have been identified as extracellular signaling molecules, involved in multiple functions of astrocytes including neuronal-glial network communication. Experiments were conducted to characterize the expression of functional P2Y/P2X nucleotide receptors in astrocytes of mixed cerebrocortical cell cultures. The vast majority of the glial cells were GFAP-positive and these astrocyte-like cells express predominantly P2Y1,2 as well as P2X4,6,7 receptor subtypes. Weak immunoreactivity was also found for P2Y4,6,13,14 and P2X5 receptors. In Ca2+ microfluorimetric studies, pressure application of ATP as well as a wide range of other P2 receptor active nucleotides caused a concentration-dependent increase in intracellular Ca2+ concentration ([Ca2+]i). In contrast, α,β-meATP, a P2X1,3 receptor agonist, induced no Ca2+ transients and high extracellular K+ (50 mM) was also essentially ineffective. The non-selective P2 receptor antagonist PPADS and the selective P2Y1 receptor antagonist MRS2179 inhibited the ATP-induced Ca2+ response, whereas the selective P2Y12 receptor antagonist AR-C69931MX (Cangrelor) showed no effect. Experiments with Ca2+-free solution and cyclopiazonic acid, an inhibitor of the endoplasmic Ca2+-ATPase, revealed that the Ca2+ response to most nucleotides, except for ATP and 2´,3´-O-(benzoyl-4-benzoyl)-ATP (BzATP), arose primarily by the release of Ca2+ from intracellular stores. Cross-desensitization experiments indicated the co-existence of various P2Y receptors. Prolonged superfusion with high ATP or BzATP (300 µM each for 60 min) did not induce pore formation or marked cell death. In whole-cell patch-clamp recordings, astroglial cells responded to BzATP, ATP and 2MeSATP with inward currents; α,βmeATP, ADPβS, UTP, UDP and UDP-glucose were ineffective. The ATP (BzATP)-evoked inward currents showed properties characteristic for P2X7 receptors. These currents were more potently activated by BzATP than by ATP, amplified in Ca2+/Mg2+-free solution, and inhibited by oxidized ATP, brilliant blue G, Zn2+ and calmidazolium. Suramin or ivermectin were ineffective. Taken together, our findings provide direct evidence that astrocytes in mixed cell cultures express various functional P2Y receptors and most likely also the P2X7 subtype, which are involved in modulating [Ca2+]i and may play an important role in cell-to-cell signaling.

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55

P2Y1 receptor-mediated increase of intracellular Ca2+ occurs independently of proliferation and dopaminergic differentiation in human neural precursor cells

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When combined with the mitogens epidermal growth factor and fibroblast growth factor 2, UTP but not ATP or ADP boosted proliferation of human mesencephalic neural stem/precursor cells (hmNPCs). UTP-induced proliferation was abrogated by the preferential P2Y receptor blocker pyridoxal phosphate-6-azophenyl-2',4'-disulphonic aicid (PPADS). UTP also stimulated dopaminergic differentiation as indicated by an increased number of tyrosine hydroxylase-positive cells. We aimed at elucidating whether the increase of intracellular Ca2+ ([Ca2+]i) is involved in the effect of UTP. Whole-cell patch-clamp recordings documented in a subpopulation of cells inward current responses to AMPA and muscimol, but not to ATP or UTP. By contrast, various nucleotides induced [Ca2+]i transients with the rank order of potency, ATP > ADP > UTP > UDP, with an about 100 fold difference between the IC50 values of ATP and UTP. The omission of external Ca2+ only slightly depressed, whereas cyclopiazonic acid markedly decreased the effect of ATP and especially that of UTP. The former manipulation is expected to interfere with the entry of Ca2+ through the plasma membrane, whereas the latter one is known to deplete intracellular Ca2+ stores. Hence, ATP/UTP may release Ca2+ from its storage sites via the activation of a certain subtype of P2Y receptor. The use of pharmacological antagonists (MRS 2179: PPADS, P2Y1; AR-C 6993: P2Y12,13) identified the ATP- and UTP-sensitive receptors as belonging to the P2Y1 subtype. Long-lasting superfusion with subthreshold ATP concentrations led to propagated Ca2+ oscillations, indicating an extracellular signalling function of this nucleotide. In conclusion, UTP in high concentrations may non-selectively stimulate the ATP/ADP-sensitive P2Y1 receptor in hmNPCs. However, this effect is certainly not related to the promotion of proliferation/differentiation, which has been shown to be mediated by the extracellular signal-regulated kinase (ERK) pathway. In fact, UTP unequivocally stimulated the ERK1/2 phosphorylation as well as dopaminergic differentiation, which were both inhibited by 1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio] butadiene (U0126), a selective ERK kinase inhibitor.

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56

Role of the C terminal tail of the P2X7 receptor in receptor trafficking and function

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P2X7 receptors belong to seven member P2X receptor family of ATP-gated cation channels each having intracellular amino and carboxyl termini and two transmembrane segments connected by a large ectodomain. Activation of the P2X7 receptor has been implicated in inflammatory responses resulting from pro-inflammatory cytokine release, cell proliferation, and apoptosis. A special feature of the human P2X7 receptor (hP2X7R) is the large C terminal endodomain of about 240 amino acids. This C terminal endodomain has been suggested to be involved in the induction of a cytolytic pore permeable to large organic cations after prolonged application of high ATP concentrations. Additional roles distinct from cytolytic pore formation that have been assigned to the carboxyl tail include regulation of the trafficking of the hP2X7R to the plasma membrane. We observed previously that C terminally truncated hP2X7R mutants mediated significantly smaller current amplitudes in response to extracellular ATP than the wild type hP2X7R. To identify the underlying nature of this current reduction, we assessed the cell surface expression of wild type and truncated hP2X7R mutants in X. laevis oocytes. Deletion of a few residues from the extreme C terminal end abolished surface expression, whereas larger truncations of up to 187 residues facilitated surface expression. Co-expression of a carboxyl tail domain comprising residues 434-595 of the full-length hP2X7R with tailless hP2X7R mutants significantly reduced the surface expression of two tailless hP2X7R mutants (residues 1-436 and 1-505), but stimulated the current amplitudes to near wild-type hP2X7R levels. By chemical cross-linking, we could demonstrate that the co-expressed C terminal tail domain interacted physically with tailless hP2X7R truncation mutants. Co-expressed tailless hP2X7R mutants assembled with full length hP2X7R to non-functional heterotrimeric receptors in a dominant-negative manner. We conclude from these data that the C terminal tail is not only important for hP2X7R trafficking, but also for hP2X7R gating.

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56a

The pH sensitivity of the hP2X3 receptor is regulated by N-linked glycosylation

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ATP-gated, cation-permeable P2X receptor-channels form a family of at least seven subunits, referred to as P2X1 through P2X7. The subunits have two transmembrane α-helices, a large extracellular loop containing the nucleotide binding domains (NBD-1, -2, -3, -4), as well as intracellular N and C terminal tails. Homomeric receptors oc-cur as stable trimers of three identical subunits. All P2X subunits have consensus sequences for N-linked glycosylation, some of which are necessary for trafficking to the cell surface. P2X3 receptors are localized at the peripheral terminals of small-diameter dorsal root ganglion (DRG) neurons and have been shown to be involved in pain sensation. We have recorded by the whole-cell patch-clamp technique current responses to the P2X3 receptor agonist α,β-methylene ATP (α,β-meATP) in HEK293 cells transiently transfected with the human (h) P2X3 receptor or its mutants. We found that neither acidification (pH=6.5) nor alkalinisation (pH=8.0) of the extracellu-lar solution from a normal pH of 7.4 altered the EC50 value of α,β-meATP at wild-type (WT) hP2X3 receptors. At the same time, the maxima of the concentration-response curves of α,β-meATP were gradually depressed by consecutively decreasing the pH from 8.0 to 6.5. The WT P2X3 subunit sequence has four glycosylation sites contain-ing conserved Asn residues (N139, N170, N194, N290). We successively mutated these residues to Asp, in order to clarify their possible role in the pH-induced regula-tion of P2X3 current amplitudes. Pronounced changes occurred only when the Asn-290 residue was mutated in the immediate neighbourhood of NBD-4. Substitution of Asn by the neutral Ala (N290A) or Thr (N290T) resulted in a loss of the pH-induced modulatory effect. By contrast, the replacement of Asn by the negatively charged Asp (N290D) led to a reversed pH sensitivity; under these conditions, the maximum of the concentration-response curve of α,β-meATP was the largest at a pH of 6.5 and de-creased at both lower and higher pH values. In conclusion, we suggest that glycosy-lation at N290 is necessary for a proper regulation of P2X3 receptor function by ex-tracellular pH.

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57

Integrins mediate ERK1/2 activation by delta-opioid receptors in NG108-15 cells

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Stimulation of δ-opioid receptors (DOR) has been shown recently to enhance integrin-dependent adhesion of monocytes to fibronectin. To examine whether a similar opioid effect occurs in neuronal cells, we determined DOR-mediated integrin-dependent adhesion in neuroblastoma x glioma hybrid (NG108-15) cells. Indeed, incubation with [D-Ala, D-Leu] Enkephalin (DADLE) and etorphine both enhances binding of NG108-15 cells to fibronectin. This effect is blocked by the opioid receptor antagonist naloxone as well as by integrin blocking RGDT peptides. Moreover, opioid-triggered binding of NG108-15 cells to fibronectin is also prevented by inhibitors of phospholipase C (U73122, Eto-18-OCH3), suggesting that PLC regulates cell adherence after DOR activation. An increase of integrin-mediated cell adhesion has been shown to be associated with activation of ERK/MAP kinase signalling. Thus, we further examined whether the control of ERK activity by DORs depends on integrin-mediated adherence of NG108-15 cells. Whereas treatment of adherent cells with DADLE and etorphine results in activation of ERK1/2, inhibition of integrins by RGDT peptides and incubation of suspended NG108-15 cells with opioids prevents DOR-mediated ERK signalling. Inhibition of opioid-triggered cell adhesion by the PLC blocker also interferes with stimulation of ERK1/2. Although ERK activation by an integrin-dependent pathway implicates stimulation of receptor tyrosine kinases, such as epidermal growth factor receptors (EGFR), incubation of NG108-15 cells with EGFR inhibitors AG1478 and BPIQ-II failed to affect opioid-induced ERK activation. In contrast, blockade of neuronal receptor tyrosine kinase TrkA by AG879 abolishes MAP kinase activation by DOR. These findings demonstrate that stimulation of DOR triggers integrin-dependent

adhesion of NG108-15 cells, which brings about activation of ERK/MAP kinases by cross-activation of TrkA receptors.

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58

Pituitary adenylate cyclase-activating polypeptide (PACAP) is up-regulated after cerebral ischemia and protects neurons from ischemic damage

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The protective effect of pituitary adenylate cyclase-activating polypeptide (PACAP) in stroke models is poorly understood. We studied patterns of PACAP and the PACAP-selective receptor PAC1 after middle cerebral artery occlusion and neuroprotection by PACAP in cortical cultures exposed to oxygen/glucose deprivation (OGD). Within hours, focal ischemia caused a massive, NMDA receptor (NMDAR)-dependent up-regulation of PACAP in cortical pyramidal cells. PAC1 mRNA showed ubiquitous expression in neurons and astrocytes with minor changes after ischemia. In cultured cortical neurons PACAP27 strongly activated Erk1/2 at low and p38 MAP kinase at higher nanomolar concentrations via PAC1. In astrocyte cultures, effects of PACAP27 on Erk1/2 and p38 were weak. During OGD, neurons showed severely reduced Erk1/2 activity and dephosphorylation of Erk1/2-regulated Ser112 of pro-apoptotic Bad. PACAP27 stimulation counteracted Erk1/2 inactivation and Bad dephosphorylation during short-term OGD but was ineffective after expanded OGD. Consistently, PACAP27 caused MEK-dependent neuroprotection during mild but not severe hypoxic/ischemic stress. While PACAP27 protected neurons at 1-5 nmol/L, full PAC1 activation by 100 nmol/L PACAP exaggerated hypoxic/ischemic damage. PACAP27 stimulation of astrocytes increased the production of Akt-activating factors and conferred ischemic tolerance to neurons. Thus, ischemia-induced PACAP may act via neuronal and astroglial PAC1. We propose that PACAP confers direct protection to neurons by preventing ischemic shut-off of Erk1/2 signaling via neuronal PAC1. Indirect neuroprotection may arise via astroglial PAC1 and the stimulation of astroglial production of neuroprotective factors acting via the Akt pathway.

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59

Endogenous opioids inhibit focal ischemia-induced hippocampal neurogenesis via the mu opioid receptor

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It is established that adult hippocampal neurogenesis is regulated by pathological and physiological stimuli including brain lesion, environmental complexity, learning and mental disorders. Little is known about factors that regulate adaptive changes in neurogenesis. Using mu opioid receptor (MOP) knockout mice we asked whether endogenous opioids influence hippocampal neurogenesis after focal ischemia. Permanent middle cerebral artery occlusion (MCAO) caused similar corticostriatal infarcts in MOP knockout and wildtype mice. Analyses of BrdU/DCX-colabeled cells in the granule cell layer 14 d after MCAO showed that ischemic knockouts contained more immature neurons generated during days 9 - 11 than wildtypes. After 29 d, similar amounts of BrdU/NeuN-labeled cells were found in ischemic knockout and wildtype mice, suggesting that granule cells that were formed in excess during days 9 - 11 in the knockouts were eliminated until day 29. Neurogenesis was similar in knockout and wildtype mice subjected to sham operation. In addition to the transient increase in neurogenesis, MCAO caused a transient upregulation of pre-prodynorphin and pre-proenkephalin mRNA expression in the granule cell layer. Our findings suggest that signaling of endogenous opioids via the MOP is activated and decreases enhanced neurogenesis after ischemic corticostriatal lesions.

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60

Regulation of TRPV1 activity by sensory neuron-specific G protein-coupled receptor-4

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SNSR (sensory neuron-specific G protein-coupled receptors) have recently been described as a new family within the superfamily of GPCR. SNSR are characterized by their exclusive expression in primary sensory neurons. Although pain enhancing effects of SNSR selective peptides (BAM8-22) in rats have been established, the molecular mechanism leading to SNSR-mediated regulation of nociception remain completely unknown. Thus, we set out to explore putative downstream effectors of SNSR signaling in HEK293 cells stably expressing SNSR-4 (HEK293-SNSR-4 cells). In these cells, we found that BAM8-22 exclusively activates Gαq proteins, as indicated by the blocking effect of specific antibodies against Gαq proteins on agonist-induced GTPγ35S incorporation. In line with this notion, BAM8-22-induced calcium release or IPn formation was insensitive to the Gαi/o specific inhibitor pertussis toxin. Next, we explored a putative SNSR-mediated regulation of proteins known to be involved in nociception and that are regulated by the Gαq/PKC/PLC pathway. The heat- and proton-sensitive vanilloid(TRPV1) receptor belongs to the family of transient receptor potential (TRP) channels and has been shown to be co-expressed with SNSR-4 in rat dorsal root ganglia. Using a HEK293 cell line stably co-expressing SNSR-4 and TRPV1 (HEK293-SNSR-4/TRPV1 cells), we could demonstrate that the BAM8-22-induced calcium increases in these cells are significantly prolonged when compared to the corresponding signal observed in HEK293-SNSR-4 cells, indicating a direct activation of the TRPV1 channel by SNSR-4. Fura-2-quenching experiments with extracellular manganese provided further evidence for the direct activation of TRPV1 by SNSR-4 and additionally revealed that SNSR-4 also sensitizes ligand-induced TRPV1 activation when the TRPV1 specific agonist capsaicin was used. In conclusion, we report here first data on the regulation of the TRPV1 cation channel by SNSR-4, which could be a new signaling pathway involved in nociception in mammals.

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61

Structural features for natriuretic peptide degradation by neutral endopeptidase

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Natriuretic peptides like atrial (ANP), B-type (BNP) and C-type natriuretic peptide (CNP) are cyclic peptide hormones with relevance to cardiovascular, endocrine and renal homeostasis. In all three peptides an intact 17 amino acid disulfide-linked loop is the essential structural prerequisite for their biological activity. Two different mechanisms are involved in the clearance of the NP: (1) binding to specific receptors (C-type) with the subsequent internalization and (2) degradation by extracellular peptidases. The neutral endopeptidase (NEP), a membrane bound type-II metallopeptidase, is generally regarded to be the main enzyme which initially catabolizes natriuretic peptides by cleavage within the loop at the Cys-Phe bond. In this study we compared the degradation of various mature, truncated, and recombinant natriuretic peptides by NEP. The degradation was clearly dependent on the length of N- or C-terminal peptide tails, but also on distinct sequence differences within the essential loop structure of the natriuretic peptides. Based on these findings, we developed a model for the interaction of NEP and natriuretic peptides that enables new insights in the mode of action and prediction of substrates of NEP. Features which support the degradability of peptides by NEP refer to (a) the size of substrates, and (b) a “recognition site” in the interior cave of NEP which interacts with the substrate, fixes it, and alleviates the catalytic attack of NEP. These structural features give rise to the development of more potent synthetic natriuretic peptides with reduced degradability by endogenous peptidases.

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62

Direct stimulation of receptor-controlled phospholipase D1 by phospho-cofilin

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The activity state of cofilin, which controls actin dynamics, is driven by a phosphorylation-dephosphorylation cycle. Phosphorylation of cofilin by LIM-kinases results in its inactivation, a process supported by 14-3-3z and reversed by dephosphorylation by slingshot phosphatases. Here we report on a novel cellular function for the phosphorylation-dephosphorylation cycle of cofilin. We demonstrate that muscarinic receptor-mediated stimulation of phospholipase D1 (PLD1) is controlled by LIM-kinase, slingshot phosphatase as well as 14-3-3z and requires phosphorylatable cofilin. Cofilin directly and specifically interacts with PLD1 and upon phosphorylation by LIM-kinase1 stimulates PLD1 activity, an effect mimicked by phosphorylation-mimic cofilin mutants. The interaction of cofilin with PLD1 is under receptor control and encompasses a PLD1-specific fragment (aa 585-712). Expression of this fragment suppresses receptor-induced cofilin-PLD1 interaction as well as PLD stimulation and actin stress fibre formation. We report here on a novel molecular link between the actin cytoskeleton and PLD1, and provide evidence that inactive phospho-cofilin exerts an unexpected biological function. By its stimulation of PLD1, known to regulate many early and late cellular functions, from calcium mobilization, glucose transport, mitogenesis to apoptosis, phospho-cofilin is most likely an active signaling component, and may control essential cellular functions.

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63

Shift of spinal prostanoid synthesis and its functional consequences on nociceptive behaviour after mPGES-1-deletion

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Cyclooxygenase-2 (COX-2)-dependent prostaglandin (PG) E2 synthesis in the spinal cord plays a major role in the development of inflammatory hyperalgesia and allodynia. Microsomal PGE2 synthase-1 (mPGES-1) isomerizes COX-2-derived PGH2 to PGE2. Here, we evaluated the effect of mPGES-1-deficiency on the nociceptive behaviour in various models of nociception that depend on PGE2 synthesis. Surprisingly, in the COX-2-dependent zymosan-evoked hyperalgesia model, the nociceptive behaviour was not reduced in mPGES-1-deficient mice despite a marked decrease of the spinal PGE2 synthesis. Similarly, the nociceptive behaviour was unaltered in mPGES-1-deficient mice in the formalin test. Importantly, spinal cords and primary spinal cord cells derived from mPGES-1-deficient mice showed a redirection of the PGE2 synthesis to PGD2, PGF2a and 6-keto-PGF1a (stable metabolite of PGI2). Since the latter PGs serve also as mediators of nociception they may compensate the loss of PGE2 synthesis in mPGES-1-deficient mice.

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64

Decisive role of cyclooxygenase-2 and lipocalin-type prostaglandin D synthase in chemotherapeutics-induced apoptosis of human cervical carcinoma cells

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The role of cyclooxygenase-2 (COX-2) in cancer remains controversial. Using cervical carcinoma cells (HeLa), the present study investigates the involvement of COX-2 in apoptosis elicited by the chemotherapeutics paclitaxel, cisplatin and 5-fluorouracil. Each compound led to a profound induction of COX-2 expression and prostaglandin (PG) synthesis, accompanied by a substantial decrease of viability and enhanced apoptosis. Cells were significantly less sensitive to apoptotic death when either COX-2 expression or its activity was suppressed by small interfering (si) RNA and by the selective COX-2 inhibitor NS-398, respectively. Experiments performed to clarify how COX-2 leads to

apoptosis revealed a profound proapoptotic action of PGD2 and its dehydration product, 15-deoxy-D12,14 PGJ2 (15d-PGJ2). In line with these findings, chemotherapeutics-induced apoptosis was prevented by siRNA targeting lipocalin-type PGD synthase (L-PGDS), which catalyzes the isomerization of PGH2 to PGD2. Moreover, apoptosis by chemotherapeutics, PGD2 and 15d-PGJ2 was suppressed by the peroxisome proliferator-activated receptor γ (PPARγ) antagonist, GW-9662, or PPARγ siRNA. Finally, a COX-2-dependent apoptotic mechanism of all investigated chemotherapeutics was confirmed in human lung cancer cells (A549) as well as in another cervical carcinoma cell line (C33A). Collectively, this study suggests COX-2 induction and synthesis of L-PGDS-derived, PPARγ-activating PGs as a decisive target by which several chemotherapeutics induce apoptosis. COX-2 is therefore suspected to sensitize cancer cells to apoptotic death under certain circumstances, suggesting that COX-2 inhibition during cancer therapy could diminish its efficacy.Supported by DFG (SFB 539, TP BI.6) and Deutsche Krebshilfe e.V. Institute of Toxicology and Pharmacology, University of Rostock, Schillingallee 70, 18057 Rostock, Germany

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65

Induction of neuronal nitric oxide synthase during maturation of human dendritic cells

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Dendritic cells (DC) are the most potent antigen presenting cells and crucial mediators of immune defence and tolerance. Their specific function within the immune system depends on their stage of maturation and activation. Immune mediators and cytokines further influence the maturation and functions of DC. The inducible nitric oxide synthase (iNOS) has been shown to be important for maturation and function of mouse DC. However, little is known about the role of NOS in human DC. We thus investigated the expression of genes involved in the arginine-NO pathway in human DC generated from peripheral progenitor cells in the presence of IL-4 and GM-CSF (immature DC) and, thereafter, cultured in the presence of IL-1, IL-6, TNF-α and PGE2 for 2 days (mature DC). At the mRNA level, we found expression of endothelial NOS (eNOS) in immature DC, and a pronounced expression of neuronal NOS (nNOS) in mature DC. In contrast, no considerable expression of iNOS could be detected at any stage of DC maturation. The expression of the major arginine transporter (CAT-1) and of arginase I and II stayed the same during maturation. The induction of nNOS during DC maturation could also be demonstrated at the protein level. Inhibitors of NOS (L-NAME) or of the major NO target, soluble guanylate cyclase (ODQ) inhibited the maturation of DC as demonstrated by reduced expression of CD80, CD83, CD86 and HLA-DR. In addition, L-NAME and ODQ treatment of DCs altered DC-mediated T cell activation. Taken together, our data demonstrate nNOS induction during DC maturation and suggest that nNOS has an important role in the regulation of DC function.

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66

Regulation of pro- and anti-oxidative genes by resveratrol in the cardiovascular system

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Resveratrol, an important antioxidant found in grapes and wine, is likely to contribute to the potential of red wine in preventing human cardiovascular disease. In addition to its known (direct) antioxidant effect, we have found that resveratrol also regulates gene expression of pro-oxidant and anti-oxidant enzymes in human endothelial cells. NADPH oxidases (Nox) are the predominant producers of superoxide in the vasculature, whereas superoxide dismutase (SOD) and glutathione peroxidase 1 (GPx1) are the major enzymes responsible for the inactivation of superoxide and hydrogen peroxide, respectively. Incubation of human umbilical vein endothelial cells (HUVEC) and HUVEC-derived EA.hy 926 cells with resveratrol (1 to 60 micromol/L) for 3 to 48 hours resulted in a concentration- and time-dependent upregulation of GPx1 and SOD1 mRNA- (quantitative real-time PCR) and protein expression (Western blot). The same resveratrol regimen downregulated the expression of Nox1 and Nox4. Treatment of apolipoprotein E knockout mice with resveratrol (30 or 100 mg/kg) reduced superoxide production in the heat. The cardiac levels of (6R)-5,6,7,8-tetrahydro-L-biopterin, the cofactor of nitric oxide synthase which is sensitive to oxidation, were increased by resveratrol. Thus, in addition to its direct antioxidant properties, resveratrol also possesses antioxidant effects at the genomic level. Enhancement of anti-oxidant genes (such as SOD1 and GPx1) and suppression of pro-oxidant genes (such as NADPH oxidases) may be an important component of the vascular protective effect of resveratrol.

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67

Anti-atherosclerotic effects of small molecular weight compounds enhancing eNOS expression and preventing eNOS uncoupling

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Many cardiovascular diseases are associated with reduced levels of bioactive NO and an uncoupling of oxygen reduction from NO synthesis in endothelial NO synthase (eNOS uncoupling). In human endothelial EA.hy 926 cells, two small molecular-weight compounds with related structures, AVE9488 and AVE3085, enhanced eNOS promoter activity in a concentration-dependent manner; with the responsible cis-element localized within the proximal 263bp of the promoter region. RNA interference-mediated knockdown of the transcription factor Sp1 significantly reduced the basal activity of eNOS promoter, but did not prevent the transcription activation by the compounds. Enhanced transcription of eNOS by AVE9488 in primary human umbilical vein endothelial cells was associated with increased levels of eNOS mRNA and protein expression, as well as increased bradykinin-stimulated NO production. In both wild type C57BL/6J mice and apolipoprotein E-knockout (apoE-KO) mice, treatment with AVE9488 resulted in enhanced vascular eNOS expression. In apoE-KO mice, but not in eNOS-knockout mice, treatment with AVE9488 reduced cuff-induced neointima formation. A 12-week treatment with AVE9488 or AVE3085 reduced atherosclerotic plaque formation in apoE-KO mice, but not in apoE/eNOS-double knockout mice. Aortas from apoE-KO mice showed a significant generation of reactive oxygen species. This was partly prevented by NOS inhibitor L-NAME indicating eNOS uncoupling. Treatment of mice with AVE9488 enhanced vascular content of the essential eNOS cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin and reversed eNOS uncoupling. The combination of an upregulated eNOS expression and a reversal of eNOS uncoupling is likely to be responsible for the observed vaso-protective properties of this new type of compounds.

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68

Differential roles of NOS isozymes in cardiotoxicity and mortality following chronic doxorubicin treatment

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Inhibition of cardiac nitric oxide synthases (NOS) has been proposed as a strategy to reduce the cardiotoxicity (CT) associated with anthracycline (AC) treatments. We investigated the effects of a chronic treatment with doxorubicin (DOX) on knockouts of the individual NOS isozymes and on transgenic mice with myocardial overexpression of eNOS. Fractional shortening (FS) was reduced in untreated homozygous nNOS and iNOS knockouts, and in eNOS transgenics. DOX-induced FS decrease in wild-type mice (21%) was attenuated in eNOS knockouts. No worsening of contractility was observed in DOX-treated eNOS transgenics and iNOS knockouts. In contrast, although the surviving nNOS knockouts exhibited no further impairment in contractility, most (72%) animals in this group died within 7 weeks after treatment onset. In comparison to untreated wild-type hearts, the nitric oxide (NO) level was lower in hearts from DOX-treated wild type mice and in all three untreated knockouts. DOX treatment had no effect on NO in the knockouts. We found no evidence of oxidative stress (OS) in DOX-treated animals. nNOS was overexpressed in untreated eNOS knockouts. These data indicate highly differential roles of the individual NOS in DOX-induced cardiotoxicity, which appear to be unrelated to OS. The protective effects of eNOS deletion may be mediated by a compensatory overexpression of nNOS. Any NOS inhibition-based prevention of AC-induced CT should be eNOS-selective, simultaneously avoiding inhibiting iNOS and, especially, nNOS.

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69

Deletion of NO-sensitive guanylyl cyclase in smooth muscle is sufficient to cause hypertension

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By catalyzing the production of the intracellular signaling molecule cGMP NO-sensitive guanylyl cyclase (NO-GC), as the major receptor for NO, has a key function within the NO/cGMP cascade. The pharmacological importance of the enzyme is reflected by NO donors used for the therapy of coronary heart disease. NO-GC is made up of one b subunit and one a subunit. As there are two α subunits (a1 and a2), two different GC isoforms are known to exist (a1b1 and a2b1). In the cardiovascular system, vasorelaxation and inhibition of platelet aggregation are mediated by the a1b1 GC. As the a2 subunit is mainly found in nerve cells of the CNS, the a2b1 heterodimer is believed to participate in synaptic plasticity. The role of NO-GC in the gastrointestinal tract is still unclear. NO is contributing to non-adrenergic non-cholinergic (NANC) relaxation of gastrointestinal smooth muscle. Using mice deficient in the b1 subunit we investigated the role of NO-GC in vascular and intestinal tissues. These mice do not express any of the a subunits and reveal no detectable cGMP synthesis upon NO stimulation. Thus mice lacking the b subunit are in fact total NO-GC knock out mice. Whereas mice heterozygous for the b1 subunit of NO-GC were phenotypically undistinguishable from WT, homozygous GC-KO mice died prematurely. 3-week-old homozygous GC-KO mice exhibit considerable growth retardation shown by a 40% reduced body weight. KO mice surviving until day 18+ are hypertensive and die from gastrointestinal dysmotility leading to ileus and perforation. By substituting normal rodent chow with fiber-free diet we were able to rescue GC-KO mice. In order to find out the relative contribution of smooth muscle cells to the GC-KO phenotype we are currently investigating smooth muscle specific GC-KO mice (SMKO). Tamoxifen was used for induction of the tissue-specific KO. SMKO mice do not reveal the reduced life expectancy of total GC-KO mice. However, tamoxifen-injected SMKO animals develop hypertension within several weeks. This model of a slowly developing hypertension further underlines the importance of constitutively released endothelial NO as major regulator of blood pressure.

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70

Anemia and splenomegaly in cGKI knockout mice

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To explore the function of cGMP-dependent protein kinase type I (cGKI) in the regulation of erythrocyte survival, gene targeted mice lacking cGKI were compared to their control littermates. By the age of 10 weeks, cGKI-deficient mice exhibited pronounced anemia and splenomegaly. Compared to control mice, the cGKI mutants had a significantly lower red blood cell count, packed cell volume and hemoglobin concentration. Anemia was associated with a higher reticulocyte number and an increase of plasma erythropoietin concentration. The spleens of cGKI mutant mice were massively enlarged and contained a higher fraction of Ter119+ erythroid cells, whereas the relative proportion of leukocyte subpopulations was not changed. The Ter119+ cGKI-deficient splenocytes showed a marked increase in annexin V-binding pointing to breakdown of phosphatidylserine asymmetry, a hallmark of suicidal erythrocyte death or eryptosis. Indeed, cGKI-deficient erythrocytes showed in vitro a higher sensitivity to eryptosis than control cells. According to Fluo-3 fluorescence, the cytosolic Ca2+ concentration, a known trigger of eryptosis, was increased in erythrocytes from cGKI knockout mice. Transfer experiments with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled erythrocytes showed that the in vivo clearance of cGKI-deficient erythrocytes was faster than that of control cells. Together, these results identify a novel role of cGKI as mediator of erythrocyte survival and extend the emerging concept that cGMP/cGKI signaling has an anti-apoptotic/pro-survival function in a number of cell types in vivo.

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71

cGKI modulators: old and new tools for the analysis of cGMP signalling

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Signal transduction via the second messenger cGMP plays an important role in biology. By using distinct target molecules, cGMP can exert many different, and even opposing, effects. An attractive cGMP receptor is the cGMP-dependent protein kinase type I (cGKI). Many studies on the role of cGKI as downstream mediator of cGMP signalling are based on the use of pharmacological “cGK inhibitors” to distinguish between cGKI-dependent and cGKI-independent actions of cGMP. We have analysed the effects of two cGMP isomers that are frequently used as “cGKI inhibitors”, Rp-8-Br-PET-cGMPS and Rp-8-pCPT-cGMPS, on the growth of wild-type and cGKI-deficient cells as well as on the kinase activity of cellular and purified cGKI. Interestingly, neither compound could abolish the cGKI-mediated effects in intact cells and in vitro both drugs acted as partial agonists rather than as antagonists of purified cGKI. The lack of reliable cGKI inhibitors prompted us to develop a new class of cGKI modulators. Initial results with these novel agents will be discussed.

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72

Protein kinase G regulates differentiation of mesenchymal stem cells

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The NO/cGMP signaling cascade plays an important role in many physiological processes such as smooth muscle relaxation and platelet aggregation. Three cGMP receptors exist in mammalian cells: phosphodiesterases, ion channels and cGMP-dependent protein kinases (PKG). Here, we analyzed the role of PKG in the differentiation process of mesenchymal stem cells (MSC). MSCs derived from brown adipose tissue (BAT) were induced to differentiate into adipocytes and treated with 200µM of the cGMP analogue 8-pCPT-cGMP. After 7 days post induction, 8-pCPT-cGMP treated cells showed an increased accumulation of fat droplets as determined by RedO staining and triglyceride (TG) content (62% increased TG content versus untreated cells). In addition, 8-pCPT-cGMP treated cells exhibited a significantly increased mitochondrial content as determined by MitoTracker fluorescence and FACS analysis. mRNA levels of the adipocyte specific markers UCP-1, PGC-1α, PPAR , Perilipin and HSL were elevated after 8-pCPT-cGMP treatment as compared to untreated cells. Lentiviral overexpression of a mutant form of PKG that is constitutively active led to a similar increase of triglyceride accumulation (45%) and mitochondrial content (14%) in these cells as compared to cells infected with empty vector. The constitutively active mutant also induced an increase of UCP-1 and PPAR expression levels similar to cGMP treatment of wild-type cells, indicating that the cGMP effects in MSCs are mediated by PKG. In MSC isolated from BAT of PKG knockout mice, differentiation into adipocytes was impaired (60% reduced TG content as compared to wild-type), mitochondrial content was reduced and treatment with 8-pCPT-cGMP showed no effect. The mRNA levels of the adipocyte specific markers UCP-1, PGC-1α, PPAR , Perilipin and HSL were strongly reduced in PKG-deficient cells. Taken together, these data suggest a so far unknown role of PKG in mesenchymal stem cell differentiation.

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73

Regulation of iNOS expression in stable human C28-I/2 chondrocytes

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The expression of inducible NO synthase (iNOS) is regulated by transcriptional and post-transcriptional mechanisms. Analyses of the signalling pathways leading to iNOS induction have shown major differences between cell types and species. The present study investigated major protein kinase pathways for their involvement in the induction of human iNOS expression in C28-I/2 chondrocytes. Similar to other human cells, C28-I/2 chondrocytes required a mixture of cytokines of TNF-α, IL-1β and IFN-γ for maximal iNOS induction. In addition, induction of iNOS expression was cell-density dependent in this cell line.To investigate the effect of different pathways on iNOS induction, superconfluent C28-I/2 cells were treated with several inhibitors, which target these pathways. In C28-I/2 chondrocytes, inhibition of the p38 MAPK pathway by SB203580 resulted in a reduction of iNOS mRNA. Furthermore, cytokine-induced iNOS mRNA expression was inhibited by treatment with the JAK-STAT inhibitor AG490. In contrast, inhibition of other major kinase pathways (JNK, ERK and NF-κB) had no effect on iNOS induction (mRNA and protein) in this cell line.In addition, treatment of C28-I/2 chondrocytes with S-Curvularin, a new STAT-1α inhibitor isolated from fungi, clearly inhibited iNOS mRNA expression. Also iNOS protein expression and iNOS-mediated NO production was reduced in a concentration-dependent manner.Our results suggest that induction of iNOS in superconfluent human C28-I/2 chondrocytes is dependent on activation of the p38 MAPK and the JAK-STAT pathway.

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74

Post-transcriptional regulation of human iNOS expression involves control of nucleocytoplasmic iNOS mRNA transport

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Regulation of human inducible nitric oxide synthase (iNOS) expression occurs on the transcriptional and on the post-transcriptional level. Several RNA binding proteins have been identified which contribute to the modulation of iNOS mRNA stability. Another mechanism involved in the regulation of gene expression is the control of the nucleocytoplasmic mRNA transport. Most cellular mRNAs are exported from the nucleus via the TAP/Nxt complex of export proteins. However, some RNAs (often containing AU-rich elements) are transported in a different way involving the nuclear export receptor CRM1.In this study, we investigated the mechanism of human iNOS mRNA export. Therefore, different cell lines were incubated with leptomycin B (LMB), a specific inhibitor of CRM1-mediated nuclear export. Inhibition of CRM1-mediated transport by LMB considerably reduced cytokine-induced iNOS mRNA expression. This effect could be confirmed by an anti-CRM1 RNA interference approach. Further analyses showed that the expression of several other AU-rich mRNAs (coding for TNF-α, COX-2 or tristetraprolin) is reduced as well under these conditions.Since CRM1 itself does not possess any RNA binding affinity, an adaptor protein is needed to mediate CRM1-dependent mRNA export. The eukaryotic translation initiation factor eIF4E has been described to promote nuclear export of the cyclin D mRNA. In order to identify the adaptor protein for iNOS mRNA export, cells were incubated with Ribavirin, a specific inhibitor of eIF4E-dependent mRNA transport. Blocking the export function of eIF4E, we could show that iNOS mRNA levels decrease in the cytoplasm while the amount of nuclear iNOS mRNA is increased. Moreover, incubation of the cells with Ribavirin had no effect on iNOS promoter activity or iNOS mRNA stability.In conclusion, the human iNOS mRNA appears to be exported from the nucleus in a CRM1-dependent manner. The eukaryotic initiation factor eIF4E seems to play an important role in this transport.

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75

Nitric oxide inhibits the TPA-induced matrix metalloproteinase-9 expression in human breats cancer cells

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Matrix metalloproteinases (MMPs) can have different effects in cancer cell invasion and metastasis. Besides their ability to degrade the extracellular matrix, MMPs have various other functions including the release of membrane-bound growth factors. Nitric oxide has been described as a mediator of pro- and anti-tumorigenic properties. We investigate the impact of nitric oxide on MMP-9 expression in MCF-7 breast cancer cells. By use of different NO donors we tested for modulatory effects of exogenous NO on MMP-9 expression and activity induced by the phorbolester TPA. Using gelatine zymography we observed that all NO donors cause a strong reduction in the extracellular MMP-9 content. Concomitantly, the intracellular protein level of MMP-9 was inhibited by all NO donors tested. Cycloheximide experiments revealed that the negative influence on MMP-9 was not caused by an increased protein degradation. By contrast, NO strongly reduced levels of MMP-9 mRNA in total polysomal fraction. Realtime-PCR analysis further proved that the treatment with NO attenuated the steady-state MMP-9 mRNA level. However, the reduction in the MMP-9 mRNA was not due to a decrease in the mRNA stability shown by actinomycin D experiments. Reporter-gene assays exhibited a reduction in the TPA-induced MMP-9 promoter activity. To test for possible epigenetic modifications induced by NO the HDAC (histone deacetylase) inhibitor Trichostatin A (TSA) was used. We found that TSA reduced the TPA-induced MMP-9 expression while it partially reversed the NO dependent reduction of MMP-9 expression. In summary, our data suggest, that the inhibition of MMP-9 by NO occurs at different levels, including transcriptional and epigenetic mechanisms.

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76

Regulation of gene expression by the organic nitrates GTN and PETN

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Given acutely, organic nitrates such as nitroglycerin (GTN) have excellent antiischemic effects. During long-term treatment their clinical usefulness is limited due to the development of tolerance and the induction of endothelial dysfunction. Nitrate tolerance was observed first in response to treatment with GTN but seems to be shared by other organic nitrates. One exception is pentaerithrityl tetranitrate (PETN); it induces less or no tolerance.Organic nitrates have to be bioactivated in order to produce vasodilatation. Bioactivation of GTN and PETN are mediated by the mitochondrial aldehyde dehydrogenase (ALDH-2). During biotransformation of GTN an increase of reactive oxygen and nitrogen species (ROS/RNS) is observed. This leads to inactivation of the ALDH-2 and thus impairs biotransformation of GTN. This may contribute to the development of nitrate tolerance and endothelial dysfunction. Also during PETN bioactivation ROS production occurs, but this is not accompanied with a loss of susceptibility to organic nitrates. In this study we demonstrated, that PETN but not GTN is able to induce the expression of antioxidative proteins like heme-oxygenase 1 or ferritin heavy chain in EA.hy 926 cells. This phenomenon could explain the differences of both compounds concerning their abilities to induce nitrate tolerance. Then we were interested if these substances also differentially regulate other genes. Therefore we compared gene profiles of rat heart tissues of male Wistar rats infused for four days with the organic nitrates GTN or PETN or the corresponding solvent controls using rat total genome arrays containing more than 28000 different oligonucleotides. In general most of the analyzed genes were not regulated. In the hearts of GTN treated animals the major part of the regulated genes is down-regulated, whereas in PETN treated animals the number of up-regulated genes is higher. So in summary both substances seem to have different effects on total gene expression in rat hearts.

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77

Paraoxonase-2 reduces intracellular superoxide levels independent of its lactonase activity

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The paraoxonase (PON) family of enzymes comprises three known proteins, PON1, PON2 and PON3. Although the three PONs show high homology at the amino acid level, they differ significantly in their enzymatic activities, substrate specificities and localization. While PON1 is mainly found as high density lipoprotein particle-associated protein, the cell-based PON2 mainly localizes to the endoplasmic reticulum (ER) and protects cells from ER stress-induced caspase-3 activation. Moreover, all three PONs possess anti-atherogenic properties that may be a result of their anti-oxidative functions. It is largely unknown however, which of the major reactive oxygen species found in diseased blood vessels was reduced by PON2. In the current study we demonstrate that PON2 reduces superoxide-, but not peroxynitrite levels. We further investigated whether lactonase activity of PON2, the best established catalytic function of the enzyme, is required for its anti-oxidative and anti-apoptotic properties. We inserted a single point mutation into the putative active site of PON2. This mutation abrogated lactonase activity of the enzyme. However, the anti-oxidative function of PON2 was completely unaffected by this mutation. Also, the ER targeting of PON2 as well as its protective function against ER stress-induced apoptosis remained unchanged. Our data thus provide evidence that PON2 reduces a major radical involved in cardiovascular damage, which is facilitated independent of its lactonase activity.

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78

VIP induces relaxation of mouse aorta via NO/cGMP

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The NO/cGMP signalling cascade is involved in the regulation of a variety of physiological responses and has an established importance in the vascular system. Here, the endothelium is the main source of NO produced by endothelial NO synthase. The most important receptor for NO, NO-sensitive guanylyl cyclase (NO-GC), is localized in smooth muscle cells. By the stimulation of NO-GC, NO plays a central role in the regulation of blood pressure. Mice lacking NO-GC are hypertensive, underlining the importance of NO in the regulation of smooth muscle tone in vivo. Vasoactive intestinal peptide (VIP), a 28-amino acid neuropeptide, exerts its action through two G protein-coupled receptors (VPAC1 and VPAC2). VIP is involved in a wide variety of biologicalactivities, including vaso- and bronchodilation, smooth muscle relaxation and stimulation of secretion. The activation of VPAC receptors in many cells results in the production of cAMP. But there is controversy as to whether or not VIP action also involves NO/cGMP-mediated signalling.In this study, we sought to determine the involvement of the NO/cGMP cascade in VIP signalling. The relaxing effects of VIP were investigated using aorta from general and smooth muscle-specific KO mice for NO-GC. Using L-NAME and ODQ, we found that VIP signalling is dependent on eNOS and smooth muscle GC. The VPAC1-specific agonist (Ala11,22,28)-VIP led to a response similar to that seen with authentic VIP. In mice lacking NO-GC, VIP induced aortic relaxation was practically absent. In sum, VIP relaxes the mouse aorta mainly through VPAC1 and NO/cGMP.

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79

Divergent roles of Gα subfamily members as full and partial agonists at a site mediating Rho GTPase activation

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Expression of the human cytomegalovirus (HCMV)-encoded chemokine receptor homolog pUS28 in mammalian cells results in ligand-dependent and -independent changes in the activity of multiple cellular signal transduction pathways. Ligand-independent constitutive activity of pUS28 causing stimulation of inositol phosphate formation has been correlated with the coupling of pUS28 to G proteins of the Gq family. In addition cells expressing pUS28 also showed a constitutive induction of serum-response-factor (SRF)-mediated gene transcription. This effect was observed in the absence of chemokines known to interact with pUS28 and was specifically mediated by endogenous Gq and/or G11 as well as RhoA and/or a closely related Rho GTPase. Interestingly, the stimulatory effect of pUS28 and Gαq/11 was markedly sensitive to inhibition by expression of Gα16, thus identifying Gα16 as an antagonist of Gαq/11 and implying that both proteins either competed for receptor interaction or for interaction with a downstream effector. To delineate the molecular mechanisms of the inhibitory effect of Gα16, we expressed pUS28 together with two constitutively active variants of Gα16, Gα16R186C or Gα16Q212L, and analyzed their influence on inositol phosphate formation and induction of SRF activity in COS-7 cells. The results showed that coexpression of either constitutively active Gα16 protein had no inhibitory effect on pUS28-mediated SRF-activation, but instead caused a slight to moderate increase in this activity when expressed alone. Likewise, formyl-peptide-receptor-mediated activation of wild-type Gα16 caused only a small increase of SRF activity. Taken together, these observations support a model in which both Gαq/11 and Gα16 are activated by pUS28 to either indiscriminately activate inositol phosphate formation or activate SRF with markedly different efficacy and/or potency. Thus, the relative abundance of the Gq subfamily members in a given plasma membrane may be a critical determinant of signal allocation in this compartment, ranging from activation of a single Gαq effector, phospholipase C-β, in the presence of Gα16 to activation of several Gαq effectors, including SRF, in its absence.

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80

Nucleoside diphosphate kinase B/Gbetagamma complex formation controls heterotrimeric G protein expression in the zebrafish

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Heterotrimeric G proteins are involved in a broad variety of physiological and pathological processes. The canonical activation of G proteins receptor and receptor-independent pathways has been studied in detail. However, little is known concerning the regulation of the cellular content of G proteins, providing an additional mechanism that modulates G protein signaling. Here, we show that the nucleoside diphosphate kinase B (NDPK B) complexed with the G$\beta\gamma$-dimer is essential for the stability and function of G proteins in the zebrafish. Selective depletion of zebrafish NDPK B, but not NDPK A, by antisense-morpholino oligonucleotides results in a severe impairment of heart function and blood vessel formation. This knockdown of NDPK B was associated with a strong reduction in Gb1g2 dimers expression. In addition, the protein levels of Ga-subunits representing all of the four G protein subfamilies, Gs, Gi, Gq/11, G12/13, were substantially reduced. Vice versa, the knockdown of the zebrafish Gβ1 orthologs, Gβ1 and Gβ1like, caused a very similar cardiovascular phenotype to that of NDPK B depletion. The Gb1/1like knockdown was similarly associated with loss of Ga-subunits, and most importantly, NDPK B. Our data therefore reveal the importance of the NDPK B/G$\beta\gamma$ complex formation in vivo. As the mRNA levels of all proteins studied remain unaffected, our results define a novel role for the interaction of NDPK B with G$\beta\gamma$-dimers in regulating G protein expression on the post-translational level.

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81

Interaction of active G-proteins with active alpha2a G-protein coupled adrenergic receptors

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Recently, a reduction of GIRK channel currents by saturating agonist concentrations compared to non saturating agonist concentration (in terms of recpetor binding) has been described for different Galphai/o coupled receptors (Bösche, L, J Physiol 2003; Leaney, J, Am J Physiol Cell Physiol 2004). Analyzing different hypothesizes for the cause of this finding, we have developed a kinetic mass-action model of the G-Protein cycle. The parameters of the model were adjusted to explain kinetic data measured by means of FRET for receptor activation, receptor G-protein interaction and G-protein activation. In order to explain existing data, the model predicts, that active G-proteins must be able to bind to the ligand bound/ activated receptor. Potentially, this interaction leads to an inhibition of the G-protein signal, depending on the reaction constants for this interaction, the concentration of ligand bound/ activated receptor and the speed of G-protein cycling at the receptor.We have tested these predictions for Galphao using FRET measurements between tagged G-protein subunits and GIRK channel measurements. HEK-293 cells stably expressing alpha2a receptors at high concentration show an inhibition of GIRK channel currents and a decrease of G-protein subunit FRET upon stimulation with agonist at saturating concentrations (in terms of receptor binding) compared to stimulation with agonist at non-saturating concentrations. The inhibition was depending on the expression level of the receptor in these cell lines. RGS insensitive Galphao subunits also exhibited an inhibition of the signal with all measured cells being basally fully activated as expected. Furthermore, the inhibition exhibits an agonist dependent response similar to the concentration response curve of agonist binding to the receptor. Irreversible activation of G-proteins by GTPgammaS did not prevent agonist induced receptor G-protein interaction. We therefore conclude, activated G-proteins can reversible bind to active receptors, potentially leading to an

inhibition of G-protein activity and subsequently effector activity depending on the receptor subtype.

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82

Phosducin prevents hypertension by control of the sympathetic nervous system

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Hypertension and its cardiovascular complications represent and contribute to the leading causes of mortality worldwide. As a significant proportion of blood pressure variability is due to genetic factors the search for susceptibility genes is fundamental. We have recently identified phosducin (Pdc) as a new hypertension-related gene. By means of the respective knockout mouse model and by means of genetic association studies in humans we found Pdc to be essential for blood pressure control especially in stress situations. Now, we sought to focus on the quantification of the influence of polymorphisms within Pdc on blood pressure and the mechanism of hypertension in the Pdc-deficient mouse model. In candidate-gene based association studies in two different populations of African American (AA) and French-Canadian (FC) origin we found several SNPs to be significantly associated with blood pressure phenotypes. One SNP in the intronic region of Pdc was accompanied by an increase in average wake systolic blood pressure of 12 mmHg (AA) or 15 mmHg (FC) in individuals homozygous for the G allele compared to those carrying two copies of the A allele. Pdc-deficient mice displayed higher blood pressure and elevated catecholamine turnover in peripheral sympathetic tissues. Interestingly, we observed a gene-dosage effect upon hemodynamic examination of heterozygous mice (Pdc+/-). Moreover, isolated postganglionic sympathetic neurons from the superior cervical ganglion (SCG) from Pdc-/- mice, as opposed to those from wild-type mice, showed prolonged action potential firing after stimulation with acetylcholine and higher firing frequencies during membrane depolarization by current injection. Control experiments however showed, that this was not due to altered presynaptic inhibition of release or to alterations in the subtype expression of the nicotinic acetylcholine receptor. In summary, phosducin is a hypertension-related gene in which distinct human haplotypes are associated with a significant increase in systolic blood pressure. In Pdc-deficient mice, hypertension is most likely due to inadequate control of sympathetic firing.

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83

Activation of Gi by Pasteurella multocida toxin

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Pasteurella multocida toxin (PMT) is one of the strongest activators of Gaq-dependent phospholipase C (PLC) b to induce inositoltrisphosphate production, Ca2+ mobilization and formation of diacylglycerol. The toxin also activates the small GTPase RhoA, resulting in formation of stress fibers and focal adhesions. The activation of RhoA depends on Gαq and Gα12/13. Interestingly, Gα11, a member of the Gq family is not stimulated by PMT. In addition, PMT induces MAP kinase and STAT activation.Here we report that PMT is not specific for Gq or G12/13 but potently activates Gαi. PMT inhibits basal, isoproterenol and forskolin-stimulated cAMP accumulation in target cells. Inhibition of cAMP accumulation caused by lysophosphatidic acid via EDG receptors is enhanced by the toxin. By gene deletion it is shown that the inhibition of cAMP production is independent of PMT-activated Gαq and/or Gα12/13. PMT treatment of cells inhibits forskolin-stimulated adenylyl cyclase activity also in membrane preparations. GTPase activity of Gi stimulated by lysophosphatidic acid is inhibited by PMT.The study reveals a broad spectrum of heterotrimeric G proteins including Gi, which is activated by PMT.

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84

An obligatory requirement of the heterotrimeric G protein Gαi3 in the anti-autophagic action of insulin in the liver

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Autophagic proteolysis represents the major self-degradative process in eukaryotes and plays a fundamental role in stress, development and disease. Central to this process is the formation of autophagosomal vesicles, which sequester and deliver portions of the cytoplasm to lysosomes for degradation. Heterotrimeric G proteins of the pertussis toxin (PTX)-sensitive Gai family have previously been implicated in the autophagic sequestration pathway. The Gαi family includes three major isoforms, Gαi1-3. The expression of Gαi1 is restricted to the neuronal system, whereas non-neuronal organs like the liver predominantly express Gαi2 and Gαi3. Here, we have investigated the role of individual Gαi proteins in hepatic autophagic proteolysis. Employing in situ mouse liver perfusion experiments, autophagy was rapidly induced under conditions of metabolic stress, and was potently inhibited by insulin. The inhibition of autophagic proteolysis by insulin was PTX-sensitive, suggesting the involvement of Gi proteins in this organ. Interestingly, mice lacking Gαi3 were deficient in the inhibitory action of insulin on autophagic proteolysis, whereas mice deficient in Gαi2, the major PTX substrate in hepatic tissue, were indistinguishable from wild type animals. Conversely, Gαi3-deficient mice were indistinguishable from wild type mice in glucose tolerance tests, suggesting that insulin sensitivity is not generally impaired. Using affinity-purified antibodies monospecific for either Gαi2 or Gαi3, we have analyzed the subcellular distribution of Gαi2 and Gαi3 in isolated mouse hepatocytes by confocal laser scanning microscopy. Whereas Gαi2 is predominantly detected at the plasma membrane, Gαi3 localizes to autophagosomes upon starvation-induced autophagy, and is redistributed to the plasma membrane upon insulin stimulation. Employing subcellular fractionation techniques, we have further confirmed that Gαi3 localizes to autophagosomal membranes upon autophagy induction. These data clearly demonstrate that Gαi3 is crucial for the anti-autophagic action of insulin, and suggest an as yet unrecognized function for Gαi3 on autophagosomal endomembranes.

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85

Active Ras acts as an indispensable adapter and regulator for receptor induced activation of the novel phosphoinositide 3-kinase γ isoform, p110γ/p87PIKAP

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The heterodimeric G-protein-coupled-receptor (GPCR)-regulated phosphoinositide 3-kinase γ (PI3Kγ) controls fundamental processes such as neutrophil chemotaxis, mast cell degranulation and cardiac function. Until recently, only one PI3Kγ isoform was known, consisting of the catalytic subunit p110γ and a non-catalytic Gβγ adapter, p101. The identification of a second non-catalytic subunit, termed p84 or p87PIKAP, has raised interest in its role for PI3γ functions. Initial data have suggested that the degree of GPCR- or Gβγ-induced stimulation of PI3Kγ is slightly higher with p101 than with p87PIKAP. To elucidate the role of p87PIKAP, we have employed complementary approaches using a reconstituted system with purified proteins, as well as confocal live cell imaging of HEK293 cells expressing fluorescently labeled proteins. Surprisingly, and in contrast to p101, p87PIKAP does not significantly contribute to the recruitment and activation of PI3Kγ by Gβγ, regardless of whether purified proteins or transfected cells were analysed. However, we observed that H-Ras was able to recruit all PI3Kγ-isoforms to the membrane via direct interaction with p110γ in a GTP-dependent manner. This suggests that active Ras functions as an adapter for p110γ. In line with these findings, the stimulation of p110γ/p87PIKAP by receptor agonists in cells overexpressing H-Ras was as efficient as the stimulation of p110γ/p101 in the absence of exogenous H-Ras. Vice versa, following inactivation of endogenous Ras though overexpression of the Ras-GAP NF-1, GPCR-induced activation of p110γ/p87PIKAP, but not of p110γ/p101, was blunted. Interestingly, the presence of p87PIKAP was essential to reach full activation of p110γ following GPCR-stimulation in the presence of overexpressed H-Ras. We therefore conclude that Ras is indispensable for the recruitment and activation of the heterodimeric p110γ/p87PIKAP, whereas the non-catalytic subunit p87PIKAP is suggested to additionally act as a regulator of the enzymatic activity of p110γ.

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86

Role of non-catalytic p101 subunit in regulation of phosphoinositide 3-kinase γ enzymatic activities

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Class IB phosphoinositide 3-kinase γ (PI3Kg) is one of the G-protein bg (Gβγ) effectors that regulates such important cellular processes as cell growth, proliferation, apoptosis, and cytoskeletal functions. Receptor-released Gβγ dimers recruit PI3Kγ to the plasma membrane and stimulate its lipid and protein kinase activities. The catalytic p110γ and the non-catalytic p101 subunits of PI3Kγ bind Gβγ in vitro, although p101 does so with much higher affinity. We have previously proposed a dual and complementary mode of Gβ1γ2-dependent activation of PI3Kγ, such that Gβ1γ2 recruits PI3Kγ to the plasma membrane by binding to p101, and subsequently stimulates its catalytic activity. According to these findings, the p101 subunit acts as an adaptor protein that confers Gβ1γ2-sensitivity to p110γ. Nevertheless, the exact role of p101 for the regulation of PI3Kγ has remained obscure. To investigate the function of the non-catalytic p101 subunit for PI3Kγ activation, we have designed Gβγ-free phospholipid vesicles containing similar amounts of recombinant monomeric (p110γ) and dimeric (p110γ/p101) PI3Kg. To exclude the possibility that the enzymatic activity of monomeric p110γ, which is less stable than the dimeric enzyme, was partially lost during the purification procedure, we have performed a set of reconstitution assays with purified p101. Co-incubation of p110γ with separately purified p101 leads to an enzymatic state of the monomeric catalytic subunit that was identical to the intact dimeric kinase. This shows that p110γ purified as a monomer possesses its full intrinsic activity. Interestingly, we observed that the basal lipid kinase activity of phospholipid vesicle-associated p110γ/p101 was considerably higher than for p110γ alone, whereas the basal protein kinase activity of p110γ was drastically reduced in complex with p101. Hence, p101 functions to simultaneously maintain the lipid kinase activity of p110γ in a high activity state, and the protein kinase activity in a low activity state. We conclude that p101 is important not only as an adaptor for Gβ1γ2, but that it is also directly involved in regulating the quality of p110γ enzymatic activities.

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87

shRNA-mediated gene targeting reveals a critical role of G-alpha12 and G-alpha13 for human small cell lung cancer cell proliferation and in vivo tumor growth

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The autocrine stimulation of G-protein-coupled neuropeptide receptors, which engage Gq/11- and G12/13-dependent signalling pathways, is of critical importance for small cell lung cancer (SCLC) cell growth. The contribution of the Gq/11-PLC-β cascade to the mitogenic effect of neuropeptides in these cells is well established; however, the relevance of G12/13 signalling in SCLC is still elusive. We employed shRNA-mediated

targeting of Gα12, Gα13, or both, in H69 and H209 cells to investigate whether G12/13-dependent pathways contribute to the malignant phenotype of SCLC. The use of a lentiviral expression system rather than non-viral transfection resulted in robust and specific knockdown of both target proteins. Upon a marked decrease in Gα12 or Gα13 levels, we observed inhibited proliferation of H69 and H209 cells in normal culture medium as well as reduced colony formation in semi-solid medium. Using specific clostridial toxins inhibiting Rho proteins or inhibitors of downstream Rho kinase revealed that the regulation of the proliferative capacity in SCLC cells by G12/13 signaling was independent of the Rho/Rho kinase pathway. Moreover, we showed that the downregulation of Gα12 or Gα13 resulted in decreased tumor growth in a subcutaneous tumor xenograft mouse model, with the underlying effect being reduced tumor cell proliferation. Strikingly, double knock-down of Gα12 and Gα13 completely abolished tumorigenicity in mice. We conclude that, in contrast to previous findings in breast and prostate cancer cells, in vitro proliferation of SCLC cells and in vivo tumorigenicity critically depend on intact G12/13 signalling.
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88

PMT inhibits the GTPase activity of Gαi
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An infection with the bacterium Pasteurella multocida leads to pasteurellosis in man and animals. The causative agent of atrophic rhinitis in pigs is Pasteurella multocida toxin (PMT). Known intracellular target proteins activated by PMT are the heterotrimeric proteins of the Gαq and Gα12/13 family. Gαq stimulates the phospholipase Cβ leading to an increased production of inositolphosphates, Ca2+ mobilization and formation of diacylglycerol. Furthermore, activated Gαq and Gα12/13 proteins stimulate the small GTPase RhoA causing formation of stress fibers and focal adhesions. The gene expression altering effect of PMT is due to activation of MAP kinases and STAT transcription factors. To show that PMT also activates the Gαi protein the GTPase activity in membranes obtained from PMT-treated Swiss 3T3 cells was measured. The GTPase activity of Gαi was inhibited by PMT in time-dependent and concentration-dependent manner. Stimulation of the steady state GTPase activity of Gαi by lysophosphatidic acid (LPA) was inhibited by pretreatment of cells with PMT. To confirm this observation the effect of PMT on Gαi was compared with the effect caused by pertussis toxin. Also PMT reduced basal GTPase activity and blocked LPA-stimulated GTP hydrolysis. In addition, GTPase activity was measured after adding RGS proteins (regulator of G-protein signalling) to the reaction mix. This study indicates that PMT affects GTPase function of heterotrimeric G proteins.
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89

Influence of Gβγ on *Pasteurella multocida* toxin-induced activation of heterotrimeric G proteins
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The intracellularly acting *Pasteurella multocida* toxin (PMT) affects several signal transduction pathways by stimulating heterotrimeric G proteins. PMT has been shown to activate the small GTPase Rho, the MAP kinase ERK and STAT proteins via the stimulation of two G protein families, Gαq and Gα12/13. PMT action also results in an increase in inositol phosphates, which is due to the stimulation of PLCβ. Recent studies indicate that PMT can distinguish between the highly related Gαq and Gα11. IP3 accumulation only depends on the activation of Gαq. Recently, it was shown that Gβγ subunits are essential for the signal transduction of Gq. The influence of PMT on the composition of the G protein complex is still unknown. To address the issue whether PMT stimulates Gq signaling via the monomeric α subunit or the heterotrimeric complex, the Gβγ subunits were sequestered by the C-terminus of β adrenergic receptor kinase (βARK-CT). Moreover, the two mutants GαqI25A,E26A (incapable of binding βγ subunit) and GαqG208A (incapable of uncoupling βγ subunit) were used to determine if Gβγ subunits are necessary as positive effectors for PMT-induced activation.Additionally, we could show that not only Gα but also Gβγ subunits are activated by PMT. Therefore we studied HEK293 cells co-expressing PtdIns-3,4,5-P3- (PIP3) sensitive pleckstrin homology (PH) domains fused to GFP together with phosphoinositide 3-kinases (PI3K) γ. Gβγ activates PI3Kγ leading to formation of PIP3 as indicated by the recruitment of PIP3-binding PH domain-containing proteins to the plasma membrane. In the presence of PI3Kγ, PMT, but not the inactive PMT-mutant, enhanced the redistribution of PH domains to the plasma membrane indicating the activation of Gβγ by PMT.The data indicate that Gβγ subunits are essential for signal transduction by *Pasteurella multocida* toxin.
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90

Sensory G protein subtypes in mammalian spermatozoa
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Although it is widely accepted that chemotaxis is a guiding mechanism which directs spermatozoa to the mature oocyte within the oviduct, the molecular transduction processes linking the detection of attracting factors to swimming responses are still ambiguous. Recently, we documented that gustducin, a G protein α-subunit that has been demonstrated to play a key role in "bitter", "sweet" and "umami" taste sensation is expressed in mature spermatozoa of different mammalian species. Its expression was found to be especially enriched in the connecting piece between the sperm head and tail section, the mitochondria-rich midpiece region as well as the proximal part of the principal region of the sperm flagellum, whereas no labeling was detectable in the end piece region. In the present work we addressed the question whether the cognate Gbγ subtypes of α-gustducin in taste sensory cells, notably Gβ3 and Gγ13, are also expressed in mammalian sperm cells. Immuncytochemical approaches performed with isolated epididymal spermatozoa of different mammalian species revealed no specific labeling for antibodies recognizing Gβ3 or Gγ13, respectively. To test the hypothesis whether transduction mechanisms of other chemosensory systems may exist in mammalian sperm, the expression of Gβγ-subunits found in pheromone-sensitive vomeronasal neurons were analyzed. The results revealed that Gβ2 and Gγ2 which are both selectively expressed in V1R1 positive neurons of the apical region of the vomeronasal epithelium were also detectable in mature spermatozoa from mouse to humans. Interestingly, assessing the subcellular distribution pattern, this sensory Gβγ dimer was mainly found in a small region connecting the sperm head and tail. Although the identified Gβ2γ2 dimer shows only partial coexpression with α-gustducin in the sperm flagellum, it will be challenging to determine the functional role of these chemosensory G protein subunits in mammalian spermatozoa.
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91

Structural requirements for the regulation of pertussis-toxin-insensitive signalling of CC chemokine receptor 2b by tumor protein D52
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The carboxyl-terminal portions of several GPCRs, including chemokine receptors, have been shown to interact with multiprotein complexes made up of heterotrimeric G protein subunits and non-G-protein-components. This interaction may affect intrinsic receptor functions, regulate the interaction of the receptors with G proteins, or give rise to non-canonical, i.e. G-protein-independent, signal transduction processes. We have previously identified the tumor protein D52 (TPD52) as a novel interaction partner of the intracellular, carboxyl-terminal-most portion of the human CC chemokine receptors CCR2a and CCR2b, and have found that coexpression of TPD52 together with CCR2 in transfected COS-7 cells resulted in a reduction of the MCP-1-dependent, CCR2a- and CCR2b-mediated increases in inositol phosphate formation and serum response factor (SRF)-dependent transcriptional activation of a luciferase reporter gene. Furthermore, we showed that both responses, inositol phosphate formation and SRF-dependent gene transcription, were synergistically enhanced by coexpression of pertussis-toxin-insensitive Gaq-subfamily members and that this enhancement was sensitive to inhibition by TPD52. To further characterize the structural requirements of the CCR2 receptors to interact with TPD52, glutathione S-transferase fusion proteins of the CCR2 carboxyl-terminal portions were produced in E. coli and examined for their ability to interact with TPD52 in coprecipitation analyses. The results showed that a juxtamembrane octapeptide shared between the two human CCR2 receptor variants and representing the amino-terminal portion of the putative 'eighth helix' is important for the interaction with TPD52. Furthermore, we observed that alanine replacement within this octapeptide resulted in a loss of cell surface expression of the mutant receptors. Taken together, these results imply that TPD52 inhibits CCR2 function by interacting with a site on CCR2 that is critically involved in Gq protein activation and/or intracellular trafficking of the receptor polypeptides.
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92

Lack of GNB3 exon polymorphism in diabetes mellitus dogs
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Diabetes mellitus and hypertension in man are associated with a T allele variant of the C5500T Single Nucleotide Polymorphism (SNP) in the G protein b3 subunit (GNB3). In this study we searched for an analogous GNB3 polymorphism that might also be linked to diabetes mellitus in dog. We have previously described the complete nucleotide sequence of canine genomic GNB3 was. We found that exon 9 of the canine GNB3 gene is analogous to the human exon 10, which hosts the proposed human diabetes mellitus and hypertension associated C5500T SNP. The entire exon 9 of the GNB3 gene from 36 diabetes mellitus and 36 control dogs was amplified by PCR and the corresponding PCR products were analyzed by direct DNA sequencing. Additionally, PCR-RFLP analysis of 600 control dogs (representing 90 distinct breeds) was performed in order to study whether a rare race specific analogous C5500T SNP in dog exists. In contrast to man, we failed to identify any polymorphism in the entire canine exon 9. Furthermore, we reported recently the absence of a hypertension associated polymorphisms in the analogous exon of primary hypertensive dogs. In studies with human beings, only the human diabetes mellitus and primary hypertension associated thymine was detected at the analogous GNB3 canine nucleotide site, regardless of whether the dogs suffered from diabetes mellitus and primary hypertension or not. Owing the lack of allele variance in all examined dogs we conclude, that diabetes mellitus and primary hypertension are not associated with a GNB3 allele variant in the human analogous nucleotide site or in the respective exon. The hypothesis that the extremely conserved canine and human GNB3 polypeptide homology would make it possible to identify a polymorphism in GNB3 exon 9 linked to diabetes mellitus and / or primary hypertension in dogs was shown to be wrong. Thus, it remains to be proven whether a unique SNP in a single gene may be responsible for complex multifactorial diseases such as diabetes mellitus and primary hypertension.
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93

Processing of the *Escherichia coli* cytotoxic necrotizing factor 1 (CNF1)
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The cytotoxic necrotizing factor 1 (CNF1) is a protein toxin mainly produced by uropathogenic *Escherichia coli* (UPEC) strains. It belongs to the family of Rho-activating bacterial protein toxins. Other members of this family are the *E. coli* toxins CNF2 and CNF3, the Y. pseudotuberculosis CNFy and the dermonecrotic toxin (DNT) produced by

B. pertussis. The activation of Rho GTPases occurs by deamidation (transglutamination by DNT) of a specific glutamine, which is crucial for GTP hydrolysis, resulting in a constitutively protein. This causes extensive stress fiber formation and multinucleation of cultured cells.CNF1 is taken up into mammalian cells by receptor-mediated endocytosis in a clathrin and caveolin independent manner, and is delivered from late endosomes into the cytosol. Up to date, it was generally accepted that the toxin reaches the cytosol as a full-length toxin. By immunoprecipitation with an antibody against the catalytic portion, we could identify a ~ 55 kDa fragment of CNF1. This C-terminal fragment is only present in the cytosol. Its processing requires an acidic pH and insertion of the toxin into the endosomal membrane. Furthermore we show that this cleavage is performed by a serine protease which is either localized in the endosomes or within the toxin itself. In addition, we define the region of toxin-cleavage by mutational analysis.
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94

The specific nuclear import of the small GTPase Rac1 is mediated by the direct interaction with karyopherin α2
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Rho proteins comprise a family of more than 20 members including three Rac GTPases. They participate in a broad range of physiological and pathophysiological processes, including the regulation of the actin cytoskeleton, gene transcription, and cell cycle progression. Rac GTPases function as molecular switches, cycling between an inactive GDP-bound state and an active GTP-bound state. While Rac1 is ubiquitously expressed, Rac2 is largely restricted to hematopoietic tissues and Rac3 to the developing brain. Although the three isoforms share between 89 and 93% sequence identity, they are involved in different physiological functions and interact with a specific subset of effectors. Recent data point out that the small GTPase Rac1 is not only involved in multiple cytosolic functions but also translocates to the nucleus to perform further tasks. In the yeast two-hybrid system, we identify the nuclear import receptor karyopherin a2 (KPNA2) as a direct interaction partner of Rac1. The C-terminal polybasic region of Rac1 contains a nuclear localization signal (NLS), whereas Rac2 and Rac3 lack a functional NLS and do not bind to KPNA2. The presence of the NLS in Rac1 determines the specificity of the interaction and is a prerequisite for the nuclear import. Although this interaction is independent of the Rac1 GDP/GTP-loading, the induction of the translocation requires Rac1 activation. The activation of Rac1 via the cytotoxic necrotizing factor 1 and the concurrent inhibition of its proteasomal degradation are crucial for the nuclear accumulation of both, Rac1 and KPNA2. Conversely, the reduction of KPNA2 expression inhibits the nuclear import of Rac1. For the first time, our results demonstrate a direct interaction between Rac1 and KPNA2 and argue for a KPNA2-dependent nuclear import of Rac1. In the nucleus, Rac1 may participate in a variety of signalling pathways and may be prepared for its proteasomal degradation.
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95

Rac1 phosphorylation attenuates the effect of *Clostridium difficile* toxin A on the epithelium barrier function
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Clostridium difficile toxins A and B are single chain proteins that monoglucosylate Rho GTPases. One major function of Rho GTPases is the regulation of the actin cytoskeleton. The filopodia, lamellipodia, membrane ruffles and stress fibers are regarded as typical phentotypes of the activated Rho GTPases. Their glucosylation leads to functional inactivation and to subsequent breakdown of the actin cytoskeleton.
In this study, we investigated the effect of Rac1 phosphorylation at serine 71 on cell morphology as well as on actin cytoskeleton. Treatment of cells with EGF (epidermal growth factor) induces Akt-mediated phosphorylation of Rac1 at serine 71. Treatment of Hep2 cells with EGF led to increased formation of filopodia and alteration of the actin cytoskeleton, as shown by phalloidin-staining of actin-filaments. Formation of filopodia and actin reorganization was also observed in Hep2 cells transfected with the phosphomimetic mutant Rac1 S71E but not with wild-type Rac1. Overexpression of the constitutive active Rac1 (Rac1 G12V) showed the typical formation of membrane ruffles but not filopodia like structures. The cell morphology of Rac1 S71E transfected cells strongly differed from cells transfected with the dominant negative Rac1 (Rac1 T17N). In addition to the morphological changes Rac1 S71E protected Hep2 cells against *Clostridium difficile* toxin A-induced morphological changes. This finding is in correlation with the protective effect of EGF on the TcdA-induced alteration of the transepithelial electrical resistance of intestinal epithelial CaCo-2 cells. From this data we conclude that phosphorylation at serine 71 does not inactivate Rac1 but mediates local actin cytoskeleton rearrangements. Since it is not substrate for TcdA, phosphorylated Rac1 attenuates toxin-induced morphological changes of cells.
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96

Up-regulation of the immediate-early gene product RhoB by the cytotoxic necrotizing factor 1 from Escherichia coli
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The cytotoxic necrotizing factor 1 (CNF1) from uropathogenic Escherichia coli activates the low molecular mass GTP-binding proteins Rho, Rac, and Cdc42, thereby causing actin re-organization and multi-nucleation. This study is based on the concept that Rac1 positively regulates RhoB, an immediate early-gene product that has been suggested to negatively regulate multi-nucleation. We show that Rac1 is strongly activated in CNF1-tretaed cells, using PAK CRIB pull-down assay. RhoB is strongly up-regulated in CNF1-treated fibroblast. RhoB up-regulation is based on transcriptional activation, as it was responsive to inhibition by either actinomycin D or cycloheximide. Furthermore, RhoB up-regulation is reflected by activation of the rhoB promoter and an increased level of rhoB mRNA. The function of RhoB was studied in mouse embryonic fibroblasts (MEF). CNF1 induced multi-nucleation more efficiently in MEF RhoB (-/-) than in either MEF RhoB (+/-) or NIH3T3 fibroblasts, suggesting that RhoB suppresses multi-nucleation. Furthermore, CNF1 induced cell death in MEF RhoB (-/-) but not in MEF RhoB (-/+) or NIH3T3 fibroblasts, suggesting that RhoB signalling is required for cell survival. Our data suggest that RhoB plays a protective role in CNF1-treated fibroblasts, i.e. it suppresses multi-nucleation and cell death.
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97

Upregulation of the immediate-early gene product RhoB by the Ras-glucosylating *Clostridium sordellii* lethal toxin
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The immediate early gene product RhoB is involved in the regulation of vesicle trafficking, the actin-based cytoskeleton, and the cellular response to toxic stress. It is the only member of the Rho family of GTPases known to be subject to transcriptional regulation. We recently reported that treatment of cells with *Clostridium sordellii* lethal toxin (TcsL) caused an up-regulation of RhoB. TcsL modifies Rac1 and the Ras-GTPases H/K/N/R-Ras. *Clostridium difficile* toxin B from strain 1470, which modifies Rac1 and R-Ras, does not cause RhoB upregulation. Thus, RhoB up-regulation was a consequence of the inactivation of H/K/N-Ras. The up-regulation was inhibited by cycloheximide and actinomycin D, indicating that it was due to transcriptional activation. Accordingly, an increase of the rhoB promoter activity and mRNA level was observed after treatment of cells with TcsL.Stress kinases such as JNK or p38 MAPK may be involved in the regulation of the RhoB gene expression level. Treatment of cells with TcsL caused an increase of the level of phosphorylated p38 MAPK, indicating its activation. Inhibition of p38 MAPK attenuated TcsL induced RhoB up-regulation. Accordingly, RhoB protein up-regulation was lower in p38 -/- fibroblasts compared to wild type fibroblasts. This finding was reflected on the mRNA level. While the half life of rhoB mRNA was unchanged, rhoB promoter activity was attenuated in p38 -/- fibroblasts. Thus, the p38 MAPK pathway positively regulated the RhoB level by increasing its promoter activity. The cellular level of RhoB protein is also regulated by degradation. In TcsL treated fibroblasts, the RhoB level was governed by proteasomal- and caspase-dependent degradation. The activity of RhoB was determined by Rhotekin pulldown assay. RhoB activity increased strongly in TcsL-treated cells. Treatment of fibroblasts with TcsL for 6 h caused a loss of cellular viability. Inactivation of RhoB by *Clostridium limosum* C3 protected cells from this early loss of viability. Thus, RhoB positively regulated the initiation of TcsL-induced cell death.
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98

Difference in the formation of multi-nuclei induced by *Clostridium difficile* toxin B from strain VPI10463 and strain 1470
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Toxin B (TcdB) from C. difficile is a mono-glucosyltransferase that glucosylates and thereby inactivates the low molecular mass GTP-binding proteins RhoA, Rac1, and Cdc42. On cultured cells, glucosylation of Rho proteins causes actin re-organization ("cytopathic effect") and programmed cell death ("cytotoxic effect"). The formation of multi-nuclei has been described as the hallmark of toxins that activate Rho family proteins, among them the cytotoxic necrotizing factors from Escherichia coli and Yersinia pseudotuberculosis. Here, we show that inactivation of Rho proteins by the Rho-glucosylating TcdB or the Rho ADP-ribosylating exoenzyme C3 from C. botulinum induces the formation of multi-nuclei in several cultured cell lines. Multi-nucleation was analyzed by fluorescence microscopy of Hoechst33342-stained nuclei and polyploidy was detected by FACS analysis of propidium iodide stained cells. Whereas TcdB-treated NIH3T3 fibroblasts were bi-nucleated (4N population), macrophage-like J774A.1 cells exhibited up to 8 nuclei (16N population) after TcdB treatment for 48 h. In contrast, the Rac/Ras-glucosylating toxin B from the "variant" C. difficile serotype F strain 1470 (TcdBF) did not induce multi-nucleation. We analyzed DNA de novo synthesis in toxin-treated fibroblasts using BrdU incorporation assay and found that TcdBF but not TcdB inhibited DNA synthesis. Thus, the formation of multi-nuclei in toxin-treated cells correlated with intact DNA de novo synthesis. The inhibition of DNA de novo synthesis is a yet non-identified activity of TcdBF.
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99

Rac1 glucosylation is critical for actin re-organization induced by *Clostridium difficile* toxin B
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Glucosylation of Rho proteins by C. difficile toxin B (TcdB) causes actin re-organization (cell rounding, „cytopathic effect“). TcdB is classified as a broad-spectrum inhibitor of Rho proteins. In this study, we identified that Rho protein whose glucosylation is critical for the cytopathic effect. Therefore, the Rho substrate specificity of TcdB and Toxin B from the variant C. difficile serotype F strain 1470 (TcdBF) was re-analyzed in detail using [14C]glucosylation of recombinant Rho proteins. TcdB glucosylated Rho(A,B,C), Rac1, Cdc42, RhoG, and TC10. TcdBF glucosylated Rac1, Cdc42, RhoG, and TC10. Both TcdB and TcdBF induced the cytopathic effect, leading to the assumption that glucosylation of either Rac1, RhoG, Cdc42, or TC10 is critical for actin re-organization. This hypothesis challenges the general notion that actin re-organization induced by TcdB is due to glucosylation of RhoA, deduced from the observation that ADP-ribosylation of RhoA by C3 exoenzyme is sufficient to cause actin re-organization. The cytopathic effect of TcdB was analyzed in fibroblasts that ectopically expressed (non-glucosylatable) constitutively active Rho proteins. The cytopathic effect of TcdB was

prevented in cells expressing Rac1-Q61L but not in cells expressing RhoA-Q63L, suggesting that Rac1 glucosylation was critical for actin re-organization. Furthermore, cells expressing the Rac1 mimic IpgB1 (but not the RhoA mimic IpgB2) were protected from the cytopathic effect of TcdB, confirming that Rac1 rather than RhoA glucosylation was critical for actin re-organization. Finally, Rac1 glucosylation was analyzed in TcdB- and TcdBF-treated fibroblasts using Rac1-mAb(clone 102), an antibody that reacts with non-glucosylated but not with glucosylated Rac1. Rac1 glucosylation correlated with the cytopathic effect of either TcdB or TcdBF, further supporting that Rac1 glucosylation is critical for TcdB-induced actin re-organization.

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100

Selective and specific uptake of the C3-transferases from *Clostridium botulinum* and *Clostridium limosum* into the cytosol of macrophage-like cells require acidic endosomes

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The C3 transferases from *Clostridium botulinum* (C3bot1) and *Clostridium limosum* (C3lim) specifically mono-ADP-ribosylate and thereby inactivate RhoA, -B and –C. Therefore these enzymes are widely used as specific inhibitors to study Rho mediated cell signalling. Current state-of-the-art is that these C3 transferases lack a binding domain thus their uptake into cells most likely occurs via unspecific pinocytosis and is very poor. To overcome the poor accessibility of C3 proteins a recombinant fusion toxin, C2IN-C3lim, was constructed in our laboratory earlier. C2IN-C3lim essentially requires the transport protein C2IIa for its uptake into all yet tested cell types. Having this in mind we discovered that macrophage-like mouse cells (J774.A1) and non-differentiated human leukaemia cells (HL-60) are severely affected by low concentrations of C2IN-C3lim (100 ng/ml) even without the transport component C2IIa. This effect was not observed in any other tested cell type. On closer examination we found that J774.A1 cells respond to C2IN-C3lim, as well as on C3lim and C3bot1, in a time and concentration dependent manner with a dramatic morphological change. The cytopathic effect of C3 proteins strictly depended on the intracellular ADP-ribosylation of Rho mediated by the C3 transferases. We confirmed this by looking on the ADP-ribosylation state of Rho or using enzymatic inactive C3bot1E174Q. Bafilomycin A1, which specifically inhibits the vacuolar ATPase proton pump, blocked the release of C3 proteins into the cytosol. Thus C3 requires endosomal acidification for translocation. Moreover both C3 proteins were able to bind to J774.A1 cells. No considerable effect concerning apoptotic cell death was detectable even after 48h of incubation with C3. We can state that J774.A1 and HL-60 cells are specific targets for clostridial C3 transferases. Furthermore their uptake into the cytosol of these cell lines happens to be specific.

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101

Formins in cancer cell invasion

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Formins and RhoGTPase-regulated Diaphanous formins are a widely expressed protein family characterised by the presence of their conserved formin homology 2 (FH2) domain. So far, in mice and in human there have been 15 formins identified that can be subdivided into 7 subfamilies. Formin proteins control actin- and microtubule-dependent processes such as cytokinesis, cell shape and cell motility. Recently, we showed that the Diaphanous formin Dia1 binds to the leukaemia-associated Rho-GEF (LARG) through RhoA-dependent release of Dia1 autoinhibition. Our results revealed that Dia1 is necessary for LPA-stimulated Rho/ROCK signalling and bleb-associated cancer cell invasion, showing that formins might have crucial functions in invading cancer cells. However, only very few formins have been characterised for their biochemical and cellular function and thus little can be said about the principal and specific role of formins in cancer cell invasion. Using an siRNA based screen against all human formins followed by analysis in different models of cell invasion assays we study the impact of formin proteins on tumour cell invasion into 3D matrices. Current results of this study will be discussed.

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102

A new modulator of Formin-induced SRF activity controls cancer cell invasion and acts as a potential tumor suppressor

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Serum Response Factor (SRF) belongs to the MADS box family of transcription factors and controls a wide array of gene expression via interactions with TCF family members through MAPK signaling or by Rho/mDia1-induced alterations in actin dynamics that regulate the SRF coactivator MAL. MAL, in turn, instructs SRF-dependent transcription of a large set of cytoskeletal target genes involved in cell motility and adhesion. Here we describe a novel factor, which interferes with Rho-actin signaling to SRF to modulate transcription. Its effects are facilitated by binding to MAL and subsequent formation of a nuclear ternary complex with SRF. This novel factor is able to lead to changes in the expression profile of SRF target genes and has been found to have a strong impact on cancer cell invasiveness. Interestingly its transcript levels appear to be downregulated in different human cancers, pointing towards a potential role in tumor suppression.

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103

p63RhoGEF, Trio and Kalirin/Duet form a subfamily of guanine nucleotide exchange factors mediating Gq-dependent RhoA activation – detailed insights by co-crystalization of the Gq/p63RhoGEF/RhoA complex

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We have recently described the guanine nucleotide exchange factor p63RhoGEF as a specific and direct mediator of the Gq protein family-dependent activation of the monomeric GTPase RhoA. By gradual truncation of p63RhoGEF, we could identify the essential binding site of Gaq and generate a minimal fragment allowing the binding of Gαq and the activation of RhoA. Co-crystalization of this minimal fragment together with Gαq and RhoA in one complex gave detailed information about structural determinants mediating this interaction. By site directed mutagenesis, we further confirmed the obtained data in in vitro and in cell based assays and we could demonstrate that a small region of about 10 amino acids following the Pleckstrin homology (PH) domain of p63RhoGEF is essential for the interaction of the G protein and the GEF. Sequence comparisons unearthed two other GEFs, Trio and Kalirin/Duet, which are also containing this Gαq-binding region. Therefore we analysed the ability of truncated and full length versions of both GEFs to mediate Gαq-dependent RhoA activation and Gαq binding. Trio and Kalirin are both multi-function proteins containing two sets of the catalytical Dbl-homology (DH) /PH tandem motifs of which the C-terminal one is specific for RhoA. Both GEFs exists in various splice variants including TrioE and Duet (variant from Kalirin) containing only the RhoA specific DH/PH motif. We could show that these proteins can bind to and be activated by Gαq.

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104

K-Ras-induced expression of the neural adhesion molecule NCAM perturbs E-cadherin-mediated cell-cell adhesion

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Inhibition of cellular adhesion between epithelial cells represents an early step in tumor metastasis and perturbation or down-regulation of E-cadherin-mediated adherens junctions is an essential requirement in this process. The influence of oncogenic Ras on epithelial to mesenchymal transition (EMT) is well documented. However, the role of individual Ras isoform, especially K-Ras in EMT and E-cadherin-mediated cellular adhesion is poorly characterized. Here we report that expression of oncogenically activated K-Ras leads to induction of highly polysialylated neural adhesion molecule NCAM in pancreatic carcinoma and kidney-derived HEK293 cells, a molecule which is usually detected in neural cells. Co-immunoprecipitation analyzes reveal that polysialylated NCAM (PSA-NCAM) binds directly to the E-cadherin/catenin tumor suppressor complex. The increased association of PSA-NCAM with the E-cadherin complex correlates with decreased cell-cell aggregation and elevated cell migration of pancreatic carcinoma cells. Enzymatic removal of polysialic acid from NCAM as well as down-regulation of expression of polysialyltransferases by siRNA results in reduced association between NCAM and E-cadherin, which subsequently increases E-cadherin-mediated cell-cell aggregation. Our data suggest that the induction of PSA-NCAM by oncogenic K-Ras results in reduced E-cadherin-mediated cellular adhesion, most likely by sterical hindrance of homophilic interaction of E-cadherin proteins, even in the presence of high amounts of E-cadherin. Altogether, these data suggest a novel molecular mechanism by which oncogenic K-Ras might contribute to the high invasiveness of pancreatic adenocarcinomas. This work was supported by the Deutsche Krebshilfe and the DFG (SFB518).

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105

ARF-related protein1 (ARFRP1) is required for sufficient targeting of E-cadherin to the cell surface

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ADP-ribosylation factor related protein 1 (ARFRP1) is a member of the ARF-family of GTPases which act as molecular switches. We have recently shown that ARFRP1 controls the ARL1-mediated Golgi recruitment of GRIP domain proteins and therefore plays a specific role for the Golgi function. Deletion of the mouse Arfrp1 gene is embryonic lethal during early gastrulation presumably due to an adhesion defect. Recently we presented data demonstrating that in the Arfrp1-/- embryos E-cadherin is dislocalized in intracellular compartments while in control embryos E-cadherin is located at the cell surface of trophectodermal and ectodermal cells. Here we show that in enterocytes of intestine-specific Arfrp1 null mutants (Arfrp1vil-/-) E-cadherin is associated with intracellular membranes or in punctae close to the cell surface while it was exclusively located in the lateral membranes of control enterocytes. In MDCK (Madin-Darby canine kidney) cells stably expressing myc-ARFRP1, ARFRP1 was co-immunoprecipitated in a complex with E-cadherin, a-catenin, b-catenin, g–catenin and p120ctn, indicating that a direct interaction of ARFRP1 with the E-cadherin complex is required for correct targeting of this adhesion protein. Immunohistochemical analysis showed that the organization of the trans-Golgi network (TGN) was impaired in Arfrp1vil-/- enterocytes because ARL1 and the trans-Golgi marker TGN38 were dislocated from Golgi membranes. In contrast, no difference in the pattern of the cis-Golgi marker GM130 was observed between control and knockout cells. Therefore, we suggest that ARFRP1 plays an essential role for processing and/or targeting of the E-cadherin complex through the TGN.

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106

Altered glucose metabolism in mice lacking ARFRP1 in the liver

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The liver plays an important role for the maintenance of glucose homeostasis. In order to analyse the relevance of protein trafficking through the trans-Golgi for the function of the liver we investigated the role of the ADP-ribosylation factor related protein1 (ARFRP1) in liver-specific null mutants. We have shown recently that the Ras-related membrane-associated monomer GTPase ARFRP1 plays an important role in the organisation and function of the trans-Golgi network. GTP-bound ARFRP1 co-localizes with ARL1 at the Golgi apparatus and recruits ARL1 and its effector Golgin-245 to the trans-Golgi network indicating that ARFRP1 is required for the organisation of this intracellular compartment. For a liver-specific deletion of Arfrp1 (Arfrp1liv-/-) Arfrp1flox/flox mice were crossed with mice expressing the Cre recombinase under the control of the hepatocyte-specific albumin promoter (Alb-Cre). Arfrp1liv-/- mutants show an early significant growth retardation (week 5: Arfrp1flox/flox 22.1 ± 2.5 g; Arfrp1liv-/- 16.0 ± 2.1 g; $p < 0.001$) accompanied by significantly reduced liver weight (Arfrp1flox/flox 1.28 ± 0.26 g; Arfrp1liv-/- 0.83 ± 0.18 g; $p < 0.001$). In addition, Arfrp1liv-/- mice exhibited decreased blood glucose and glycogen levels in the liver. mRNA levels of glycolytic enzymes and of glucose transporters GLUT2 and GLUT9 were increased. In contrast, protein levels of both transporters were remarkably reduced in Arfrp1liv-/- livers indicating that uptake and/or release of glucose were affected. Our results indicate that Arfrp1 plays an important role in the function of the liver and in glucose metabolism. We suggest that defects in translational control or processing of proteins like GLUT2 are responsible for the phenotype of liver-specific Arfrp1 null mutants.

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107

Structural requirements for isozyme-specific activation of phospholipases C-beta-2 and -gamma-2 by Rac GTPases

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We have recently described a novel mechanism of phospholipase C (PLC) activation by Rho GTPases. Two PLC isozymes, PLCβ2 and PLCγ2, are specifically sensitive to this activation. PLβ2 is activated by members of the Rac and Cdc42 subfamilies, whereas PLCγ2 is specifically activated by Rac. To determine the structural requirements of the Rho-PLC-interaction, several mutants of Rac2 and Cdc42Hs were functionally reconstituted with PLCβ2 and PLCγ2 in intact cultured cells and in a cell-free system made up of recombinant proteins. The results show that carboxyl-terminal isoprenylation is both necessary and sufficient for Rac2-mediated activation of PLCβ2 and PLCγ2 in intact cells and in the cell-free system. A tryptophan specifically present at the N-terminal end of the switch II region of Rac, but not of Cdc42, has previously been suggested to be an important element of the intermolecular interface between Rac1 and PLCβ2 [Jezyk et al. (2006) Nat. Struct. Mol. Biol. 13:1135]. Furthermore, the presence of a phenylalanine in the corresponding position of Cdc42 has been proposed to be responsible for the relatively low potency and efficacy of Cdc42 to activate PLCβ2. Surprisingly, we found that Rac2(W56F) was indistinguishable from wild-type Rac2 in its ability to activate PLCβ2 in intact cells, but was largely ineffective as an activator of PLCγ2. In addition, Cdc42Hs(F56W) was not appreciably different from wild-type Cdc42 as an activator of PLCβ2 in intact cells, and both proteins were incapable of activating PLCγ2. The results suggest that the structural requirements and the molecular mechanisms of Rac-GTPase-mediated activation of PLCβ2 and PLCγ2 are similar, if not identical, with respect to the C-terminal posttranslational modifications, but distinct in terms of the activation-dependent interactions mediated by the switch II region of Rac.

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108

Rac2 regulates its effector phospholipase C-γ2 through interaction with a split PH domain

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Small GTPases like Rac2 regulate diverse signalling pathways by interacting with distinctive downstream effectors. We have identified phospholipase C-γ2 (PLCγ2) as a new effector of Rac. In order to identify the structural elements of PLCγ2 that are necessary for its interaction with Rac2, we constructed chimeric proteins of PLCγ2 and PLCγ1 with either their N-terminal pleckstrin homology or their split pleckstrin homology (spPH) domains swapped. The results of the former experiments suggested that the spPH domain of PLCγ2, rather than its N-terminal PH domain, serves as an effector site for Rac. To validate the role of the PLCγ2 spPH domain as the site of Rac interaction, we purified the isolated spPH domains of PLCγ2 and PLCγ1 to carry out interactions studies. By the use of isothermal titration calorimetry, we found that Rac2 in its GTP-, but not in its GDP-bound form, binds directly to the PLCγ2 spPH domain. The binding of Rac2 to the spPH domain was isoform-specific, as no measurable interaction with the PLCγ1 spPH domain could be detected. In order to determine the specific site on the PLCγ2 spPH domain that interacts with Rac2, we carried out titration NMR experiments and mutational analyses. We identified three amino acid residues around β strand 5 and within the α helix of the C-terminal half of the PLCγ2 spPH domain as important for activation by Rac2. Interestingly, these residues are all clustered on the surface of the spPH domain. Taken together, our results suggest that the two PLC isozymes sensitive to activation by Rac GTPases, PLCγ2 and PLCβ2, both interact with activated Rac2 through a PH domain, but that the site (internal spPH versus N-terminal PH domain) and the mechanisms of interaction are distinct.

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109

Effect of the cytotoxic necrotizing factors from Escherichia coli and Yersinia pseudotuberculosis on cell division and cell viability

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The cytotoxic necrotizing factor 1 (CNF1) is produced by uropathogenic Escherichia coli strains (UPECs). The toxin interferes with various cellular functions as it permanently activates the Rho family GTP-binding proteins Rho, Rac and Cdc42, by catalyzing their deamidation at a specific glutamine residue. Another CNF isoform, the cytotoxic necrotizing factor from Yersinia pseudotuberculosis (CNFY), has been shown to selectively activate Rho. A hallmark of CNF1 and CNFY is the induction of multinucleated cells, reflecting the role of Rho proteins in cell cycle progression. In this study we analyzed the effect of CNF1 and CNFY on cell division, cell viability and protein content in NIH3T3 fibroblasts. In a proliferating population of cells, the number of cells exponentially increased up to 48 h, until cell division is blocked by contact inhibition. In a population treated with either CNF1 or CNFY, the number of cell is almost unchanged for several days, as the toxins blocked cell division and prevented cell death. Interestingly, the viability (as determined by WST-1 assay) and the protein content (determined by Bradford test) comparably increased in CNF1/CNFY-treated and untreated (proliferating) cells. In the CNF populations, this increase is apparently based on increased metabolic activity, whereas this increase in proliferating cells is based on the increased number of cells. These findings challenge the notion that the CNF toxins promote cellular proliferation.

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110

Formation of multi-nuclei by the cytotoxic necrotizing factor 1 from Escherichia coli

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Escherichia coli causes infections of the urinary tract. Uropathogenic E. coli strains produce a number of virulence factors, among them the cytotoxic necrotizing factor 1 (CNF1). CNF1 enters its target cell by receptor-mediated endocytosis and permanently activates low molecular weight GTP-binding proteins of the Rho subfamily by catalyzing de-amidation of Gln-63 in RhoA or RhoB (or the respective Gln-61 in Rac1 or Cdc42) into Glu. This activation modulates a high number of cellular functions, including the reorganization of the actin cytoskeleton, the promotion of cell spreading, and multi-nucleation.In this study we analyzed the CNF1-induced formation of multi-nuclei in different cell lines. We found that NIH3T3 fibroblasts were highly susceptible to multi-nucleation, whereas CNF1 less efficiently induced multi-nuclei in African green monkey epithelial (Vero) cells. We further investigated the cellular level of Rho proteins in CNF1-treated cells using Western blot analysis and found that RhoA was degraded in fibroblasts but not in Vero cells. This observation is likely based on the fact that Vero cells are deficient in Smurf-1, an ubiquitin-ligase, required for the proteasomal degradation of RhoA and RhoB. We conclude that RhoA and RhoB suppress the formation of multi-nuclei in CNF1-treated Vero cells.

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111

Programmed cell death induced by *Clostridium sordellii* lethal toxin in macrophage-like J774A.1 cells

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Clostridium sordellii causes rapidly progressing myonecrosis in obstetric patients and necrotizing fascitis in injection drug users, in severe cases accompanied by a fulminant toxic shock syndrome. The severity of *C. sordellii*-associated diseases is due to the release of soluble virulence factors, most toxic (in terms of the LD50) among them the Lethal Toxin (TcsL) and the Hemorrhagic Toxin (TcsH). On cultured cell lines TcsL and TcsH cause actin re-organization ("cytopathic effect") and programmed cell death ("cytotoxic effect").In this study, the cytotoxic effect of TcsL was analyzed in S-phase macrophage-like J774A.1 cells, in terms of up-regulation of pro-apoptotic RhoB, phosphatidylserine exposure (Annexin V staining), and the reduction of metabolic activity (WST-1 asssay), and the fragmentation of nuclei. The cytotoxic effect was responsive to inhibition by SB203580, an inhibitor of p38 MAP kinase. Unlike other clostridial glucosylating toxins such as the *Clostridium difficile* Toxin B (TcdB), TcsL did not cause multi-nucleation. TcsL (but not TcdB) inhibited DNA de novo synthesis (as determined by BrdU incorporation assay), which offers an explanation for the lack of multi-nucleation in TcsL-treated cells. Inhibition of DNA de novo synthesis, however, is a not yet described aspect of the cytotoxic effect of TcsL.

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112

The expression of *Clostridium difficile* toxin E affects bacterial growth

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Clostridium difficile is a grampositiv anaerobic bacterium that produces two single chain protein toxins A and B. These toxins are glucosyltransferases that inhibit small GTPases of the Rho subfamily. These toxins are causative for the observed clinical symptoms of diarrhea and pseudomembranous colitis.The pathogenicity locus of C. difficile exhibits an open reading frame (tcdE) located between the genes for the toxins A and B encoding the 19 kDa protein toxin E. Toxin E (TcdE) is a highly hydrophobic protein of unknown function. It shows significant sequence homology to bacteriophage-encoded

holins which are lytic proteins causing cell death of bacterial host cells. This finding arose the hypothesis that TcdE cause cell lysis to facilitate the release of the toxins A and B to the extracellular environment. This hypothesis is based on simultaneous expression of the three toxins. Western blot analysis showed the appearance of TcdE in pathogenic C. difficile (strain VPI10463, 1470, 75, 196) as well as in C. sordellii (strain 6018, G5, VPI9048) which correlated with the accumulation of the respective glucosyltransferases. We further investigated the effect of TcdE on prokaryotic cells to get insight into its functional ability. An expression construct encoding recombinant histidin-tagged toxin E was generated. TcdE expression in Escherichia coli resulted in a sudden loss of culture turbidity and a transient inhibition of bacterial growth already within one hour after induction. For specific detection of TcdE we generated polyclonal anti-TcdE serum. The expression of TcdE in E.coli lysates was detected by Western blot analysis and was in strong correlation with inhibitory effects on the growth of bacteria. Further constructs containing smaller fragments of TcdE were generated giving the possibility to locate the functional regions of the protein. Expression in a eukaryotic system will help to characterize a direct effect of TcdE on host cells in addition to the postulated facilitation of the export of Rho modifying toxins from the pathogenic C. difficile.
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113

Multiple regulatory roles of the phospholipase C-γ split pleckstrin homology domain

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Phosphoinositide-specific phospholipase C-γ isoforms (PLCγ1 and PLCγ2) are enzymes that catalyze the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate into the second messengers diacylglycerol and inositol 1,4,5-trisphosphate, thereby governing diverse cellular processes. Both PLCγ isoforms contain two pleckstrin homology (PH) domains, an amino-terminal one and one located between the two catalytic subdomains and split by a SH2-SH2-SH3-tandem. This peculiar "split" PH domain has been found to be an effector site for Rac GTPases in PLCγ2, to contribute to membrane lipid binding, to regulate PLCγ activity via an autoinhibitory mechanism, and to regulate TRPC3 (transient receptor potential, canonical, type 3) cation channel location and function by a novel mode of protein-protein-interaction, in which two PH domain fragments associate to form an intermolecular PH domain. Here, we report findings on the regulatory role of the split PH domain of PLCγ. The split PH domains of PLCγ1 and PLCγ2 were expressed and purified either individually as domain fragments or contiguously as fusion proteins lacking the SH2-SH2-SH3-tandem. Binding of these fragments to phospholipids was assayed. In fluorescence correlation experiments, PH domain fragments bound to purified full-length PLCγ isozymes. Most intriguingly, PLCγ activity was increased in vitro in the presence of PH fragments, supporting the concept that intermolecular PH domains can form between PLCγ and other proteins. Furthermore, our results suggest that PLCγ activity is directly affected by such a mechanism, presumably by a relieve of the enzyme from autoinhibition upon PH domain unfolding.
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114

Activation of Rho GTPases by the human CC chemokine receptors 2a and 2b

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Chemokines constitute a growing superfamily of small cytokines involved in regulating a wide array of leukocyte functions, including chemotaxis, adhesion, and transendothelial migration. Transmembrane signaling of chemokines is mediated by chemokine receptors, members of the G protein-coupled receptor (GPCR) family, which are coupled to pertussis toxin-sensitive and in certain cases, also pertussis toxin-insensitive G protein(s). As previously reported, expression of the human CC chemokine receptors CCR2a and CCR2b in COS-7 cells induced a MCP-1-dependent increase in inositol phosphate formation, which is coupled to the activation of G proteins of the Gq family. It is well known that activation of Gq proteins by cell-surface receptors is linked to activation of the RhoGTPase RhoA, which in turn increases the activity of the transcription factor serum response factor (SRF). The marked activation of Gq proteins by CCR2a and CCR2b prompted us to investigate the activation of SRF and the involvement of RhoGTPases in this process. The results presented herein demonstrate that expression of either CCR2a or CCR2b in COS-7 cells caused a ligand-dependent vigorous induction of SRF activity. This effect was specifically mediated by Gaq and Gα14, but not by Gα11 and Gα16. Rho guanine nucleotide exchange factors (RhoGEFs) and RhoGTPases were involved in the stimulation of SRF activity by CCR2a and CCR2b, since coexpression of the Gαq-specific RhoGEF p63RhoGEF and/or RhoA led to synergistic effects on SRF-induced gene transcription and coexpression of *Clostridium botulinum* ADP-ribosyltransferase C3 abolished the stimulatory effect of the receptors. In addition, as shown by coprecipitation experiments ligand-induced activity of CCR2a and CCR2b largely increased the cellular amount of activated RhoA in HEK293 cells. Taken together, our data indicate that CCR2-induced SRF-dependent gene transcription is specifically mediated by the pertussis toxin-insensitive G proteins Gaq and Gα14 via a RhoGEF- and RhoAGTPase-dependent pathway.
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115

Bimodal control of RhoGDI by the FERM-domain-containing proteins merlin and ezrin

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pUS28, a chemokine receptor homologue encoded by the human cytomegalovirus (HCMV) genome, has been shown to regulate a broad spectrum in signalling pathways related to migration and proliferation. For example, pUS28 is able to constitutively activate the transcription factor serum response factor (SRF), which is involved in controlling many cellular functions, including cytoskeletal organization. The pUS28-triggered SRF activation is mediated by Gq family members and by RhoGTPases. The activation and deactivation as well as the subcellular distribution of RhoGTPases is regulated by Rho GDP dissociation inhibitors (RhoGDIs). RhoGDIs have previously been shown to interact with Merlin (foe moesin, ezrin, and radixin-like protein), although the functional consequences and the pathophysiological relevance of this interaction remained elusive. Merlin is a tumor suppressor and the protein product of neurofibromatosis type 2 gene (NF2). It is a member of 4.1-protein superfamily, which includes ezrin, radixin, and moesin and other proteins characterized by a conserved FERM (four-point-one, ezrin, radixin, and moesin) domain at their amino termini. FERM domain containing proteins regulate actin remodelling, cell contact events, and others cellular functions. The current study was undertaken to investigate the effects of exogenous RhoGDIα, an ubiquitously expressed RhoGDI variant, and Merlin-1 on pUS28-mediated activation of SRF in transfected COS-7 cells. Our results showed that expression of RhoGDIa caused a marked inhibition of pUS28-mediated SRF activation and that coexpression of Merlin-1 enhanced this inhibitory effect. On the other hand, expression of an active variant of ezrin, ezrin (T567D) reduced the inhibitory effect of RhoGDIa. The results suggest that RhoGDI proteins are subject to bimodal, stimulatory and inhibitory, control by FERM domain-containing proteins such as Merlin-1 and Ezrin.
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116

Transgenic mice ubiquitously expressing cAMP sensor Epac1-camps for in situ and in vivo cAMP imaging

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Fluorescence resonance energy transfer (FRET) using genetically encoded biosensors is a powerful technique to monitor signalling events in living cells. Recently, we have developed several small but highly sensitive cAMP sensors based on single cyclic nucleotide binding domains fused to cyan and yellow fluorescent proteins. More recently, we have generated transgenic mice with the heart-specific expression of one of these sensors to study spatio-temporal dynamics of cAMP signalling in cardiac myocytes.Here, we demonstrate that our cAMP sensor Epac1-camps can be ubiquitously expressed in transgenic mice under the control of the β-actin promoter, not interfering with their proper development, life expectancy and physiology. These animals express the sensor in virtually all tissues and can be used for the real-time cAMP imaging in different types of freshly isolated primary cells, including fibroblasts, neurons, cardiac myocytes, macrophages and pituitary cells. Finally, we show that these mice can serve as a tool for in vivo imaging of cAMP by monitoring FRET in living pituitary slices. In conclusion, the FRET-based cAMP sensor Epac1-camps was successfully expressed in ubiquitous manner in transgenic mice, which can now be used for real-time studies of the spatial and temporal dynamics of this important second messenger in situ and in vivo.
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117

Ca2+-mediates regulation of cAMP levels in endothelial cells

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Endothelial cells form a semipermeable barrier to separate circulating blood elements from underlying tissue. The permeability of endothelial cells is regulated both by Ca2+ and cyclic nucleotide signals. An acute rise in intracellular Ca2+ catalyses the formation of intercellular gaps that is blocked by a rise in cAMP at cell membranes. Therefore Ca2+- and cAMP-signals have opposing effects regarding endothelial barrier function. As an impact of Ca2+ signals on cAMP gradients is widely accepted, analysis of these signals in living cells is very important. To study the crosstalk of Ca2+ and cAMP signals in endothelial cells, we utilized a FRET-based sensor (Nikolaev et al., JBC, 2004) to detect cAMP gradients in single living cells. Therefore HUVECs were transfected with an Epac1-based FRET sensor via electroporation. For investigation of Ca2+ signals and kinetic analysis of Ca2+ and cAMP gradients, Ca2+ signals were imaged in Fura-2AM loaded cells. To analyse a potential impact of a rise in intracellular Ca2+ on basal cAMP levels of less than 0.2µM, we stimulated HUVECs with ATP. Superfusion of single cells with ATP resulted in a fast transient rise in intracellular Ca2+. Subsequently a several fold increase in cAMP concentrations was detected. Kinetic analysis of Fura and FRET ratios revealed a delay of 43.5s (t1/2) between Ca2+ and cAMP peaks. This positive impact of Ca2+ on cAMP signals was also verified by stimulation of cells with the Ca2+ ionophor A23187. However, when cAMP levels were elevated above 2.5µM, an ATP-mediated Ca2+ release resulted in robust inhibition of cAMP levels. The transient rise in Ca2+ led to an approximately tenfold decrease in cAMP levels with a delay of 17.6s (t1/2). Overall these results reveal an opposing impact of Ca2+ signals on cAMP gradients depending on intracellular cAMP levels. This can be due to activation or inhibition of different Ca2+-dependent adenylylcyclases or due to activation of Ca2+-dependent PDE1. The mechanisms involved in the opposing effects of Ca2+ signals will be investigated in future.
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118

A fluorimetric assay for adenylyl cyclase activity facilitating real-time enzyme kinetics and high throughput screening

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Adenylyl cyclases (ACs) catalyze the production of the second messenger cAMP from ATP in mammalian cells with pyrophosphate (PPi) being formed as a byproduct. In mammals nine membrane-bound and one soluble AC isoform is known to date. Due to many different functions of ACs in various cell types, the AC isoforms are potential drug

targets. We have developed a fluorescent AC assay which is applicable to high throughput screening and kinetic determinations, thereby directly monitoring the turnover of the substrate ATP. A fluorescence assay was designed implementing the Tb(III)-norfloxacin (TbNflx) complex for monitoring enzyme activity. TbNflx shows a five-fold increase in fluorescence upon addition of 1:1 ATP:Tb(III), a five-fold decrease upon addition of 1:2 PPi:Tb(III) and no change in fluorescence characteristics upon addition of cAMP. Therefore, a decrease in lanthanide luminescence is observed upon conversion of ATP to cAMP and PPi by AC. As model the Ca(II)- and calmodulin (CaM)-dependent AC toxin edema factor (EF) from Bacillus anthracis was used because of its high catalytic activity and good water-solubility. This new fluorescent assay allows the determination of the Michaelis-Menten-constant KM and the v0max-value of ATP turnover as well as the determination of IC50-values for inhibitors of EF activity. The TbNflx assay is applicable to high throughput screening of enzyme regulators, allows real-time monitoring of AC activity and does not substitute the substrate ATP by fluorescent derivatives. Furthermore, the new assay renders the application of radioactively labeled substrates such as [a-32P]ATP or fluorescently labeled antibodies like anti-cAMP redundant. The TbNflx assay is inexpensive, straightforward and fast, and enables screening of libraries of potential therapeutics in order to select drug candidates.

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119

Neuronal AKAP150 coordinates PKA and EPAC mediated PKB/AKT phosphorylation

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In diverse neuronal processes ranging from neuronal survival to synaptic plasticity cyclic adenosine monophosphate (cAMP)-dependent signaling is tightly connected with the protein kinase B (PKB)/Akt pathway but the precise nature of this connection remains unknown. In the current study we investigated the effect of two mainstream pathways initiated by cAMP, cAMP-dependent protein kinase (PKA) and exchange proteins directly activated by cAMP (Epac1 and Epac2) on PKB/Akt phosphorylation in primary cortical neurons and HT-4 cells. We demonstrate that PKA activation leads to an inhibition of PKB/Akt phosphorylation, whereas activation of Epac has the opposite effect. PKA, PKB/Akt and Epac2, but not Epac1, were all shown to establish complexes with the neuronal A-kinase anchoring protein 150 (AKAP150). Activation of Epac2 increased phosphorylation of PKB/Akt complexed to AKAP150, and silencing of cellular Epac2 diminished this cellular response. From experiments using PKA-binding deficient AKAP150 and the AKAP inhibitory peptides, we conclude that AKAP150 act as a key regulator in the two cAMP pathways to control PKB/Akt phosphorylation.

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120

Different mechanisms of cGMP-mediated relaxation in colon and jejunum smooth muscle from mice

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cGMP dependent protein kinase I (cGKI) in intestinal smooth muscle may induce relaxation via several mechanisms that include inhibition of intracellular Ca2+ release by phosphorylation of the inositol-1,4,5-phosphat receptor associated G-kinase substrate (IRAG) and increasing myosin phosphatase activity by phosphorylation of the myosin phosphatase targeting subunit 1 (MYPT1). In the present study, we investigated whether one or both mechanisms are involved in cGMP-mediated inhibition of carbachol-stimulated contraction of colon and jejunum. In simultaneous recordings of tension and intracellular Ca2+ signals, 8-Br-cGMP reduced both carbachol-induced tension and intracellular Ca2+ signals in colon muscle. In contrast, in jejunum muscle, 8-Br-cGMP reduced tension but did not change intracellular Ca2+ signals. In colon muscles from mice exhibiting a mutated IRAG (IRAGΔ2), 8-Br-cGMP did not reduce carbachol-induced tension and Ca2+ signals. Inhibition of phosphatase activity by calyculin A abolished relaxation of carbachol-induced contraction by 8-Br-cGMP in jejunum but not in colon muscle. Western blot analysis showed a larger signal for phosphorylated MYPT1 in carbachol–stimulated jejunum than in colon muscle. These result suggest that cGMP/cGKI induces relaxation mostly by inhibition of intracellular Ca2+ release in colon and by activation of myosin phosphate in jejunum. Supported by a grant from Deutsche Forschungsgemeinschaft and from SFB 391.

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121

Dual acylation of PDE2A splice variant 3: targeting to synaptic membranes

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Cyclic nucleotides play an important role as second messengers in many signal transduction pathways. Therefore, a tight control of their intracellular concentration via regulation at the level of synthesis and degradation is required. The enzymes responsible for the elimination of cAMP and cGMP are the cyclic nucleotide phosphodiesterases. (PDEs) Of these enzymes 11 different families have been identified, with each family having several different isoforms and splice variants.PDE2A, a dual substrate PDE, hydrolyzes both cGMP and cAMP. Three splice variants have been described in different tissues and cellular compartments. Although the divergent N termini of the splice variants have been suggested to determine their subcellular localization, the mechanism for the membrane attachment remains unknown. Here, we show that the splice variant PDE2A3 is targeted to the plasma membrane via dual acylation of its unique N-terminal sequence. Myristoylation of Gly2 is required to target the newly synthesized protein to the Golgi where Cys5 and possibly Cys11 are palmitoylated. Palmitoylation serves as a signal for membrane targeting. Mutation of the respective residues led to a cytosolic distribution of the otherwise membrane bound holoenzyme in HEK 293 cells. Furthermore, the fusion protein of the N terminus of PDE2A3 and CFP expressed in HEK 293 and PC12 cells was clearly confined to ER/Golgi and the plasma membrane, whereas the myristoylation site mutant (G2A) was cytosolic as observed by fluorescence microscopy. Substitution of the putatively palmitoylated cysteines-5 and -11 impeded plasma membrane targeting, but did not interfere with Golgi localization. Our tissue distribution analysis revealed PDE2A3 as the main PDE2A enzyme in mouse brain, where it was found at synaptic membranes. As the described targeting mechanism appears to hold true for primary hippocampal pyramidal cells, we suggest that dual acylation enables synaptic targeting of PDE2A3.

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122

Inhibition of adenylyl cyclases by anthraniloyl-group derived nucleotides

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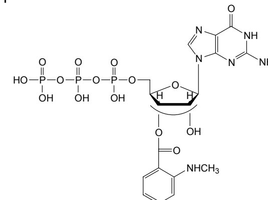
Monosubstituted 2´ (3´)-(methylanthraniloyl-) ribosyl-nucleotides (MANT-nucleotides) constitute a novel class of competitive adenylyl cyclase (AC) inhibitors [1] (scheme1). Originally used as a fluorophore applicable for a broad variety of biochemical experiments the MANT-group itself causes the inhibitory effect. Therefore the MANT-substituted nucleotides are highly suitable compounds for fluorescence based experiments, including investigations on soluble Bordetella pertussis CyaA adenylyl cyclase toxin and for the determination of binding affinities [2]. In this study newly synthesized MANT-nucleotides were tested for their potency and selectivity at mammalian AC and at CyaA toxin. At the mammalian membranous AC isoform V the tested compounds showed strong inhibition at nano molar concentrations. Compared to the monosubstituted nucleotides, the di-substitution of the ribose at both hydroxyl positions results in unique fluorescence characteristics for the testing of CyaA toxin due to an improved signal to noise ratio. Moreover these derivatives exhibit even higher inhibitory potencies compared to the mono MANT-nucleotides.



Scheme 1: mono substituted MANT-nucleotide with the nucleobase guanine (MANT-GTP). [1] Gille, A.; Seifert, R. J. Biol. Chem. 2003, 278, 12672-12679 [2] Göttle, M.; Dove, S.; Steindel, P.; Shen, Y., Tang, W.-J.; Geduhn, J.; König, B.; Seifert, R. Mol Pharmacol 2007, 72, 526-535

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123

Cyclic nucleotide analogs as probes for signaling pathways

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Introduction: The important feature of this work was to measure 13 of the most commonly used cyclic nucleotide analogues on protein kinase A I and II (PKA I and II), protein kinase G Iα, Iβ and II (PKG Iα, Iβ and II), and the guanine nucleotide exchange factor Epac I to address the question of possible target cross reactivity. The study was extended to eight cyclic nucleotide hydrolysing phosphodiesterases (PDE IA, IB, IC, II, IV, V, VI and X), thereby introducing microcalorimetry to overcome radioactive labeling of the tested analogs and allowing analysis of the kinetic parameters Km, Ki and Vmax. Results and Discussion: We successfully used isothermal titration microcalorimetry (ITC) for analysing the kinetic parameters of eight PDEs by various cAMP- and cGMP-analogs. The measured data reflect very well the reported literature and the technique offers significant advantages over traditional microbiological methods. The measuring of both, kcat and Km, provide additional insight into protein active sites by determining competitive, non-competitive and linear-mixed inhibition of the PDEs by the cyclic nucleotides. To our surprise, some accepted hydrolysis-stable analogs became degraded by the newer phosphodiesterases or show high inhibitory effects. For example, 8-pCPT-2`-O-Me-cAMP, a highly selective analog for Epac in vitro was hydrolysed by PDE5 and PDE10 with efficiency similar to cGMP and inhibited PDE2 and PDE6 with a Ki comparable to the Km for the hydrolysis of cAMP or cGMP. The often used PKG activator 8-pCPT-cGMP was hydrolysed by PDE5 and PDE10 and also by PDE4. Cross activation was seen, for example, with the potent activator of PKA, Sp-5,6-DCl-cBIMPS, This analog activated Epac with a higher affinity than cAMP. The defined specificity profiles of the tested cyclic nucleotides are presented in clearly arranged tables and will help to interpret in vivo data in the light of the cyclic nucleotide receptor proteins present in a given cell. An extended overview of the data is accessible to the scientific community at: www.cyclic-nucleotides.org.

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124

Pharmacological characterization of a phosphodiesterase 2A reporter cell line

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Cyclic nucleotide-specific phosphodiesterases (PDEs) play an essential role in cellular signal transduction by regulating the intracellular levels of cAMP and cGMP and, therefore, are important pharmacological targets. We report here the generation and pharmacological characterization of a PDE 2A reporter cell line. A plasmid construct encoding human PDE 2A was stably transfected in a parental cell line expressing the atrial natriuretic peptide (ANP) receptor and the cyclic nucleotide-gated (CNG) cation channel CNGA2, acting as the biosensor for intracellular cGMP. In this reporter cell line, cGMP levels can be monitored in real-time via aequorin luminescence stimulated by calcium influx through the CNG channel.By using different PDE inhibitors, we could show that our reporter assay specifically monitors PDE 2A inhibition. The PDE 2A selective inhibitors erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA), 9-(6-Phenyl-2-oxohex-3-yl)-2-(3,4-dimethoxybenzyl)-purin-6-one (PDP) and 2-(3,4-dimethoxybenzyl)-7-{(1R)-1-[(1R)-1-hydroxyethyl]-4-phenyl-butyl}-5-methylimidazo[5,1-f][1,2,4]triazin-4(3H)-one (BAY 60-7550) alone did not increase basal luminescence levels in this experimental setting. However, these inhibitors induced concentration-dependent lumines-cence signals in combination with ANP. The PDE 2A inhibitors significantly potentiated the intra-cellular cGMP levels generated by ANP stimulation and induced leftward shifts of the corresponding concentration-response curves. In contrast, in the parental ANP receptor cell line PDE 2A inhibitors had no effect on ANP-stimulated luminescence signals. In addition, we also tested other PDE inhibitors including vinpocetine, milrinone, rolipram, sildenafil, zaprinast, BRL 50481 and BAY 73-6691. However, even in the presence of ANP, these inhibitors did not stimulate luminescence signals on our PDE 2A reporter cell line. The results imply that this novel PDE 2A reporter assay is well-suited for the characterization of the cellular activity of PDE 2A inhibitors and may also be used for lead structure identification by ultra-high-throughput screening (uHTS).

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125

Cytidylyl cyclase activity of *Bacillus anthracis* adenylyl cyclase toxin

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Bacillus anthracis adenylyl cyclase toxin edema factor (EF) exhibits anti-inflammatory effects, weakening immune response and, thereby, promoting the pathogenesis of anthrax infection. We have developed EF inhibitors with high potency and selectivity with respect to mammalian adenylyl cyclase isoforms. Comparing the inhibitory potencies of 2´,3´-N-methylanthraniloyl- (MANT) substituted nucleotides on EF catalytic activity, we found MANT-CTP to be the most potent MANT nucleotide (Ki = 60 nM), and molecular modeling studies revealed a unique binding mode of MANT-CTP. Investigating the inhibitory potencies of natural nucleoside 5'-triphosphates, we found CTP to inhibit EF with a surprisingly high potency (Ki = 5 µM). Taken together, these findings raised the question whether EF may also possess cytidylyl cyclase activity. Therefore, we incubated EF with [α-32P]CTP and actually found cytidylyl cyclase (CC) activity which could be stimulated by calmodulin (CaM), the endogenous activator of EF. The EC50 value of CaM stimulated CC activity was 600 pM and maximal turnover number (Vmax) was 7 s-1. In Michaelis-Menten saturation experiments, we found a Km value of 10 µM CTP and a Vmax of 10 s-1. The identity of cCMP was confirmed by mass spectrometry. Based on these findings and the fact that cCMP is known to inhibit host immune response, we propose that bacterial adenylyl cyclase toxins also form cCMP, increasing infection severity.

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126

Analysis of adenylyl cyclase activity in rat kidney membranes

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Adenylyl cyclases (ACs) play an important role in transmembrane signalling events of the G-protein-coupled receptor (GPCR) cascade. They are responsible for the conversion of ATP into the second messenger cAMP. For a better understanding of diseases involving modified kidney function like diabetes insipidus and polycystic kidney disease it is important to study regulation of AC activity in kidney. As physiological model we used rat kidney cortical membranes for our studies. In the AC activity assay using Mg2+ we examined the efficacies and potencies of various neurotransmitters and hormones such as isoproterenol, vasopressin, and glucagon. Glucagon was the most efficient GPCR agonist with a 175% increase of AC activity. The additional presence of isoproterenol in different concentrations showed an additive but not synergistic effect. The analysis of AC activation in rat renal inner medulla showed a 4-fold increase of AC activity by direct stimulation of the enzyme with forskolin (100 µM). In contrast, the GPCR agonists were ineffective. The most potent GPCR agonists for AC stimulation in rat kidney cortical membranes were vasopressin (EC50 9 nM) and isoproterenol (EC50 22 nM). Inhibition data were obtained in the AC assay after full stimulation of renal ACs with forskolin and the G-protein activator guanosine 5'-[g-thio]triohosphate using Mn2+. Ki-values of N-methyl-anthraniloyl- (MANT)-ATP, MANT-CTP and MANT-GTP were 68 nM, 46 nM and 12 nM, respectively. MANT-ITP and MANT-UTP showed Ki-values of 5 nM and 22 nM, respectively. Saturation experiments with Mn2+ yielded a KM-value of 16 µM; with Mg2+, the KM-value was 84 µM. In conclusion, (i) glucagon is the most effective AC activator in kidney cortex, pointing to a crucial role of this hormone in renal homeostasis. (ii) Glucagon and isoproterenol activate different Gs-protein- and/or AC pools. (iii) AC activation via GPCRs is restricted to renal cortex. (iv) The kinetics of AC activators and inhibitors resemble those of recombinant AC 5.

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127

Regulation of human platelet cyclic nucleotide levels – experimental data and in silico modeling

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Cyclic nucleotides are the key mediators in human platelet function regulation. Elevated intracellular cAMP or cGMP concentration reduce platelet responsiveness to exogenous factors and causing inhibition of platelet aggregation. In cardiovascular diseases cyclic nucleotide elevating pathways are severely impaired thus increasing the risk for thrombotic events. Consequently agents stimulating cyclic nucleotide production or preventing their degradation are potent anti-platelet drugs. Especially drugs inhibiting cyclic nucleotide degrading phosphodiesterases have received remarkable attention in recent years. Though cyclic nucleotide regulation by the isolated enzymes is well understood regulatory mechanisms in cells are poorly described. This is because most cells express a variety of cyclic nucleotide regulating enzymes (e.g. cyclases and phosphodiesterases) with distinct selectivity and activity interacting and counter-regulating each other. Hence understanding of the cellular regulation of cyclic nucleotides in particular cross regulation is rather incomplete. This study aims at an improved comprehension of the underlying mechanisms and, on basis of the data gathered, development of a model allowing for a prediction of the intracellular regulation of cyclic nucleotide levels and cyclic nucleotide dependent pathways. Here we present the first data on human platelet cyclic nucleotide regulation with respect to the participating cyclases and phosphodiesterases. Data on cyclic nucleotide levels under basal conditions and after treatment with cylcase stimulation or phosphodiesterase inhibition were collected from human platelets. Phosphodiesterase concentrations were determined by protein assay. On basis of these data an in silico model for cyclic nucleotide regulation was developed. This includes predictions for the activity of the involved enzymes and the expected levels of cyclic nucleotides. Application examples from the model involve pharmacological effects (e.g. inhibition) which are compared with and refined by our experimental data. Insights into the different signaling arms via cAMP and cGMP are possible (e.g. regarding cross-talk) and will be discussed.

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128

Dual regulation of cAMP levels by CA2+ in vascular smooth muscle cells

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Cyclic AMP represents a crucial regulator of blood pressure and vascular smooth muscle tone. Typically it antagonizes smooth muscle contraction induced by intracellular Ca2+. In addition to the antagonistic effect on the vascular tone, there are reports of direct effects of Ca2+ on intracellular cAMP concentrations. We attempted to study the dynamics of this interplay between intracellular Ca2+ and cAMP in single living aortic smooth muscle cells. To measure cAMP we utilized the FRET-based cAMP sensor Epac1-camps (Nikolaev et al, JBC, 2004). Changes in intracellular Ca2+-concentrations were observed by loading the same cells with FuraII. Thereby we now had the opportunity to observe changes in cAMP and changes in intracellular Ca2+ at the same time. This provided also the possibility to analyze the spatio-temporal relationship between Ca2+ release and cAMP changes inside the cell. The Ca2+-release was evoked by treatment with ATP or Ionophor (A23187). Simultaneous measurements of Ca2+ and cAMP revealed a Ca2+ induced rise in cAMP. cAMP elevations in response to Ca2+ signals were characterized by a delay of T0.5=45.2±5.4s. If cAMP was elevated above approximately 5 µM ATP-mediated Ca2+ release resulted in a substantial decrease of cAMP within 60 s (T0.5=60.13±10.9s). In summary we found a clear relationship between Ca2+ release and cAMP concentrations in smooth muscle cells. At low cAMP concentrations Ca2+ caused an increase in cAMP. In contrast, at high cAMP levels intracellular Ca2+ signals reduced cAMP levels severalfold. The underlying molecular mechanisms will be analyzed in future studies.

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129

A small molecule for interference with compartmentalised cAMP signalling

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Small organic molecules for disruption of protein-protein interactions are valuable tools to study cellular signalling processes. Of particular interest are direct protein-protein interactions mediated by scaffolding proteins, which position protein kinases, protein phosphatases, phosphodiesterases and other signalling proteins at defined sites within cells. This compartmentalisation facilitates temporally and spatially coordinated cellular signalling and thereby specific responses of cells to given external stimuli. Compartmentalisation of protein kinase A (PKA) is achieved by direct interaction of its regulatory subunits (preferentially RII) with a class of scaffolding proteins termed A kinase anchoring proteins (AKAPs). AKAP-RII interactions are involved in many physiologically relevant processes, including the regulation of cardiac myocyte contractility and vasopressin-mediated water reabsorption in renal collecting duct principal cells. Screening of a small molecule library containing 20,000 compounds led to the identification of nine compounds that inhibit AKAP-RII interactions. Analysis of the effects of the compounds in cardiac myocytes suggested that AKAP-dependent protein-protein interactions are suitable targets for disruption with small molecules.

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130

Activation of cGKI in primary vascular smooth muscle cells promotes adhesion

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Nitric oxide (NO) regulates many cellular functions via the cGMP/cGMP-dependent protein kinase type I (cGKI) pathway. The analysis of isolated vascular smooth muscle cells (VSMCs) suggested that activation of cGKI can promote cell "growth", in contrast to many studies on passaged smooth muscle cell lines that reported an anti-proliferative role for cGKI. Stimulation of primary but not subcultured wild type VSMCs with 8-Br-cGMP strongly promotes "growth". This effect depends on cGKI since it is absent in cGKI-deficient cells. Confirming the previous results by others, cGKI was found to mediate an anti-proliferative effect in repeatedly passaged VSMCs. To resolve the underlying mechanism of the growth promoting effect of cGKI, the detailed analysis of proliferation, apoptosis, and cytoskeletal dynamics indicated that 8-Br-cGMP increased cell adhesion in primary wild type VSMCs. This pro-adhesive effect of cGKI is mediated via an inhibition of the RhoA/Rho kinase pathway. The inhibition of the RhoA/Rho kinase pathway enhanced b1 and b3 integrin mediated adhesion. In conclusion, a yet unknown effect for cGKI-signalling in primary VSMCs was revealed. Furthermore, these results show that the opposing effects of cGK-signalling depend on the phenotypic context of VSMC.

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131

Analyzing the possible roles of endogenous cGKI in pancreatic islet cells on glucose homeostasis

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The cGMP-dependent protein kinase type I (cGKI) is involved in various (patho-)physiological processes and mediates many effects of the nitric oxide (NO)/cGMP pathway. To our knowledge, a functional role of endogenous cGKI has not been described in the regulation of glucose metabolism. Prkg1, the cGKI gene, encodes two isozymes, cGKIa and cGKIb that differ only in their individual amino-terminal sequences. Recently, cGKIa and cGKIb rescue mice have been generated which express either the Ia or the Ib isozyme selectively in smooth muscle cells (SMCs) on a cGKI-deficient genetic background1. These animals are an improved model to investigate the in vivo roles of cGKI in non smooth muscle tissues since in comparison to conventional cGKI knockouts rescue animals show a prolonged life expectancy and normal SMC functions. In the present study, the possible role of cGKI on glucose homeostasis was analyzed. Therefore, insulin and glucose tolerance tests were carried out in cGKIa-rescue mice and their littermate controls. After 12 h basal fasting glucose levels and serum glucagon levels of cGKIa-rescue mice were significantly elevated. Insulin (0.5 mU/g) induced glucagon secretion and glucose clearance were normal in the cGKIa-rescue animals, whereas the response to oral application of glucose (2 mg/g) was disturbed. To identify the cellular basis for these disorders we performed RT-PCR, Western blot, and immunohistochemical analysis of pancreatic islets. We show that cGKI is expressed in glucagon secreting pancreatic A-cells of control mice, whereas no cGKI was detectable in islets of rescue animals or the conventional cGKI knockouts. In conclusion, our data indicates that A-cell cGKI has an inhibitory effect on glucagon secretion under low glucose conditions.1Weber et al. Rescue of cGMP kinase I knockout mice by smooth muscle specific expression of either isozyme. Circ Res. 2007;101(11):1096-1103.

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132

Associative fear memory and LTP of synaptic transmission in the lateral amygdala is supported by cGMP-dependent protein kinase I

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Nitric oxide (NO) and cGMP have been reported to modulate fear memory and long-term potentiation (LTP) of synaptic transmission in the lateral amygdala (LA) likely through cGMP-dependent protein kinase (cGK). The two isoforms cGKI and cGKII are both expressed in the amygdala and both have been implicated in synaptic plasticity. Here we investigated the function of cGKI and cGKII for associative learning and long-term potentiation (LTP) in the LA using fear conditioning experiments and extracellular recordings of postsynaptic field potentials.We initially examined LTP in the LA of wild type mice and null mutants of cGKI (cGKI-/-) and cGKII (cGKII-/-). cGKI-/- mice showed reduced LTP both in the cortical and in the thalamic input to the LA while LTP was normal in both inputs in cGKII-/- mice. In line with this, cGKII-/- showed normal fear conditioning behaviour, i.e. neither short-term nor long-term memory was altered in these mutants.Because cGKI-/- mice die prematurely due to severe smooth muscle dysfunction, we used a mouse model (cGKI rescue) for behavioural analysis in which the expression of cGKI is rescued selectively in smooth muscle cells. These mice live to adulthood allowing to examine whether cGKI-deficiency causes a behavioural defect in parallel to the observed impairment of synaptic plasticity. Therefore, we analysed amygdala-dependent cued fear and contextual fear behaviour in wild type and cGKI rescue mice at different time points after auditory footshock conditioning. There was no difference in short-term memory between the two genotypes in freezing behaviour to the conditioned tone. In contrast, behavioural testing after 24 hours and after 7 days revealed a significant reduction in cued fear in cGKI rescue mice compared with control. Yet, freezing in the conditioning environment was not different between the genotypes indicating that contextual fear memory was normal. In summary, these results point out that cGKI deficient mice exhibit a specific defect in long-term memory formation. We suggest that cGKI supports fear memory consolidation in the LA. The underlying mechanisms remain to be elucidated.

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133

CRP4 is a downstream effector of cGKI in nociceptive processing

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The cGMP/cGMP-dependent protein kinase I (cGKI) signaling pathway plays an important role in spinal nociceptive processing. However, downstream targets of cGKI in this context have not been identified to date. Using a yeast two-hybrid screen, we isolated cysteine-rich protein 4 (CRP4) as a novel cGKI interactor in the spinal cord. CRP4 is expressed in laminae I and II of the mouse spinal cord and is colocalized with cGKI, calcitonin gene-related peptide and isolectin B4. Moreover, the majority of CRP4 mRNA-positive dorsal root ganglion (DRG) neurons express cGKI and peripherin. CRP4 is phosphorylated in a cGMP-dependent manner and its expression increases in the spinal cord and in DRG after noxious stimulation of a hind paw. To elucidate the functional role of CRP4 in nociception we analyzed mice with a targeted deletion of CRP4. CRP4-deficient (CRP4-/-) mice demonstrate normal behavioral responses to acute nociception and following axonal injury of the sciatic nerve, but increased nociceptive behavior in models of inflammatory hyperalgesia as compared to wild-type mice. Intrathecal administration of cGMP analogs increases the nociceptive behavior in wild-type but not in CRP4-/- mice, indicating that the presence of CRP4 is important for cGMP-mediated nociception. These data suggest that CRP4 is a new downstream effector of cGKI-mediated spinal nociceptive processing and point to an inhibitory role of CRP4 in the generation of inflammatory pain.

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134

The role of class I phosphatidylinositide-3-kinases in the regulation of astroglial proliferation

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The family of phosphatidylinositide-3-kinases (PI3-Ks) consists in 3 classes. The four members of the class I, i.e. p110a, p110b, p110g and p110d, are involved in cell attachment, proliferation and mobility. Little is known about the expression of the different isoforms in neural cells of the CNS and their contribution to the regulation of proliferation.In a first step, we examined the isoforms of class I PI3-kinases expressed in cultured neocortical astroglial cells. Non-radioactive in situ hybridization, reverse transcription PCR and Western blotting showed that only p110a and p110b were expressed. To investigate the role of both isoforms in proliferation, we used the adenoviral expression of dominant negative (dn) isoforms coupled to EGFP via IRES. In addition, we applied the PI3K-isoform selective inhibitors AS-252424 (a-isoform) and TGX-221 (b-isoform). In astrocytes synchronized by transient serum withdrawal for 24 h, the PI3-K pan-inhibitor Wortmannin reduced by approximately 50% the nuclear uptake of bromo-deoxyuridine (BrdU), indicating an antiproliferative effect. Expression of dnp110a or dnp110b also reduced the nuclear uptake of BrdU by approximately 50% as did the isoform selective inhibitors AS-252424 and TGX-221.Next, we investigated the interactions of p110a and p110b with signaling pathways known to be involved in the regulation of proliferation. Extracellular-signal-regulated-kinases (ERKs), which are essential for proliferation, were reduced in their activity after inhibition of the b-isoform, whereas blockade of the a-isoform had no such effect. In contrast, both isoforms induced the phosphorylation of glycogen-synthase-kinase 3b (GSK-3b) and thereby mediated the nuclear localisation of cyclin D, which is essential for DNA synthesis. Finally, only inhibition of the PI3K a-isoform enhanced the level of p27Kip1, suggesting that this isoform alone acted at this level of the regulation of proliferation. According to these findings, both PI3-K isoforms can regulate proliferation via different signaling pathways.The financial support of the Graduiertenkolleg 843 Neuroscience Freiburg is gratefully acknowledged.

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135

Tempol and N-acetyl cysteine interfere with PDGF-signaling in vascular smooth muscle cells and inhibit neointima formation in vivo

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Growth factor- and reactive oxygen species (ROS)-induced activation of vascular smooth muscle cells (VSMCs) are important contributors to vascular disease, like neointima formation. ROS, such as H2O2, are generated in vascular lesions and by receptor tyrosine kinases (RTKs). Protein Tyrosine Phosphatases (PTPs) were identified as targets of ROS, resulting in inhibitory oxidation at the active-site cysteine. Aim was to inhibit ROS- and RTK-mediated signals by reactivating PTPs due to antioxidants, thereby consecutively inhibiting neointima formation. In vitro the impact of the antioxidants tempol and N-acetyl-cysteine (NAC) on PTP-activity, H2O2- and Platelet-Derived Growth Factor (PDGF)-signaling and cellular responses was analysed. In vivo tempol and NAC were administered to Sprague-Dawley-rats, and 14d after endothelial-denudation of the carotid artery, the PTP-expression and –activity, phosphorylation of the βPDGF-receptor and neointima formation were assessed. NAC prevented the H2O2-induced decrease in PTP-activity in VSMCs in vitro. Furthermore, H2O2-induced signaling, including phosphorylation of the βPDGF-receptor and of downstream-signaling molecules Erk and Akt, was inhibited by NAC. Both tempol and NAC concentration-dependently reduced PDGF-induced chemotaxis and BrdU-incorporation. Administration of tempol and NAC in vivo led to enhanced PTP-activity in restenotic arteries (2.16- and 2.10-fold of control, respectively, $p<0.05$). Accordingly, the phosphorylation of the βPDGF-receptor in the neointima was significantly lower in tempol- and NAC-treated animals, while receptor and PDGF-B-ligand-expression were not altered. Finally, tempol- and NAC-treatment was resulted in decrease of neointima formation (reduction of intima-media-ratio by 23.6% and 31.8%, respectively, $p<0.05$).

Antioxidants-induced reactivation of the PTP-activity results in considerable effects on PDGF-signaling and neointima formation. The lower βPDGF-receptor phosphorylation in vivo, in association with enhanced total PTP-activity, indicate an impact on βPDGF-receptor-antagonizing PTPs by NAC and tempol. The results imply, that PTPs serve as targets of antioxidant-therapy in vivo.
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136

Identification and characterization of AUM, a novel mammalian haloacid dehalogenase involved in spermiogenesis
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The haloacid dehalogenase (HAD) superfamily of hydrolases represents a ubiquitous enzyme family with approximately 2000 members, the majority of which are magnesium-dependent phosphatases. Although the existence of about 40 human HAD enzymes has been predicted, our understanding of their cellular functions remains rudimentary. We have recently described the mammalian HAD phosphatase Chronophin as a regulator of actin dynamics. Here, we report the identification and characterization of a novel HAD phosphatase. We have named this enzyme AUM, for actin remodelling, ubiquitously expressed, magnesium-dependent HAD phosphatase. To characterize the substrate preference of AUM, we have conducted in vitro phosphatase assays with 720 phosphopeptides derived from human phosphorylation sites. AUM exclusively dephosphorylated phosphotyrosine (pTyr)-containing peptides. Furthermore, only 18 pTyr peptides (~2% of all pTyr peptides investigated) acted as AUM substrates, indicating a high degree of substrate specificity. Putative AUM substrates include proteins involved in cytoskeletal dynamics, differentiation, apoptosis and tyrosine kinase signaling. In addition, overlay assays showed that AUM dephosphorylates a limited number of tyrosine-phosphorylated proteins. Overexpression or siRNA-mediated depletion of AUM in HeLa cells and subsequent immunocytochemical and electron microscopic analysis showed morphological deformities consistent with a role for AUM in actin cytoskeletal and membrane dynamics. By Northern blot, real-time PCR and Western blot analysis with a monospecific AUM antibody, we show that AUM is broadly expressed in all major tissues, with highest levels found in testes. AUM is specifically detected in maturing germ cells and its expression peaks during spermiogenesis, suggesting a role in this vital germ cell differentiation process. In summary, we have characterized AUM, a previously unidentified mammalian HAD-type protein tyrosine phosphatase. Current efforts are focussed on physiological AUM substrates and the analysis of AUM functions in maturing germ cells.
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137

Role of the cofilin phosphatase chronophin in glioblastoma pathogenesis
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Cofilin is a key regulator of actin cytoskeletal dynamics whose activity is controlled by phosphorylation of a single serine residue. Whereas cofilin inactivating kinases are well characterized, much less is understood about specific phosphatases that reactivate cofilin functions. We have recently identified Chronophin (CIN), a cofilin phosphatase of the haloacid dehalogenase family of hydrolases that is highly expressed in brain. Altered CIN activity or loss of CIN function interferes with the physiological phosphocycling of cofilin in HeLa cells and triggers cell division defects, resulting in a subsequent accumulation of aneuploid cells. Aneuploidy is a hallmark of most tumors, and an altered cofilin activity has been causally linked to the initiation and progression of malignancies. Interestingly, the CIN gene maps to human chromosome 22q13.1 in the vicinity of a region that is frequently deleted in malignant astrocytic gliomas. We have therefore analyzed the expression of CIN and other cofilin regulatory proteins in a panel of astrocytic tumor samples. Using real-time PCR and a newly developed CIN rabbit monoclonal antibody, we show that CIN expression was strongly reduced in the majority of the investigated grade III and IV gliomas, but not in grade I-II astrocytomas. In contrast, the expression of the cofilin kinase LIMK2 and its upstream activator Pak4 was markedly upregulated in CIN-deficient tumors. To investigate the potential role of CIN in glioma pathogenesis on a cellular level, we have depleted CIN by RNA interference in the astrocytoma model cell line GBM6840. The reduction of CIN levels stabilized F-actin structures, increased the percentage of aneuploid cells with nuclear deformities, and increased colony formation in soft agar. Our ongoing studies address the molecular mechanism leading to CIN depletion as well as the molecular link between CIN activity and glioma initiation and progression.
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138

Role of the calcium- and integrin-binding protein CIB1 as an essential activator of chronophin
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We have recently identified Chronophin (CIN) as a regulator of cofilin-dependent actin dynamics in dividing cells. CIN is an aspartate-dependent phosphatase that belongs to the large, ancient and ubiquitous family of proteins characterized by three haloacid dehalogenase (HAD) domains. To elucidate the mechanisms of CIN regulation, we have performed yeast two-hybrid screens. Here, we report the direct and specific interaction of CIN with the calcium- and integrin-binding protein 1, CIB1. CIB1 is an EF-hand-containing regulatory protein that modulates the functions of diverse effector proteins, including several kinases. Thus far, no phosphatases have been described as CIB1 effectors, although CIB1 shares significant structural similarity to the non-catalytic subunit of protein phosphatase 2B/calcineurin. We have mapped the binding site of CIB1 on CIN to a discrete region of 5 amino acids, and have identified a region of 13 amino acids in the C-terminus of CIB1 that is critical for CIN binding. The CIN/CIB1 interaction was confirmed in pulldown and immunoprecipitation assays. Subcellular localization studies using confocal microscopy and fluorescence resonance energy transfer analysis have demonstrated that CIN and CIB1 co-localize and interact in cells. Solid phase binding experiments revealed that the CIN/CIB1-association can be increased about four-fold in a CIB1 EF-hand-dependent manner, and is maximal in the presence of one micromolar calcium. Importantly, CIB1 markedly and calcium-dependently stimulated CIN phosphatase activity. Together, these results clearly show an important function for CIB1 as a direct activator of CIN, and link CIN activity to hormonal signaling. Interestingly, it has recently been reported that the deletion of CIB1 in mice leads to male infertility due to a defect in spermatogenesis. We are currently addressing the role of CIB1 for CIN-mediated cytoskeletal dynamics in testicular cells.
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139

Characterisation of inducible novel phosphorylation sites in TAB1, a regulatory subunit of the protein kinase TAK1
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The protein kinase TAK1 and its regulatory subunit TAB1 are important upstream molecules in the pathways activated by IL-1, TNF and toll-like receptors. TAK1 is a member of the MAP3K family and thus can activate the MAP kinases p38 and JNK as well as the NFkB signaling pathway. The Y. enterocolitica effector YopP inhibits MAPK and NFkB pathways simultaneously and we have found that this effect of YopP occurs by inhibition of TAK1. As assessed by SDS-PAGE mobility shifts and mutational studies, ectopic expression of YopP reduces phosphorylation of TAB1 at S438 of TAB1. S438 of TAB1 is one of three known phospho-amino acids that are phosphorylated by p38 MAPK. These sites are involved in negative feedback regulation of TAK1. During these analyses we also detected novel phosphorylation sites of TAB1 located within a C-terminal cluster of six serines, namely aa452-457. These sites are phosphorylated upon overexpression of TAK1 in intact cells. However, deletion of aa 452-457, or double mutants (SS452/453AA, SS456/457AA) did not affect the ability of TAB1 to coimmunoprecipitate with and activate TAK1 in vitro. Currently we are analysing these sites further by mass spectrometry and by phosphorylation-site specific antibodies. To assess a biological role of TAB1 modifications we reconstituted TAB1-deficient embryonic fibroblasts with wild type and mutant TAB1 proteins (S438A, SSS423/431/438AAA, deltaS452-457) and have been analysing the mRNA expression profiles of these cell lines by genome wide DNA microarrays and by RT-PCR. Deletion of TAK1 or TAB1 in mice is embryonic lethal but shows little overlapping phenotype indicating that e.g. TAB1 may have TAK1-independent functions. Hence, our results may provide one clue to delineate unknown functions of TAB1.
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140

I-kappaB kinases as novel targets for the pharmacotherapy of pain
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Several studies indicate that the NF-κB-activation cascade does not only play a crucial role in immune responses, inflammation and apoptosis but also in the development and processing of pathological pain. Accordingly, a pharmacological intervention into this pathway may have antinociceptive effects and could provide novel treatment strategies for pain and inflammation.An essential step in NF-κB activation is the phosphorylation of IκB proteins by IκB kinases (IKK). Therefore, our studies focussed on the role of these kinases in pain. We found that IKKα and β as well as the newly identified IKKε are all constitutively expressed in murine neuronal tissues that are relevant for nociception, specifically the dorsal root ganglia, the spinal cord and the brain. Peripheral noxious stimulation increased IKKα, β and IKKε mRNA and protein levels in the spinal cord of mice. Systemic administration of a specific and effective inhibitor of IKKα and β (S1627) reduced thermal and mechanical hyperalgesia in the zymosan induced paw inflammation model as well as thermal and tactile allodynia in the chronic constriction injury model of neuropathic pain. In conclusion, our data indicate an important role of IKKs in pain and may therefore proof these kinases as interesting novel drug targets for the treatment of pathological pain. The work is supported by the Deutsche Forschungsgemeinschaft (NI 705/2-1) and Graduate School "Biologicals" GRK 1172).
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141

Presynaptic cGMP-dependent protein kinase I facilitates spinal synaptic plasticity and inflammatory pain
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Nitric oxide (NO) has been speculated to serve as a retrograde messenger at spinal nociceptive synapses and the production of cGMP via the NMDA receptor-NO pathway has been shown to mediate central sensitization which is associated with chronic pain. However, the downstream targets of cGMP which mediate these phenomena and their precise locus in the spinal circuitry are yet unclear. Although pharmacological and

transgenic studies have suggested that the cGMP-dependent protein kinase I (PKG1) may modulate nociceptive processing, these analyses have remained unconclusive due to the lack of specificity of reagents and developmental defects in global knock-out mice. Using the Cre/loxP system for conditional gene deletion, we generated transgenic mice lacking PKG1 specifically in nociceptors (SNS-PKG1-/-), preserving expression in the spinal neurons, brain and all other organs. By using a combination of electrophysiological, behavioural and biochemical methods, we analysed these genetically modified mice in preclinical models of inflammatory and neuropathic pain and uncovered the mechanisms and downstream targets of PKG1 underlying hyperalgesia and allodynia. We conclude that (1) PKG1 expressed by nociceptors is the primary target of the NMDA-NO-cGMP pathway and critically required for hyperlagesia and allodynia in post-inflammatory states. (2) PKG1 localized in spinal terminals of nociceptive afferents mediates LTP at synapses on spinal neurons projecting to the PAG. (3) Modulation of calcium release from intracellular stores and activity-dependent phosphorylation of IP3 receptors, VASP and MLC likely mediate PKG1-induced synaptic potentiation and exaggerated pain.
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142

An essential role of classical transient receptor potential channel 6 (TRPC6) in ischemia-reperfusion injury in isolated perfused murine lungs
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Lung endothelial damage is a characteristic morphological feature of ischemia-reperfusion (I/R) injury, although the molecular steps involved in the loss of endothelial integrity and subsequent invasion of immune cells are still poorly understood. Most interestingly, isolated lungs from TRPC6-deficient mice are resistant to I/R injury, while lungs from wild-type mice show an increased capillary filtration coefficient (Kfc) resulting in pulmonary oedema formation in reperfused areas. To further elucidate the role of TRPC6 in immune and endothelial cells, TRPC6-deficient bone marrow cells were transplanted in irradiated wild-type mice and vice versa. Because isolated wild-type lungs with TRPC6-deficient bone marrow-derived immune cells were not protected from I/R injury, an important role of TRPC6 in immune cells during these pathophysiological processes seems to be unlikely. Therefore, endothelial cells from wild-type and TRPC6-deficient mice were isolated and further analyzed. TRPC1, TRPC4 and TRPC6 were the most prominently expressed TRPC isoforms in pulmonary endothelial cells and TRPC expression was not altered in TRPC6-deficient cells except for TRPC6. Moreover, primary isolated endothelial cells from TRPC6-deficient lungs show a reduced hypoxia-induced Ca2+ influx and actin stress-fiber formation, as well as a decreased transendothelial resistance compared to cells from wild-type lungs. Therefore, TRPC6 may represent a promising pharmacological target to suppress I/R injury in isolated lungs intended for transplantation.H.K. and A.S. contributed equally.
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143

The selective TRPC6 activator hyperforin induces neurite outgrowth
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The preclinical antidepressant profile of hyperforin is supported by a large body of evidence. Hyperforin is active in many relevant behavioral models, alters brain levels of serotonin, norepinephrine, dopamine and inhibits synaptosomal uptake of all three neurotransmitters in vivo. In contrast to classical antidepressants, hyperforin does not directly interact with the transporter molecules but selectively activates TRPC6 channels and thereby increases the intracellular sodium concentration. Therefore, re -uptake inhibition is the consequence of an elevation of the intracellular sodium concentration, as the re-uptake of all neurotransmitters is strongly depending on the sodium gradient. In the brain, TRPC channels are involved in growth cone guidance and neurite extension. Therefore, we applied hyperforin to PC12, analyzed the neurite length and compared it with NGF-treated cells. Hyperforin in the clinically relevant concentrations of 0.1 and 0.3 µM induce modest Ca2+- and Na+-influx. In side-by side experiments, PC12 cells were incubated for 5 days with either hyperforin or nerve growth factor (NGF) (50 ng/ml). Differentiation of PC12 cells was analyzed by measuring the neurite length of PC12 cells after 5 days. Hyperforin had similar effects on PC12 cell differentiation like NGF, both induced a significant neurite outgrowth. To determine whether or not TRPC6 channel activation is involved in hyperforin-mediated differentiation of PC12 cells, we co-incubated hyperforin (0.3 µM) with La3+ (100 µM) and Gd3+ (100 µM) as channel blockers for 5 days. Under these conditions hyperforin-induced differentiation and neurite outgrowth was abolished. To further test the role of TRPC6 channels for hyperforin-mediated differentiation, PC12 cells were transiently transfected with anti-TRPC6 siRNA or with the plasmid coding for the dominant negative TRPC6 mutant (TRPC6-DN-YFP) and subsequently treated with hyperforin (0.3 µM) for 3 days. Both independent approaches knocking down TRPC6 function in PC12 cells inhibited the Hyperforin induced neurite outgrowth. These results strongly argue for the involvement of TRPC6 in the hyperforin-induced differentiation and neurite outgrowth of PC12 cells.
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144

N-phenylcinnamides as inhibitors of TRP channels
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TRP channels involved in temperature sensation and hormonal signal cascades are modulated by a the great variety of secondary plant compounds like menthol, campher, capsaicin or hyperforin. In contrast to the selective action of these natural occurring compounds, the known TRP channel blocker are mostly unselective. The lack of specific blockers has been become evident for us during our attempts analysing the physiological role of TRPM2 channel proteins. TRPM2 is highly expressed in brain, where it is preferentially localized in microglial cells, the host macrophages of the central nervous system. An expression of TRPM2 in immune cells in general is suggested by its detection in some T-lymphocyte cell lines, monocytic U937 cells and primary human neutrophils. TRPM2 is activated by intracellular ADP-ribose (ADPR) and hydrogen peroxide (H2O2). Activation of TRPM2 channels by H2O2 has been suggested to depend on activation of poly(ADP-ribose) polymerase, an ubiquitously expressed enzyme catalysing the breakdown of NAD+ into nicotinamide and ADPR. The rather unspecific inorganic TRP channel blocker, gadolinium and lanthanum ions, are ineffective in blocking TRPM2 activity, therefore we looked for new TRPM2 blocker. Finally, we identified N-(p-amylcinnamoyl)anthranilic acid (ACA). This compound and its related structure ONO-RS-082 have been described to inhibit phospholipase A2 (PLA2) activity. N-(p-amylcinnamoyl)anthranilic acid completely inhibited ADPR-induced whole-cell currents and H2O2-induced Ca2+ signals in TRPM2-expressing HEK293 and human U937 cells. In parallel, SB366791 has been described as specific TRPV1 blocker. Both compounds, ACA and SB366791, have in common a N-phenylcinnamide core structure. The selectivity of SB366791 in blocking TRPV1 makes it likely that it is possible to develop TRP channel-specific compounds based on N-phenylcinnamide, as a new pharmaceutical lead structure.
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145

Ubiquitous inactivation of TRPM4 leads to elevated blood pressure in mice
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Recently we showed that transient receptor potential TRPM4 proteins act as Ca2+-activated nonselective cation channels in mast cells that critically limit the driving force for Ca2+ influx, release of inflammatory mediators and anaphylactic responses (Vennekens et al., Nat Immunol 8: 312-20; 2007). In addition to mast cells we could detect TRPM4 proteins in heart, aortic endothelial cells, kidney and adrenal gland from wildtype but not from TRPM4-/- mice. Earley et al. (Circ Res 95: 922-9, 2004) suggested that TRPM4 is expressed in vascular smooth muscle cells where it is supposed to act as a mechanosensor regulating vascular myogenic tone. Telemetric blood pressure measurements reveal that mean arterial blood pressure (MAP) averaged from day 9 to 13 after implantation of the transmitter is elevated in TRPM4-/- mice by 11mmHg under basal conditions compared to wild type (WT) controls. Systolic blood pressure is 122±1 mmHg and 129±1 mmHg, diastolic blood pressure is 83±1 and 95±1 mmHg in WT and TRPM4-/- mice, respectively (WT: n=11-13, TRPM4-/-: n=12-15, p<0.001). Blood pressure elevation is observed during the complete circadian period and most notable under resting conditions. Plasma aldosterone levels, renin mRNA expression in the kidney and renin plasma concentrations are not different in TRPM4-/- mice fed with regular diet and there are no differences in MAP in response to intraperitoneal application of phenylephrine, norepinephrine and L-NAME. Intraperitoneal application of prazosin (1mg/kg) abolishes the difference in MAP between both genotypes similarly like inhibition of ganglionic transmission with the nicotinergic-acetylcholine receptor blocker hexamethonium (20mg/kg i.p.) suggesting that an increased neurogenic tone contributes to the development of the hypertension.
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146

Early embryonic lethality following inactivation of the TRPM7 gene in mice
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TRPM7, like its closest homologue TRPM6, forms a bifunctional protein encoding a cation channel domain fused to a C-terminal α-kinase domain. The cation channel is permeable for divalent cations including Ca2+and Mg2+. TRPM7 is ubiquitously expressed in humans and we could identify TRPM7 transcripts in murine kidney, mast cells, heart, aortic endothelial cells, brain and embryonic stems cells using Northern blot analysis. Gating of TRPM7 is regulated by intracellular levels of Mg2+ and MgATP. In mammals, TRPM7 may mediate anoxia-induced excitotoxic neuron death, and it is also required for the viability in avian lymphocytes. To determine the physiological role of TRPM7 we inactivated its gene in mice. We used homologous recombination in embryonic stem cells to generate mice carrying an allele in which exon 2 containing the translation start site was flanked by loxP sequences. Removal of exon 2 which results in a frameshift mutation followed by an early stop codon was achieved using Cre-mediated recombination in vivo to generate a TRPM7 null allele (TRPM7 -). No homozygous (TRPM7-/-) offspring were born from heterozygous TRPM7+/- intercrosses. Systematic analysis from embryonic day (E) 0.5 to 12.5 revealed that blastocyst stage TRPM7-/- embryos (E3.5) are already frequently degenerated with the inner cell mass being detached from the zona pellucida. We examined the ability of these blastocysts to expand in vitro, but in contrast to wild type and TRPM7+/- blastocysts, TRPM7-/- blastocysts failed to hatch and no expansion of trophoblast stem cells was observed even after removal of the zona pellucida. Fluorimetric analysis of two-cell stage TRPM7+/+ and TRPM7-/- embryos revealed that basal Ca2+ and the Mg2+ levels were significantly reduced upon inactivation of TRPM7. We conclude that TRPM7 channels are essential for Ca2+ and Mg2+ entry and maturation of preimplantation-stage embryos in mice and that the lack of TRPM7 cannot be compensated by its homologue TRPM6.
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147

Molecular determinants of divalent cation permeation of the enzyme-linked TRPM channels

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The mammalian TRPM gene family can be subdivided into distinct categories of cation channels that are either highly permeable for Ca2+ (TRPM3/6/7), nonselective (TRPM2/8), or even Ca2+ impermeable (TRPM4/5). TRPM6/7 are fused to a-kinase domains, whereas TRPM2 is linked to an ADP-ribose phosphohydrolase (Nudix domain). At a molecular level, the evolutionary steps that gave rise to the structural and functional TRPM channel diversity remain elusive. Here, we provide phylogenetic evidence that Nudix-linked channels represent an ancestral type of TRPMs that can be found in various phyla, ranging from protists to humans. This means that TRPM3-like channels (TRPM3/6/7), which are present only in more recent phyla starting from cnidaria, may have evolved due to a secondary loss of the nudix domain. Hence, the kinase-fused channels TRPM6 and TRPM7 would have gained their kinase-domain in an independent step. Surprisingly, the pore-forming segments of the ancestral, invertebrate TRPM2-like proteins display high sequence similarity to those of Ca2+-selective TRPMs, while human TRPM2 is characterized by a loss of several conserved residues. Using the patch-clamp technique, Ca2+ imaging, and site-directed mutagenesis, we demonstrated that restoration of only two “ancient” pore residues in human TRPM2 (Q981E/P983Y) significantly increased its permeability for Ca2+, Ba2+ and Mg2+. Conversely, introduction of a “modern” sequence motif into mouse TRPM7 (E1047Q/Y1049P) resulted in a dramatic reduction of divalent cation permeation and a linear TRPM2-like current-voltage relationship. Additionally, an intrinsic block of monovalent outward currents by extracellular divalent cations was nearly abolished in the mutant TRPM7 (E1047Q), whereas the block by intracellular Mg2+ remained unaffected. In summary, we found that the primordial TRPM-channel seems to be formed by a TRPM2-like protein. We also demonstrated how the exchange of two amino acids in the pore-forming region influences the permeability for divalent cations in TRPM2 and, in reverse, also in TRPM7. Overall, our findings provide an integrative view on the evolution of the domain architecture and the structural basis of the distinct ion permeation profiles of TRPM channels.

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148

The Drosophila TRPM mutant is a metamorphosis-dependent lethal

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Among TRPM channels, the melastatins remain the least understood group. The unique insect TRPM is structurally most closely related to the mammalian orthologues TRPM1 and TRPM3. Since these mammalian TRPs are among the most enigmatic TRPs, the Drosophila TRPM is thus an interesting candidate model for characterizing the functions of TRPM proteins. Therefore, we generated Drosophila lines bearing mutants in this gene. The trpm1 allele contains a frame-shifting P element-mediated deletion of three exons in the N-terminal domain, and trpm2, which we generated by homologous-recombination, contains a targeted deletion of three exons. Though trpm transcripts could be detected by means of Northern blotting at a variety of developmental stages in wild-type animals, the most striking phenotype of either of the null alleles is lethality occurring at the very onset of metamorphosis. Because embryos and larvae were both viable, even after maternal transcription was precluded, trpm is the first known TRP channel whose phenotype is developmental stage-dependent lethality. In embryos trpm RNA is detectable mainly in the Malpighian tubules whereas a transgene expressing Gal4 under the control of the major trpm promoter mainly drives expression of fluorescent proteins in the rectal papillae, a resorptive organ formed from the insect hindgut epithelium during the course of metamorphosis. Since the trpm mutant larvae have a reduced tolerance for Mg2+, we propose that a defect in fluid and electrolyte balance plays a central role in causing the lethality in the trpm mutant.

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149

Functional characterization of a retinitis pigmentosa-associated CNGB1 mutation (G993V)

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The rod cyclic nucleotide-gated (CNG) channel is a heteromer consisting of three CNGA1 and one CNGB1a subunit. A point mutation in the CNGB1 gene (G to T at position nt2978 of human CNGB1), was previously described in a French family with severe recessive Retinits pigmentosa (RP). This mutation leads to a glycine-valine replacement in the cyclic nucleotide binding domain (G993V) of the CNGB1a protein. In this study, we characterized the functional consequences of the G993V mutation by combining site-directed mutagenesis, electrophysiology, biochemistry and immunocytochemistry. We transfected HEK293 cells with CNGA1 (A1) and either wild type (B1a) or mutant CNGB1a (B1aG993V). We found that the B1aG993V protein was expressed in these cells. There was also evidence for trafficking of B1aG993V to the plasma membrane. Currents recorded after transfection with A1/B1aG993V did not differ from A1/B1a currents with respect to their apparent cGMP affinity. Biochemical assays indicated that A1 and B1aG993V formed a stable protein complex. Surprisingly, however, the functional properties of A1/B1aG993V channels were virtually identical to those of homomeric A1 channels. By contrast, channel features typically conferred by B1a, such as sensitivity to L-cis diltiazem, single-channel flicker or sensitivity to Ca2+-calmodulin, were absent in A1/B1aG993V. Together, these data suggest that A1/B1a993V channels, while formed, are functionally inactive. We propose that currents observed after coexpression of A1 and B1aG993V solely derive from homomeric A1 channels that are formed along with heteromeric channels. We present data suggesting that B1aG993V can be functionally rescued by truncating its long cytosolic N-terminus. Our results indicate that the G993V mutation strongly affects the CNG channel properties. We propose that assembling of B1aG993V with A1 leads to functionally inactive channels. Thus, in a physiological setting, the G993V mutation is likely to be equivalent to the total loss of CNGB1 function explaining the severe phenotype of patients suffering from this mutation.

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150

Zonisamide block of cloned human T-type voltage-gated calcium channels

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Zonisamide (ZNS) is a multi-target antiepileptic drug reported to be efficient in both partial and generalized seizures with T-type Ca2+-channel block being one of its proposed mechanisms of action based on previous work in rat cortical neurons and human neuroblastoma NB-I cells. We systematically investigated electrophysiological effects of ZNS on cloned human Cav3.1-3.3 Ca2+-channels in a heterologous (HEK-293) expression system using the whole cell patch-clamp technique. Dose-response studies revealed a 15.4 - 30.8 % reduction of Ca2+-influx for Cav3.2 within the maximum therapeutic range (50 - 200 mM ZNS) and an IC50 of 2.1 mM, whereas Cav3.1 and Cav3.3 Ca2+-channels turned out to be even less sensitive. Furthermore, voltage- and concentration-dependence of inactivation kinetics remained unchanged in Cav3.2 VGCC, whereas Cav3.1 and Cav3.3 exhibited minor, though significant reduction of inactivation time constant. Interestingly, ZNS-block of Cav3.2 VGCCs was not affected by changes in holding potential and steady-state inactivation studies did not display a significant shift in steady-state availability of Cav3.2 channels at 100 mM ZNS (DV1/2 = 3.1 mV, p = 0.071). Our studies indicate that ZNS is only a moderate blocker of human Cav3 T-type Ca2+-channels with little or no effect on channel inactivation kinetics and state-dependence of block. The observed T-type Ca2+ channel inhibition is likely to be effective in the anti-absence activity of ZNS.

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151

Alternative splicing in the C-terminus of CaV1.3 L-type Ca2+ channels allows fine-tuning of channel gating properties

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CaV1.3 channels control excitability in sensory and central neurons and sinoatrial node function mainly by the means of their low-voltage activation. In dopaminergic neurons, CaV1.3 mediated pace-making determines neuronal vulnerability in Parkinsons' disease. We have previously discovered an intrinsic mechanism which regulates voltage- and calcium-dependent gating in CaV1.4 channels by an intrinsic C-terminal modulator (CTM). Given the high C-terminal homology and the existence of a short CaV1.3 splice variant (Cav1.3S) which lacks the potential CTM, we asked if CaV1.3 channels are likewise regulated. We therefore expressed CaV1.3S channels together with β3 and α2δ-1 in tsA-201 cells and determined their biophysical properties using whole-cell patch-clamp technique compared to the full length (CaV1.3L) channel. Ca2+ currents through CaV1.3S activated at a more negative voltage range (V0.5act: CaV1.3S: -12.9±0.8mV, n=15; CaV1.3L: -2.2±0.6mV, n=14: p<0.0001), inactivated faster (in %; 250-ms to peak voltage: CaV1.3S: 73.6±1.9, n=19; CaV1.3L: 27.5±1.9, n=16; p<0.01), showed more pronounced CDI (p<0.01) as well as a 3-fold increase in current density. Window current was shifted to more negative potentials. CaV1.3S mRNA was identified in human brain, retina and heart and several mouse brain regions. We have identified the CaV1.3 CTM within its distal C-terminus by truncation of the last 116 amino acids of CaV1.3L channels. Resulting CaV1.3DC116 channels showed gating properties similar to CaV1.3S. CaV1.3L-like behaviour was restored by co-expression of the corresponding C-terminal peptide C116 suggesting an intra-molecular protein interaction in the C-terminus of CaV1.3 channels which was also confirmed in FRET experiments. Our results demonstrate that alternative splicing leading to short C-termini results in changes in voltage and calcium-dependent activation and inactivation gating. Therefore the CTM allows CaV1.3 channels to tightly control channel gating to optimize their performance as required for the modulation of neuronal firing behaviour and sinoatrial node pace-making. Support: FWF P17159, University of Innsbruck, and the Tiroler Wissenschaftsfonds.

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152

Association of protein kinase C to the Cav1.2 Ca2+ channel improves muscarinic-induced contraction of urinary bladder

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Muscarinic agonists like carbachol induce contraction of urinary bladder. This contraction is blocked by L-type Ca2+ channel antagonists and is abolished in Cav1.2 deficient detrusor muscle from mice, demonstrating that this type of contraction depends on functional L-type Ca2+ channels. However, it is unclear how muscarinic stimulation couples to the L-type Ca2+ channel. In this study, we present several lines of evidence that protein kinase C is involved in this signalling pathway: (i) carbachol-induced contraction was attenuated by Go6850; (ii) contraction and (iii) intracellular Ca2+ signals induced by carbachol were increased after activation of protein kinase C with phorbol ester; (iv) these effects of phorbol ester were inhibited by the protein kinase C inhibitor Go6850; (v) co-immunoprecipitation using a Cav1.2 antibody and (vi) analysis of membrane preparations using blue native gelelectrophoresis revealed a predominant association of protein kinase C to the Cav1.2 channel if the muscles had been stimulated by carbachol; (vi) phosphorylation of the Cav1.2 channel was increased in carbachol-stimulated preparations and attenuated by Go6850. These results suggest that association of protein kinase C to the Cav1.2 Ca2+ channel participates, at least partially, in the signalling pathway from the muscarinic receptor to the L-type Ca2+ channel during micturition.

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153

Investigation of the interaction site between the L-type calcium channel Cav1.2 and the BKCa channel

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L-type calcium channels are involved in a large number of physiological functions such as contraction of cardiac and smooth muscle, the release of neurotransmitters and hormones and the regulation of enzymatic activities. In neurons, voltage-activated calcium channels appear to form macromolecular complexes with the unique large conductance calcium- and voltage-activated K+ channels (BKCa). The BKCa channel co-purified with the calcium channels Cav1.2, Cav2.1, Cav2.2, but not with the Cav2.3 channel. Such co-localization ensures the selective and rapid activation of the BKCa channels by the local increase of cytosolic calcium. Together these channels contribute to the action potential repolarization, to the fast phase of afterhyperpolarization and to the formation of a feedback loop that regulates the release of hormones and transmitters.In order to demonstrate a direct interaction between the BKCa and voltage-activated calcium channels and to identify the precise interaction site, we used the split-ubiquitin system, a yeast based genetic screening system. With this novel system the interaction of two proteins can be detected in situ at the cellular membrane. As a positive control we routinely used the calcium channel ß subunit, which binds to the intracellular loop connecting domain I and II. In the yeast system, the ß subunit interacts with domain I of all calcium channel constructs tested so far (Cav1.2, Cav2.1, Cav2.3). To identify the interaction site with the BKCa channel, we performed screenings of BKCa-cDNA fragments with the four different domains of the Cav1.2 a1 subunit as baits. We revealed several positive yeast clones on selective media and in the ß-galactosidase test. The results indicate a direct interaction of the BKCa channel with domain I of the Cav1.2 a1 subunit, but not with domains II to IV. We are currently narrowing down this interaction site using various transmembrane constructs of domain I.

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154

The importance of Kir6.2 for the binding of glibenclamide to the sulphonylurea receptor

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Glibenclamide (GBC) binds to the sulphonylurea receptor (SUR1), the regulatory subunit of the ATP-sensitive (KATP) channel to induce channel closure. Experimental evidence suggests that the pore-forming subunit of the channel, Kir6.2, is also part of the binding site for GBC on the channel. We thought to investigate the importance of Kir6.2 for [3H]GBC binding to SUR1. Binding experiments were performed in intact HEK cells expressing SUR1 or SUR1 + Kir6.2 at 37 °C. In equilibrium experiments, coexpression with Kir6.2 decreased KD of SUR1 3 times, from 1.5 to 0.5 nM. Kinetic experiments showed that binding of GBC to the channel followed second order kinetics. The apparent rate of association increased linearly with [3H]GBC concentration and from the slope, the association rate constant kon was determined to be 0.1 nM-1 min-1. The dissociation rate constant was koff = 0.05 min-1 and was independent of whether dissociation was induced by dilution of the complex or by addition of the unlabelled ligand up to 10 µM. From these values, KD was calculated to be 0.5 nM in excellent agreement with the value from equilibrium experiments. GBC binding to SUR1 alone proved to be more complex. The rate of dissociation elicited by dilution was koff = 0.14 min-1 and increased with the concentration of unlabelled ligand when dissociation was measured by isotopic exchange, reaching a limiting value of 3 min-1 with midpoint at 0.6 µM. This is strong evidence for the existence of a low affinity GBC site at SUR1. Occupation of this site may lead to a rearrangement of the complex with lower affinity of SUR1 for the first ligand and faster dissociation, thus representing a particular example of negative cooperativity. The apparent association rate increased rather weakly with GBC concentration and did not extrapolate towards the dissociation rate constant, thus ruling out second order binding kinetics.The study shows that Kir6.2 exerts a profound effect on GBC binding to SUR1 by changing the mechanism of binding kinetics and by shifting the affinity of the second GBC site on SUR1 from 0.6 µM to very high (>100 µM) concentrations.

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155

Dissociation of glibenclamide from the pancreatic KATP channel and recovery from inhibition

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This study investigates the relation between the recovery of the pancreatic KATP channel, (SUR1/KIR6.2)4, from glibenclamide (GBC) inhibition and the dissociation rate of GBC from the four SUR1 subunits in channel. At room temperature, inhibition of channel activity by GBC is irreversible, on the time scale of the experiment (< 1hr), following washout of the drug. In [3H]GBC binding studies the dissociation half-time of the complex (t1/2(d)) is >1 hr; at 37°C, t1/2(d) is 15 min but little recovery of channel activity is observed over 15 min. This is incompatible with partial channel reactivation following partial dissociation of GBC from the 4 SUR1 subunits. In a model where all 4 subunits must be free before the channel can reopen, recovery is predicted to follow a sigmoidal time course with a lag phase and an apparent recovery half-time, t1/2(r) = 2.6 times t1/2(d). A truncated KIR6.2, KIR6.2DN5, lacking 5 amino-terminal residues forms complexes with SUR1 that have a t1/2(d)~8 min. When (SUR1/KIR6.2 DN5)4 channels were tested in the whole cell mode at 37°C following application (5 min) and washout of GBC (30 nM), sigmoidal recovery kinetics were observed with t1/2(r) = 15 (12, 21) min (n = 4), in qualitative agreement with the model. Recovery was only partial, probably as a consequence of channel run down which leads to an apparent speed-up of the observed kinetics. We conclude that a model in which GBC must dissociate from all 4 subunits before the channel can reopen is in agreement with the experimental data.

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156

Allosteric control of ATP-induced SUR1/Kir 6.2 closure

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Allosteric interaction of the receptor binding sites for KATP channel blocking sulfonylureas and potassium channel openers has been shown to be critically affected by point mutations within the sulfonylurea binding region of the second transmem-brane domain of SUR1. The aim of this study was to analyze the effect of some of these mutants in allosteric control of ATP-in-duced SUR1/Kir6.2 closure. For this purpose SUR1 and Kir6.2 mutants were generated, sequenced to verify PCR fideli-ty, tran-siently expressed in COS1-cells and analyzed using the inside-out mode of the patch-clamp technique. The EC50 for ATP-in-duced inhibition (10µM) was slightly shifted to the right by ad-dition of Mg2+ (final free concentration = 0.7 mM; EC50 = 15 µM.) This rightwards shift was strongly increased by intro-ducing point mutations within SUR1, which egalized the nega-tive allosteric interaction between the sites for sulfonylureas and potassium channel openers (EC50 for ATP-induced channel clo-sure in the presence of Mg2+ = 100 µM). Vice versa, however, in SUR1/Kir6.2G334D-channels lacking sensitivity for inhibitory ATP these mutants induced a clear increase of MgATP´s po-tency for channel activation. These results support a complex model of allosteric interactions in which sensitivity of the in-hibitory ATP-site on Kir6.2 is regulated by two distinct sites for MgATP on SUR1.

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157

The KATP channel subunit SUR1 determines cell viability of beta cells under condtions of oxidative stress

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Oxidative stress occurs during diabetes mellitus as well as islet transplantation and leads to impaired beta cell function and cell death. In order to determine the relevant target for the destructive effects, we evaluated the impact of oxidative stress on cell viability of KATP channel deficient, SUR1 knockout (SUR1-KO) beta cells. As shown previously, low concentrations of H2O2 (0.025 and 0.1 mM) impair insulin secretion of wildtype (WT) islets. By contrast, in SUR1-KO islets hormone secretion decreased at concentrations above 0.25 mM H2O2. Electrical activity as well as calcium homeostasis are preserved in beta cells of SUR1-KO mice stressed with 10 µM H2O2 but not in WT beta cells. To investigate possible differences in cell viability, we determined apoptosis (TUNEL- and caspase3-positive beta cells) in WT and SUR1-KO mice. One hour incubation of WT beta cells with 10 or 25 µM H2O2 increased apoptotic cell death 3.0±0.4-fold (n=3, P<0.01) and 4.0±0.3-fold (n=3, P<0.01) above basal values, respectively. By contrast, 10 µM H2O2 had no effect on apoptosis in SUR1-KO beta cells. As reactive nitrogen species contribute to oxidative stress, we analysed the effect of the NO-donor SNOC on cell survival. 100 µM SNOC did not influence apoptosis in beta cells of SUR1-KO mice whereas it was augmented 2.7±0.4-fold (n=3, P<0.01) in WT mice. To elucidate the underlying mechanism, we preincubated WT cells with 25 µM tolbutamide for four hours which prevented the destructive effect of 10 µM H2O2. These data show that loss of functional KATP channels renders beta cells less sensitive to oxidative stress as cell viability and functionality are preserved under these conditions. Consequently, KATP channels are essential sites of action for reactive oxygen species in pancreatic beta cells. Thus, modulation of KATP channels may be an interesting target to protect beta cells against oxidative insults.

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158

17β-Estradiol is a ligand of the sulfonylurea receptor (SUR) and induces apoptosis in a SUR1 isoform-specific manner

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The sulfonylurea receptor (SUR) forms the regulatory subunit of ATP-sensitive K+ channels (KATP channels) and is able to interact with nucleotides or synthetic channel modulators. Previously, we have shown that the “phytoestrogen” resveratrol (RSV) specifically binds to SUR and can induce enhanced apoptosis in cells expressing the pancreatic isoform SUR1. In the present study, we investigated whether 17β-estradiol (E2) was also a ligand of SUR and triggered SUR1-specific apoptosis. E2 is an endogenously occuring estrogen which shows structural and functional analogies to RSV. Heterologous competition experiments using the [3H]-labeled KATP channel blocker glibenclamide (GBC) or the [3H]-labeled opener P1075 as the radioligands showed that E2 specifically binds to SUR with isoform-dependent differences. The interaction of E2 with GBC at SUR is dependent on the presence of MgATP. In addition, cells from the clonal β-cell lines HIT-T15 or RIN-m5F as well as HEK293 cells expressing different isoforms of SUR were treated with E2 (100 µM, 24 h). E2 induced severe cell detachment and changes in nuclear morphology in the β-cell lines or SUR1-expressing HEK cells, while HEK cells expressing the cardiac isoform SUR2A or the vascular isoform SUR2B as well as sham-transfected control-cells were less affected. Moreover, a significant increase in caspase 3-like activity was observed in SUR1-expressing HEK cells. The number of apoptotic nuclei in recombinant SUR1-cells was about 3-fold higher compared to SUR2A-, SUR2B-, or control-cells. Differences between SUR1-cells and control-cells were already observed with lower E2 concentrations after longer treatment intervals (e.g. >1 µM, 4 days). SUR1-specific apoptosis of E2-treated cells was abolished by a single mutation (M1289T) in transmembrane helix 17 of SUR1. In conclusion, E2 specifically interacts with SUR in an isoform and/or nucleotide-dependent manner and induces SUR1-specific apoptosis even to a much larger extent than resveratrol. As E2 levels are markedly elevated during pregnancy, E2-induced apoptosis in pancreatic β-cells might provide an interesting aspect concerning the etiology of gestational diabetes.

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159

(-)-Epigallocatechin-3-gallate specifically interacts with the sulfonylurea receptor (SUR) in a SUR isoform-dependent manner

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Sulfonylurea receptors (SURs) are subunits of ATP-sensitive potassium channels (KATP channels) and play an important role in channel regulation. They possess binding sites for nucleotides and several synthetic KATP channel modulators. Apart from the nucleotides little is known about naturally occuring ligands. In previous studies, we were able to show that the stilbene derivative resveratrol directly interacts with SUR and is able to induce enhanced apoptosis in a SUR1-dependent manner.In the present study, we analyzed whether (-)-epigallocatechin-3-gallate (EGCG), a polyphenol present in green tea, which reduces ATP sensitivity of KATP channels, was also a ligand of SUR. Binding of EGCG to SUR was examined in heterologous competition binding studies using the KATP channel blocker [3H]glibenclamide (GBC) or the opener [3H]P1075 as the radioligands. In order to perform comparable experiments with [3H]GBC, the mutation Y1206S was inserted in SUR2A and 2B because it endows these isoforms with higher affinity for GBC. Furthermore, the effect of EGCG on cell viability of recombinant SUR-expressing HEK293 cells was investigated by determining detachment of these adherently growing cells.[3H]GBC competition assays show that EGCG specifically interacts with SUR. SUR1 shows higher affinity for EGCG in the presence of 1 mM MgATP (inhibitory constant Ki ≈ 80 µM) than in the absence of MgATP (Ki ≈ 210 µM). In case of SUR2A(Y1206S), binding of EGCG also depends on MgATP with Ki ≈ 40 µM in the presence of MgATP and Ki ≈ 140 µM without MgATP. For SUR2B(Y1206S), [3H]GBC is displaced independent of the presence of MgATP (Ki ≈ 140 µM). Differences in binding EGCG to SUR2A and SUR2B were also visible in [3H]P1075 competition experiments. Concerning viability, EGCG treatment of sham-transfected HEK293 cells (100 µM, 6 h) leads to severe cell detachment. This detachment was significantly reduced in cells expressing SUR1 or SUR2A, but not SUR2B.In conclusion, EGCG specifically binds to SUR in an isoform-specific manner, which in case of SUR1 and SUR2A is modulated by the presence of nucleotides. The effect of EGCG on cell viability also differs in a SUR isoform-dependent manner.

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160

Different modes of antagonism by glibenclamide and 6-chloro-3-alkylamino-4H-thieno[3,2-E]-1,2,4-thiadiazine 1,1-di-oxide derivatives in SUR2B-type KATP channels

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New openers (KCO) of SUR1-type KATP channels reported by Nielsen et al. (J Med Chem 45: 4171, 2002) were characterized in SUR2B-type KATP channels as agonists if R=CH3 and antagonists if R=H (Lemoine et al., this journal 375, R45, 2007). To elucidate the question if these novel KATP channel blockers act competitively or allosterically (similar to glibenclamide) we performed experiments in rat aortic smooth muscle cells. Agonistic and antagonistic actions of compounds were measured in 12-channel fluorescence detectors with fast data sampling (1 Hz) as hyperpolarisation and as depolarisation, resp., of membrane potentials using DiBAC4(3) (1.5 µM, 488/515 nm). As test compounds we chose agonists of varying chemical structures with different dissociation constants (KD): the benzopyran KC 399 (0.3 nM), the cyanoguanidine P 1075 (6 nM) and the thiadiazines diazoxide (200 µM) and the cyclopentyl (n=3, R=CH3)-derivative C55 (100 nM)as well as the antagonists glibenclamide and the cyclopentyl (n=3, R=H)-derivative C51 (100 nM). Using receptor-saturating concentrations of KC 399 and P 1075, kinetics of hyperpolarisation were fast (t½ 1 – 3 min); restoring signals induced by 10 µM glibenclamide interestingly revealed fast kinetics (t½ 1 – 3 min), but slow kinetics (t½ 15 – 20 min) when induced with 30 µM C51. In contrast, hyperpolarisation induced with the thiadiazines could be restored with fast kinetics independently of the type of antagonist. The findings could be consistenly interpreted by a model which attributes an allosteric and a competitive mode of action to glibenclamide and C51, resp. Glibenclamide by occupying its binding site (which is different from the agonist binding site) induces the transition of the SUR2B-receptor to a low affinity state thereby inducing a fast dissociation of the agonist associated with a fast depolarisation, whereas C51 displaces the high-affinity, slowly dissociating agonist from its binding site by simple competition. Thus, it becomes clear that sulphonylurea antagonists (glibenclamide) bind to a region of the SUR2B-receptor different from the binding sites for novel thiadiazine-antagonists.

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161

Inside-out patch-clamping with KATP channels at 37 °C

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KATP channels are composed of pore-forming subunits Kir6.x and of regulatory subunits, the sulfonylurea receptors which are endowed with ATPase activity. They are gated by nucleotides (in particular ATP and ADP) and their activity is regulated by the delicate balance between inhibitory effect of ATP binding to Kir and the activatory effect of MgATP and MgADP binding to SUR. In particular Kir6.2 containing channels have been characterised intensively using the inside-out patch-clamp configuration at room temperature. This approach is common and well established for many channels, however, it may give limited information on the metabolically gated KATP channels. A striking example are the Kir6.1 containing channels which show good activity in whole-cell patch-clamp recordings at 37 °C, but are almost inactive in the inside-out recordings at room temperature. In this project we attempt to explore the effect raising temperature from 22 to 37 °C on the properties of recombinant Kir6.x/SUR2B channels in inside-out patches.First, a setup was constructed allowing defined heating of the small solution volume applied to the tip of the patch-pipette with a sewer pipe system. At 37 °C, Kir6.2/SUR2B channels showed a decreased sensitivity to inhibition by MgATP and a prominent rundown of channel activity, especially in the absence of MgATP, resulting in a lower activity in the absence of ATP compared with the presence of 100 µM MgATP. This behaviour resembles that of Kir6.1/SUR2B channels which open only in the presence of Mg-nucleotides. Preliminary experiments with Kir6.1/SUR2B channels showed an increased activity with increasing temperatures.These preliminary data show that increasing temperature shifts the balance between the inhibitory and activatory effects of MgATP and therefore exerts a profound effect on the gating of KATP channels.

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162

SK channels modulate glucose-induced stimulation of pancreatic beta cells

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Besides KATP channels voltage-activated Ca2+ and K+ channels play an important role for glucose-induced electrical activity in pancreatic beta cells. In the present study we tested whether ablation of Ca2+-regulated K+ channels of intermediate conductance (SK4 channels) affects glucose homeostasis and beta cell function. For glucose and insulin tolerance tests 2 g glucose/kg body weight (BW) and 0.7 I.U. insulin/kg BW, respectively, were injected i.p. in male SK4 channel knockout (SK4-KO) mice and wildtype (WT) littermates. Vm was measured with the patch-clamp technique. Cytosolic free Ca2+ concentration ([Ca2+]c) was determined by fura-2. SK4-KO animals showed improved glucose tolerance (2-hour plasma glucose concentration of 6.2±0.5 mM in SK4-KO vs. 9.5±1.2 mM in WT animals, n=5, p<0.5) with no alterations in insulin sensitivity (reduction of plasma glucose 60 min after i.p. injection of insulin: 47±6 % in SK4-KO vs. 39±3 % in WT littermates, n=5-6). Since these results suggest that KO of SK4 channels alters beta cell reactivity isolated beta cells were tested for differences in glucose responsiveness. Stimulation with 15 mM glucose induced Ca2+ action potentials in WT and KO beta cells. However, action potential frequency was significantly increased in SK4 channel-deficient cells (SK4-KO: 97±5/min, n=53 vs. WT: 74±12/min, n=18, p<0.05). Elevating glucose concentration from 0.5 to 6 mM induced depolarization of Vm and Ca2+ action potentials in 64 % of SK4-KO beta cells (n=11) with no stimulation in WT beta cells (n=7). Rising glucose to 8 mM depolarized 100 % of SK4-KO (n=5) but only 38 % of WT beta cells (n=8). A similar left-shift was observed for glucose-induced elevation of [Ca2+]c. In conclusion, these data show that SK4 channels contribute to glucose-mediated stimulation of pancreatic beta cells. Modulation of SK4 channel activity might provide a novel strategy to increase insulin secretion in patients with impaired glucose homeostasis.

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163

A more severe urinary bladder phenotype in time- and tissue-specific than in constitutive deletion of BK channel

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The large-conductance, voltage and Ca2+-dependent K+ (BK) channel links membrane depolarization and local increases in cytosolic Ca2+ to hyperpolarizing K+ outward currents, thereby controlling smooth muscle contractility. Constitutive deletion of the BK channel in mice (BK-/-) leads to overactive bladder associated with increased intravesical pressure and frequent micturitions which was traced back to hyperactivity of the detrusor muscle caused by depolarized membrane potential. Moreover, the time-dependent and smooth muscle-specific deletion of BK channel (SM-BK-/-) revealed a more severe phenotype than in BK-/- mice, suggesting that compensatory pathways became operative after constitutive deletion of BK channel in mice. We found an enhanced β-adrenoceptor/cAMP-mediated suppression of contractions accompanied with an increased isoproterenol-induced cAMP formation in BK-/- detrusor muscle compared to wild-type. In-depth analysis revealed an up-regulated protein expression of regulatory as well as catalytic subunit of PKA in BK-/- urinary bladder when compared to wt. This compensation was not observed after time-dependent deletion of urinary bladder BK channels. These results indicate that the time-dependent and smooth muscle-specific deletion of BK channel can unmask compensatory mechanisms, thus facilitating the analysis of the pure role of BK channels in smooth muscle.

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164

Combined IKur/Ito block by 4-aminopyridine produces a positive inotropic effect in human atrial trabeculae but provokes arrhythmias in preparations from patients in SR

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Selective block of IKur elevates the human atrial action potential plateau accompanied by a secondary increase in ICa (Wettwer et al., Circulation 2004;110:2299 resulting in a positive inotropic effect in atrial trabeculae from patients in sinus rhythm (SR) or chronic atrial fibrillation (AF). IKur blockade could be proarrhythmic due to increased Ca-influx with further aggravation by increased sympathetic activation. Here we investigated the effect of IKur/Ito block on force of contraction (Fc) in electrically stimulated (1 Hz) human atrial trabeculae from SR and AF patients. Proarrhythmic effects were assessed by occurrence of spontaneous contractions. Ten µM of 4-aminopyridine (4-AP) were used for selective IKur block, 100 µM and 1 mM for mixed IKur/Ito block, before preparations were challenged with norepinephrine (NE). Basal Fc in all-time rhythmically stable preparations was lower in AF than in SR (1.15 ± 0.12 vs. 5.55 ± 0.34 mN/mm2, n = 75/11 [trabeculae/patients] vs. n = 142/32, p< 0.001). In AF 10 µM 4-AP increased Fc by 68 % from 1.11 ± 0.22 to 1.82 ± 0.39 mN/mm2 (n = 20/10). High concentrations of 4-AP (1 mM) increased Fc close to the control level in SR (from 0.99 ± 0.16 to 4.36 ± 0.72 mN/mm2, n = 15/9). In SR, 10 µM of 4-AP increased Fc by 17% from 5.47 ± 0.61 to 6.40 ± 0.65 mN (n = 40/27, p < 0.005), 1 mM of 4-AP increased Fc from 5.26 ± 0.47 to 7.57 ±

0.65 mN/mm2, n = 27/21. Increasing concentrations of NE (range 1 nM to 100 µM) lead to significantly more spontaneous contractions in SR (25 %) than in AF (<5%). AF preparations may be protected by increased activity of constitutively active IK,ACh (Dobrev et al, Circulation 2005;112:3697). The percentage of arrhythmic SR preparations was increased to 54 % with 100 µM and to 62 % with 1 mM 4-AP, whereas only 15 % (1 mM 4-AP) of AF trabeculae became arrhythmic. In conclusion, combined IKur /Ito block improves contractile function in AF and SR. SR preparations were significantly more prone to develop spontaneous activity in response to IKur/Ito block than AF trabeculae. Thus, combined IKur/Ito block in AF treatment may be accompanied by an increased risk of arrhythmias, once SR is restored.

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165

Diminished effects of AVE0118 on potassium channels and contractility in AF-remodeled human atria

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The mixed K+ channel blocker AVE0118 restores sinus rhythm (SR) and reverses contractile dysfunction in a goat model of atrial fibrillation (AF), however, its electrophysiological effects in human atrial myocardium are controversial, possibly due to AF-induced electrical remodeling or species differences. Here we analysed the effects of AVE0118 on electrophysiological and contractile parameters of atrial myocardium from patients in SR and chronic AF. Potassium currents (Ito, IKur) were measured in isolated atrial myocytes with whole-cell voltage clamp (500 ms-long clamp steps between –60 and +50 mV, 0.1 Hz). Action potentials and contractions were recorded in isolated atrial trabeculae with standard techniques. Experimental temperature was 37°C. Transient outward currents were significantly (p<0.05) smaller in AF than in SR, i.e. at +50 mV, peak current amplitudes (Ito) were 12.3±5.9 pA/pF vs. 25.4±2.3 pA/pF (n=24/8 cells/patient), and late current amplitudes (IKur) were 7.0±0.7 pA/pF vs. 9.0±0.8 pA/pF (n=21/9), respectively. AVE0118 concentration dependently reduced outward current, however, the AVE0118-sensitive fraction was smaller in AF compared to SR (13±8% vs. 45±3% for peak and 15±5% vs. 52±5% for late current; n=23/7 vs. 7/5, respectively, p<0.05). AVE0118 (12 µM) prolonged action potential duration in AF (from 287±16 ms to 301±11 ms; n=8/8, p<0.05), but not in SR (344±6 ms and 337±7 ms, respectively, n=14/14). The most prominent effect of AVE0118 in SR consisted of elevation of plateau potential from -15.3±2.6 mV to 1.3±3.7 mV (n=16/16), that was accompanied by enhanced force of contraction (from 7.8±0.9 mN to 8.9±1.0 mN, equivalent to 25% of maximum response to high extracellular Ca2+ [8 mM] p<0.05; n=31/14). In AF, however, AVE0118 also elevated plateau potential though less prominent than in SR (from 7.8±1.3 mV to 14.4±2.4 mV, p<0.05, n= 8/8), but failed to enhance contractile force (2.4±0.3 mN vs. 2.6±0.4 mN, n=22/9, n.s.). The efficacy of AVE0118 to block Ito/IKur channels in human atrial preparations is attenuated likely due to AF-induced electrical remodeling. There is no evidence for amelioration of AF-associated contractile dysfunction in human atrial tissue preparations.

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166

SUR2B(Y1206S) lacking transmembrane domain 0 (TMD0) can still from oligomers and interact with KIR6.2

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ATP binding cassette (ABC) proteins are defined by the presence of the 'ABC unit', which comprises two short conserved peptide motifs (Walker A and Walker B), involved in ATP binding, and the 'ABC signature', a third conserved sequence located between Walker A and B. Most ABC proteins are membrane proteins with one or two transmembrane domains each followed by a cytosolic ABC unit. Several members of the MRP/CFTR subfamily, including the sulfonylurea receptors, SUR1 and SUR2, the regulatory subunits of ATP-sensitive potassium (KATP) channels, have an additional N-terminal transmembrane domain termed TMD0. TMD0 strongly associates with the KIR6.2 pore, modulates its trafficking, and with L0, the adjacent peptide segment, governs gating. We examined the role of TMD0 in the SUR2B(Y1206S)-KIR6.2 interaction and the KIR independent SUR oligomerization. Replacing Y1206 with serine allowed radioligand binding experiments utilizing [3H]glibenclamide. In the absence of ATP KIR6.2 shifted the affinity of SUR2B(YS)DTMD0 (i.e. lacking TMD0) for repaglinide from 63 nM to 40 nM (P < 0.01). Furthermore, the MgATP-dependent, biphasic displacement of [3H]glibenclamide by P1075 (N-cyano-N'-(1,1-dimethylpropyl)-N''-3-pyridylguanidine) from SUR2B(YS), without KIR6.2, hypothesized to be due to SUR2B homotetramerization, was also seen with SUR2B(YS)DTMD0. These results are supported by immunoprecipitation (IP) data: FLAG-tagged SUR2B(YS)DTMD0 will co-IP with both myc-tagged KIR6.2 and myc-tagged SUR2B(YS)DTMD0. These findings show that SUR2B(YS) lacking TMD0 can interact with KIR6.2 and can self-oligomerize in absence of KIR6.2. However we could not detect any channel activity in HEK293 expressing KIR6.2 and SUR2B(YS)DTMD0. Apparently this channel complex cannot be activated or does not reach the plasma membrane e.g. because of improper folding or insufficient shielding of ER retention signals.

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167

M2 muscarinic receptor stimulation induces a dual inhibition of BK channel activity through Gßγ and PKC

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This study investigates the mechanism by which stimulation of M2 muscarinic receptors (M2Rs) inhibits large conductance calcium-activated K+ channels (BKCa channels). In HEK293 cells stably over-expressing the M2R and transiently transfected with the pore-forming a-subunit of the BKCa channel cloned from bovine trachea, carbachol (CCh; 10 µM) inhibited BKCa currents elicited in the whole-cell patch-clamp configuration by 53% (n = 22). This inhibitory effect was abolished after pre-treatment of the cells with 500 ng/ml PTX or by over- expression of the Gißγ scavenger transducin a (TDa). The PLC inhibitor U73122 (2.5 µM) and the PKC inhibitors Ro318220 (1 µM) and Goe6976 (100 nM) partially reversed the CCh effect. In cell-attached recordings, CCh induced a transient increase in the channel open probability (NPo). This transient effect was prevented by pre-treatment of the cells with PTX, U73122, or by over-expression of TDa. In inside-out patches, 100 nM TDßg decreased NPo by 55% (n = 15). After additional application of 300 nM TDa, NPo returned to baseline. Co-immunoprecipitation experiments demonstrated a direct interaction between BKCa channels and Gβγ. In HEK293 cells stably over-expressing the Gi-coupled α2A adrenoceptor (α2A-AR) and transiently transfected with the BKCa a-subunit, 10 µM noradrenaline (NA) produced no effect. However, in HEK293 cells stably over-expressing M2Rs or a2A-ARs and transiently transfected with GIRK1/4 channels, both CCh and NA induced strong inward rectifier currents. In isolated smooth muscle cells from mouse trachea, CCh induced a PTX-sensitive inhibition of BKCa currents (by ~50%) which was partially reversed by the PKC inhibitors Goe6976 and Ro318220. The results demonstrate that Gβγ subunits released from PTX-sensitive Gi proteins by activated M2Rs, stimulate intracellular calcium release via PLC-IP3 and inhibit BKCa channel activity through a direct effect and through activation of PKC. The inhibitory effect via Gβγ on BKCa channel is receptor-specific. Inhibition of BKCa channels is a mechanism that facilitates airway contraction during cholinergic stimulation.

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168

The glucose utilization is impaired by angiotensin II in leptin resistant Zucker rats due to an adrenal mechanism

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We previously have found that angiotensin II (ANG) increased the activity of the hypothalamus-pituitary-adrenal (HPA)-axis in leptin resistant obese Zucker rats. The HPA-hyperreactivity was paralleled by an impaired glucose utilization indicating a significant function of the HPA-axis and in particular the adrenal glands within the pathophysiological scenario of the metabolic syndrome. Our aim was to verify the potential role of the adrenals for regulating the glucose homeostasis in dependency of HPA-reactivity. Obese Zucker rats were adrenalectomized (ADX) and substituted with corticosterone (4.76 mg/d, s.c.) via pellets. ADX or sham operated rats (SHAM) and were treated (24 d) with ANG (9 µg/h, s.c.) or saline (controls) using osmotic minipumps. Compared to SHAM, the body weight was decreased in ADX. In addition, aldosterone was below the detection limit and did not increase in response to ANG. Due to supplementation, plasma corticosterone did not differ between ADX and SHAM. HPA-reactivity was characterized by monitoring stress hormones before and 30 min after a forced swim test (15°C; 10 min). The stress-induced increase in ACTH was similar between saline and ANG treated rats. Selectively in SHAM, the ACTH response was paralleled by an increase in corticosterone. After stress, blood glucose selectively declined in ADX confirming the significance of the adrenals for maintaining glucose homeostasis in stress situations. To confirm this hypothesis and in particular the importance of ANG in the process, an oral glucose-tolerance-test (1 g glucose/kg) has been performed by monitoring glucose during 4 h. After glucose challenge, the plasma profiles of glucose did not differ between saline treated ADX and SHAM. In response to ANG, the glucose levels were markedly increased in SHAM, but reduced in ADX compared to the corresponding controls. In summary, we verified our hypothesis that the adrenal glands play a key role for maintaining glucose homeostasis in response to ANG. Thus, we approve findings that a reduction in HPA-hyperreactivity after AT1-blockade may participate in the diminished incidence of type-2 diabetes as seen in clinical trials.

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169

Liver-specific PPARalpha-target gene regulation by the angiotensin type 1 receptor blocker telmisartan

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Objective – The angiotensin type 1 receptor blocker (ARB) and PPARg-modulator telmisartan has been recently demonstrated to reduce plasma triglycerides in non-diabetic and diabetic hypertensive patients. The present study investigates the molecular mechanisms of telmisartans hypolipidemic actions, in particular its effect on the PPARa pathway. Research Design and Methods – Regulation of PPARa-target genes by telmisartan was studied by real-time PCR and Western immunoblotting in-vitro and in-vivo in liver/ skeletal muscle of mice with diet-induced obesity (DIO). Activation of the PPARa-ligand binding domain (LBD) was investigated using transactivation assays. Results – Telmisartan significantly induced the PPARa target genes carnitine palmitoyl transferase 1A (CPT1A) in human HepG2 cells and acyl-CoA synthetase long-chain family member 1 (ACSL1) in murine AML12 cells in the µ-molar range. Telmisartan-induced CPT1A stimulation was markedly reduced after siRNA-mediated knockdown of PPARa. Telmisartan consistently activated the PPARa-LBD as a partial PPARa agonist. Despite high in-vitro concentrations required for PPARa activation, telmisartan (3mg/kg/d) potently increased ACSL1 and CPT1A expression in liver from DIO-mice associated with a marked decrease of hepatic- and serum triglycerides. Muscular CPT1B expression was not affected. Tissue specificity of telmisartan-induced PPARa-target gene induction may be the result of previously reported high hepatic concentrations of telmisartan. Conclusions – The present study identifies the ARB/ PPARg modulator telmisartan as a partial PPARa agonist. As a result of its particular pharmacokinetic profile, PPARa activation by telmisartan seems to be restricted to the liver. Hepatic PPARa activation may provide an explanation for telmisartan's anti-dyslipidemic actions observed in recent clinical trials.

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170

Effects of angiotensin peptides on insulin secretion and 45 Ca2+ uptake in the insulinoma cell line INS-1

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We have previously shown an autonomous angiotensin-generating system in INS-1 cells. Angiotensinogen is enzymatically cleaved to the bioactive angiotensin peptides ANGI, ANGII, ANGIII, ANG1-7 and ANGIV. These observations raise the possibility that an intracellular renin angiotensin system exists in the B-cell. The AT1- and AT4-receptor (insulin regulated aminopeptidase (IRAP)) have been demonstrated in INS-1 cells by western blot. The role of these receptors with respect to their impact on the insulin secretory pathway is not well established. AT1/AT2-receptor ligands and AT4-receptor ligands are used to elucidate their role in regulating insulin secretion by insulin radioimmunoassay and 45Ca2+ uptake.ANGII as well as the non-degradable analogue sarile increase insulin release in a bell-shaped way (max. at 1 nM); the increase is completely antagonized by 1 µM losartan. The insulin release is not accompanied by an equivalent 45Ca+ uptake. The number of receptors (Bmax) for ANGII is low indicated by a small displacement of 125I-ANGII from binding sites at nM concentrations and high nonspecific binding. The AT4-receptor agonist ANGIV exhibits a weak increase in insulin release at nanomolar concentrations and significantly enhances the 45Ca2+-uptake in subnanomolar concentrations. Nle-ANGIV, a more stable analogue of ANGIV, increases insulin release at nanomolar concentration. Divalinal, an AT4-receptor antagonist, abolishes the effect of ANGIV and Nle-ANGIV on both parameters. Surprisingly we found that divalinal indicates partial agonistic properties at the AT4-receptor at micromolar concentrations leading to an increased 45Ca2+-uptake. ANGII binding has a low impact on insulin release. AT4-receptor ligands may transduce the signal initiated by ligand binding to the C-terminal domain of the AT4-receptor to the intracellular domain that interacts with several proteins like PI3K, PKB and ERK resulting in 45Ca2+-uptake and insulin secretion.

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171

Characterization of cardiac myocyte-specific deletion of the mineralocorticoid receptor gene in mice

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Introduction: The RALES and EPHESUS trials showed that treatment with the mineralocorticoid receptor (MR) antagonists spironolactone and eplerenone reduced cardiovascular mortality. However, the mechanisms involved in these beneficial effects are still not completely known. As MR is expressed in both heart and kidney, it is difficult to distinguish between direct and indirect effects mediated by aldosterone. The aim of this work was to evaluate whether cardiomyocyte-selective deletion of the MR gene is cardioprotective after chronic stimulation with deoxycorticosterone acetate (DOCA) or transverse aortic constriction (TAC).Results: At baseline, cardiomyocyte-specific MR knockout (KO) mice showed a 30% increase in myocyte size, a significant increase in the heart weight/tibia length ratio (WT: 5.9±0.1; KO: 6.8±0.4 mg/mm, n=6, p=0.03), as well as a 2-fold increase in cardiac ANP mRNA levels. Moreover, in KO mice cardiac catheterization revealed an increased left ventricular (LV) basal contractility (WT: 6314±361; KO: 7712±371 mmHg/s, n=12, p=0.001) and systolic pressure (WT: 95±4; KO: 107±3 mmHg, n=12, p=0.02). Treatment with high-salt and DOCA or TAC for 8 weeks led to significant increases in cardiac weight and dimensions with no significant differences between genotypes. Histological analysis revealed an increase in interstitial fibrosis in LV sections of mice submitted to either DOCA-salt or TAC compared to baseline. In addition, CTGF and procollagen Ia1 mRNA expression were increased after both interventions, although none of these responses was attenuated by the MR-KO. Finally, microarray analysis (Affymetrix) of WT and KO heart samples at baseline demonstrated a significant regulation (>1,5-fold) of 66 genes (p<0.05), e.g. calsequestin 1 (3.5-fold upregulated) and the ryanodine receptor 2 (1.6-fold downregulated). Differential expression of these genes was confirmed by qRT-PCR. Conclusion: Cardiac myocyte-specific deletion of the MR gene in mice did not protect from cardiac hypertrophy and fibrosis after DOCA-salt treatment or TAC intervention. Thus, the cardioprotective effect of MR antagonists may be mediated via a non-cardiomyocyte dependent mechanism.

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172

A new function of ACE - interactions with membranal co-localized carboxypeptidase M (CPM)

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Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is a membrane-bound dipeptidyl-carboxypeptidase with prominent functions as regulator of circulatory and blood-pressure relevant systems. It converts the decapeptide angiotensin I to the vasoconstrictory octapeptide angiotensin II, and inactivates vasodilators like bradykinin. Beside this typical dipeptidyl-carboxypeptidase activity of ACE, several unusual functions were described in the last years. We demonstrate with several molecular, biochemical and cellular techniques that the somatic isoform of murine ACE (mACE), stably transfected in CHO or MDCK cells, interacts with endogenous membranal co-localized carboxypeptidase M (CPM). CPM belongs to glycosylphosphatidylinositol (GPI)-anchored proteins. We describe that ACE, completely independent of its well known dipeptidase activities, has GPI-targeted actions. Our results indicate that the spatial proximity between mACE and the endogenous CPM enables an ACE evoked release of soluble CPM. These results are discussed with respect to recently published GPI-ase activity of sperm-bound ACE.

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173

Biphasic regulation of HMG-CoA reductase expresssion and activity during wound healing and its functional role in the control of keratinocyte angiogenic and proliferative responses

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We determined the regulation and potential function of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGR) during skin repair in mice. Upon skin injury, wildtype mice exhibited a biphasic increase in HMGR expression and activity with elevated levels at day 3 and 13 post-wounding. In situ hybridization revealed wound margin keratinocytes as a cellular source of HMGR expression. As those cells also represent the major source of wound-derived vascular endothelial growth factor (VEGF), we determined the functional role of HMGR for keratinocyte-mediated VEGF production by treatment of wounded wildtype mice with simvastatin. The simvastatin-mediated inhibition of HMGR activity in wound tissue did not cause any change in the VEGF mRNA levels at the wound site. By contrast, VEGF protein expression in wound tissue was severely impaired upon simvastatin treatment of mice. The observed loss of wound VEGF protein was paralleled by a strong reduction of eukaryotic initiation factor (eIF)-4 binding protein (4E-BP1) phosphorylation. Immunohistochemistry clearly confirmed keratinocytes as the prime cellular source of reduced VEGF levels at wound sites of mock- and simvastatin-treated mice. Reduced levels of wound VEGF might be partially connected to reduced numbers of wound margin keratinocytes upon simvastatin treatment of mice. Interestingly, we identified a decreased proliferation of wound keratinocytes in simvastatin-treated mice. The inhibition of HMGR activity did not impair the Ki67-positive, proliferating cells of the wound margin epithelia. However, simvastatin appeared to interfere with a mechanism temporally downstream of keratinocyte mitosis, as the Ki67-negative cell fraction was markedly reduced upon simvastatin treatment of animals.

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174

Impaired skin repair following diet-induced obesity in C57Bl/6J mice

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Obesity is defined as an increase of adipose mass resulting from a chronic imbalance between energy intake and expenditure. An epidemic of obesity-induced diabetes is on the increase worldwide. Wound healing disorders are a major complication of diabetes mellitus. In rodents, a number of autosomal genes have been implicated in the development of obesity, insulin resistance, and diabetic. We have previously shown that obese and diabetic mice which are genetically defect in their leptin system suffer from severely impaired skin repair. However, the relevance of such murine monogenic models of obesity to human obesity-and-diabetic-associated disorders such as impaired cutaneous repair is questionable. We hence investigate the course of skin repair in a dietary [high-fat diet (HFD)-induced] model of obesity. Compared with mice fed a normal chow diet (CD), mice fed a HFD gradually increased their body weight, which was paralleled by a raise of the glucose, insulin and leptin levels, indicating the onset of diabetic similar to that of humans. Upon injury, HFD-diabetic mice showed a delayed and attenuated acute inflammatory response in comparison with control CD-mice. Reduced inflammation at the wounds of HFD mice results in a decrease of tissue formation and of re-epithelialization rate that was manifested by a delay of wound closure. Finally, similar to genetic (ob/ob or db/db) models of murine obesity, resolution of inflammation and complete healing were considerably disturbed. This indicates the validity of diet-induced obesity as an additional mouse model for diabetes-impaired cutaneous wound healing.

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175

The high mobility group A1 protein - a new regulator of PPARgamma-dependent gene transcription in vascular smooth muscle cells

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Recently, PPARgamma (peroxisome proliferator-activated receptor gamma) ligands have been shown to exert deleterious cardiovascular side effects. In order to develop new PPARgamma-ligands with improved clinical safety the molecular basis for PPARgamma-activation in cardiovascular cells has to be understood. The present study aimed to identify new nuclear cofactors for PPARgamma-dependent gene transcription in human aortic smmoth muscle cells (HASMC).Using an - Oligo GEArray® Mouse Nuclear Receptors and Coregulators Microarray (Superarray®) - for gene expression profiling in unstimulated HASMC, we identified the transcriptional regulator and chromatin modifying High Mobility Group (HMG) A1 protein highly expressed in HASMC. PPARgamma-activation by pioglitazone (10µM) resulted in a 2.6-fold induction of HMGA1 protein expression in HASMC assessed by western immunoblotting. PPARgamma-dependent gene regulation was studied by analysis of PMA-induced MMP-9 (matrix metalloproteinase 9) expression ± pioglitazone (10µM). PMA (50ng/ml) stimulated MMP-9 mRNA expression by 46.3±22.3-fold (p<0.05 vs. vehicle) which was markedly blocked by pioglitazone (10µM: 17.4±4.8-fold vs. vehicle, p<0.05 vs. PMA alone). Pioglitazone also blocked PMA-induced MMP-9 promoter activity by 45% in transactivation assays using a pGL3-MMP-9 2.2 kb construct. To evaluate the role of HMGA1 in PPARgamma-mediated repression of MMP-9, gene silencing experiments with siRNA for HMGA1 were performed. Transfection of HMGA1-siRNA in HEK293 cells resulted in a 80.2% reduction of HMGA1 protein expression. HMGA1 si RNA completely abolished PPARgamma-mediated MMP9-mRNA repression (control siRNA: pioglitazone-mediated MMP-9 regulation: -59.3% vs. PMA alone p<0.05; HMGA1 siRNA: pioglitazone-mediated MMP-9 regulation vs. PMA alone: +14.7% vs. PMA alone; p=n.s..). HMGA1 siRNA also prevented inhibition of MMP-9 promoter activity by PPARgamma activation.In summary, the present study identfies HMGA1 as a nuclear cofactor in HASMC. HMGA1 is required for PPARgamma-mediated repression of MMP-9 gene transcription. Ligand-induced HMGA1-PPARgamma interactions might be an important determinant for anti-atherosclerotic actions of PPARgamma-ligands in vascular smooth muscle cells.

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176

Reactive oxygen species-induced inhibition of calcineurin and activation of the dual-leucine-zipper-bearing kinase contribute to the loss of beta-cell function and mass

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The prediabetic state is characterized by marginally elevated blood glucose levels, postprandial blood glucose peaks, and hyperinsulinemia. Periods of hyperglycemia enhance the mitochondrial production of reactive oxygen species (ROS), which have been shown to decrease insulin secretion and content within the beta-cell. Previous data demonstrated a crucial role for the calcium/calmodulin dependent phosphatase calcineurin for the maintenance of beta-cell function and mass. Therefore, in the present study the effect of ROS on calcineurin phosphatase activity was investigated.Treatment of the insulin-producing pancreatic beta-cell line HIT with hydrogen peroxide for 90 min reduced the viability of the beta-cells in a concentration-dependent way, and the amount of the anti-apoptotic protein Bcl-XL by 36 %. In transient transfections using a luciferase reporter gene under the control of the human insulin gene promoter (from -336 to +113bp) treatment with 100 µM hydrogen peroxide reduced specifically cyclic AMP plus membrane depolarisation-stimulated insulin gene transcription by 30%. Furthermore, hydrogen peroxide inhibited specifically calcineurin phosphatase activity, in a time- and concentration dependent manner. The kinase activity of the endogenous, immunoprecipitated dual-leucine-zipper-bearing kinase (DLK) was 1.5 fold enhanced by hydrogen peroxide, consistent with the notion that inhibition of calcineurin increases DLK activity. Downregulation of cellular DLK by siRNA reduced hydrogen peroxide induced beta-cell death. Considering the importance of calcineurin for the maintenance of beta-cell function and mass and the previously demonstrated beta-cell apoptosis-inducing effect of DLK, our data suggest that ROS by decreasing calcineurin phosphatase activity increase DLK kinase activity, thus leading to diminished beta-cell function and mass. This novel molecular mechanism might contribute to the ROS-induced deterioration of beta-cell function and mass, and the inhibition of DLK might prevent the progression from the prediabetic state to clinically overt diabetes mellitus.

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177

The glucose dependence of insulin secretagogues - the role of depolarisation

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Closure of the KATP channel and subsequent plasma membrane depolarisation and Ca2+ influx are currently regarded as being sufficient to elicit insulin secretion in the presence of a non-stimulatory glucose concentration. The surprisingly modest secretory response to KATP channel blockers from the class of imidazolines led us to reconsider this paradigm using KCl as a non-nutrient depolarizing agent. Using the patch clamp technique in the conventional whole cell mode, a concentration dependency for the depolarizing effect of KCl on normal mouse b-cells was set up. At 40 mM the depolarization was nearly maximal (-29,4 mV) and the half maximally effective concentration was calculated to be 15 mM. With metabolically intact b-cells (perforated patch mode) KCl had the same depolarizing strength. Thus the effect of 40 mM KCl on insulin secretion of perifused mouse islets was compared to that of 15 mM. Initially, the glucose concentration was 5 mM glucose (basal) and was then raised to 10 mM (moderate stimulatory). At 15 mM, KCl had only a modest transient effect on secretion when the glucose concentration was basal, but strongly amplified the response when glucose was raised to 10 mM. At 40 mM KCl, an immediate massive increase of secretion was observed which turned into a continuous decline after 10 min. Raising the glucose concentration during this phase produced only a small transient increase of secretion. The depolarization by 15 mM KCl was sufficient to quickly increase the cytosolic Ca2+ concentration in Fura 2-loaded perifused mouse islets. Raising the KCl concentration to 40 mM led to a fast overshooting response with a subsequent elevated plateau. The block of endoplasmic reticulum calcium pumps by cyclopiazonic acid diminished the plateau response to 40 mM KCl, but not 15 mM KCl. It is concluded that only a depolarization which leads to a Ca2+ induced Ca2+ release from the endoplasmic reticulum is sufficient to elicit a secretory response in the presence of a basal glucose concentration. The insulin secretory response to Ca2+ influx alone may be much smaller than currently believed.

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178

The dual-leucine-zipper-bearing kinase DLK contributes to TNFalpha-induced impairment of pancreatic islet beta-cells function and mass

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In obesity, the production and secretion of the proinflammatory cytokine tumor necrosis factor alpha (TNFα) from adipose tissue is enhanced. Through activation of the c-Jun N-terminal kinase (JNK) and inhibitory phosphorylation of the insulin receptor, TNFα has been shown to play a pivotal role in the development of insulin resistance. In the present study the effect of TNFα on beta-cell survival was investigated. Treatment of the insulin producing pancreatic beta-cell line HIT with TNFα for 6 h reduced beta-cell viability by 10% as measured by the MTT test. In an immunocytochemical analysis using an antibody against the cleaved caspase-3 as hallmark for apoptosis TNFα increased the number of apoptotic beta-cells in a time- and concentration-dependent manner. In an in vitro kinase assay TNFα stimulated the enzymatic activity of cellular, immunoprecipitated dual-leucine-zipper-bearing kinase (DLK) 1.5 fold. Furthermore, in the presence of overexpressed DLK the apoptotic effect of TNFα was more than additive when compared with the cytokine or the overexpressed DLK alone, whereas the presence of the kinase-dead DLK mutant prevented the apoptosis-inducing effect of TNFα. In transient transfection assays TNFα inhibited specifically the membrane depolarization-induced transcriptional activity of beta-cell protective CREB in a concentration dependent way, with maximal 80% and 60% inhibition for CRE- and CREB-directed transcription, respectively. Reduction of cellular DLK by small interference RNA diminished the TNFα-caused inhibition of membrane depolarization-induced CRE-directed transcription from 47% to 27% and reduced the TNFα-induced phosphorylation of JNK. Our data indicate that DLK contributes at least in part to the inhibitory effect of TNFα on CRE/CREB-directed transcription presumably through activation of JNK. Considering the pivotal role of CREB for beta-cell survival our data suggest, that TNFα-induced activation of DLK inhibits CREB-directed transcription, thereby leading to increased beta-cell apoptosis and contributing to the molecular pathogenesis of diabetes mellitus. Thus, inhibitors of DLK might prevent the development of this disease.

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179

Pancreatic A-cell NaV1.7 sodium channel is involved in glucagon release in mice

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The cellular mechanisms responsible for glucagon release are poorly understood. Low blood glucose levels increase the cytoplasmic calcium concentration followed by the hormone release. Calcium enters the cell via high voltage activated (HVA) calcium channels. Like B-cells pancreatic A-cells possess ATP-sensitive potassium (KATP) channels which have been shown to be pivotally involved in the regulation of glucagon secretion. Therefore it has been postulated that low voltage activated ion channels are required as a link between low glucose and KATP-channels. Sodium and T-type calcium channels were dealt with as putative candidates. In our hands neither T-type current nor mRNA were identified in single isolated mouse pancreatic islet cells. Instead we amplified NaV1.1, NaV1.3, NaV1.6 and NaV1.7 mRNA from whole islet tissue and identified NaV1.3 and NaV1.7 mRNA in single islet cells. In accordance with these results islet cells express at least two different types of sodium current. One was only active when the membrane potential was negative to -80 mV, the other persisted at more positive potentials. To analyse the specific roles of the channels on glucagon release, A-cell α-NaV1.7 knockout mice (αNaV1.7-/- mice) were generated by using the Cre/loxP approach. RT-PCR analysis revealed that NaV1.7 is only expressed in A-cells. In whole islet patch-clamp experiments 56% of cells lacked a sodium channel. In accordance with this result in 79% of isolated A-cells from aNaV1.7-/- mice the sodium current was absent. The glucagon secretion of isolated islets from αNaV1.7-/- mice was disturbed. Therefore we conclude that αNaV1.7 could be the link between KATP-channels and HVA calcium channels.

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180

Depletion of the centrosomal protein TACC3 sensitizes to paclitaxel-induced cell death and senescence

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The centrosomal protein TACC (transforming acidic coiled coil) 3 is specifically expressed during the G2/M phase of the cell cycle where it regulates mitotic spindle assembly and chromosomal alignment [Schneider et al. (2007), J. Biol. Chem., 282, 29273-29283]. Constitutive deletion of the TACC3 gene in mice leads to embryonic lethality associated with growth retardation and developmental defects [Piekorz et al., (2002), EMBO J., 21, 653-664]. Here, we employed a lentiviral-based and inducible RNA interference approach to characterize the role of TACC3 in cell cycle progression and survival. We show that down-regulation of TACC3 results in delayed mitotic progression and aneuploidy. As a consequence, TACC3-depleted cells arrest postmitotically in G1 phase due to the activation of p53 and its transcriptional target p21WAF which acts as a cell cycle inhibitor [Schneider et al. (2007), Oncogene, doi:10.1038/sj. onc.1210628]. This arrest is either transient, resulting in a strong inhibition of proliferation, or permanent, leading to rapid cellular senescence. Interestingly, we observed a comparable situation following administration of subtoxic nanomolar concentrations of paclitaxel (Taxol®), a spindle poison and antitumor agent influencing microtubule dynamics. However, and unlike in TACC3-expressing cells, exposure to paclitaxel had a significant impact in TACC3-depleted cells, resulting in either rapid polyploidization and cell death or an accelerated onset of senescence. Although paclitaxel treatment was associated with increased activation of the survival kinase Akt, inhibition of cell death was abrogated following depletion of TACC3. We therefore speculate that components of the mitotic spindle apparatus like TACC3 may represent attractive cellular targets for antitumor therapy.

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181

Identification of cyclin-dependent kinase (CDK) 6 as a novel p65 NK-kappaB Ser536 kinase

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Recent genetic evidence has challenged the classical role of cyclin-dependent kinase 6 and its close homologue CDK4 in cell cycle transition from G1 to S-Phase as most mouse cell types proliferate normally in the absence of CDK4 and CDK6. This has led to speculations that these CDKs might have effector functions unrelated to their major substrate retionoblastoma protein (Rb). Here we show, that using an unbiased approach to isolate NF-κB-phosphorylating protein kinases, CDK6 was found in a highly purified fraction of human cells that phosphorylates ser536 of p65 NF-κB. Three types of purified or recombinant CDK6 preparations phosphorylated ser536 directly in vitro. We also show that a previously described constitutively active viral cycline stimulates NF-κB-dependent transcription via CDK6-dependent phosphorylation of ser536. RNAi-mediated suppression of CDK6 severly suppresses NF-κB activation induced by the proinflammtory cytokine IL-1. The selective CDK4/6 inhibitor PD332991 partially inhibits IL-1-or cell cycle-induced ser536 phosphorylation. These results have widespread

biological implications as they define a novel cross talk mechanism by which cyclin-dependent protein kinases feed into activation of the NF-κB pathway. Persistent activation of NF-κB is associated with chronic inflammation and cancer. Based on our results aberrant CDK6 expression as frequently observed in human tumors may drive malignant transformation via NF-κB-dependent genes rather than through phosphorylation of Rb.

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182

Reduced mast cell activation and anaphylaxis in mice lacking the intermediate conductance Ca2+-activated K+ channel KCa3.1

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Mast cells are key players in allergic reactions including anaphylaxis. Activation of mast cells by antigen involves an influx of extracellular Ca2+ via calcium release-activated calcium (CRAC) channels and is followed by an opening of a Ca2+-activated K+ channel, which participates in the orchestration of mast cell degranulation. Pharmacological blockade suggests that the intermediate conductance Ca2+-activated K+ channel KCa3.1 seems to be involved in the antigen-induced degranulation of sensitized mast cells. However, due to the lack of specificity of these blockers, the contribution of KCa3.1 for mast cell activation, degranulation and subsequent for anaphylaxis is only ill-defined. To elucidate the functional role of KCa3.1 in mast cells we established a KCa3.1-deficient (KCa3.1-/-) mouse line. For in vitro experiments we used wild-type (wt) and KCa3.1-/- bone marrow-derived mast cells (BMMCs). Flow cytometry revealed a normal maturation of KCa3.1-/- BMMCs when compared to wt. Electrophysiological characterization revealed an increase of KCa3.1 currents in antigen-stimulated wt, but not in KCa3.1-/- BMMCs. Furthermore, sensitized KCa3.1-/- BMMCs lacked an hyper-polarization of the membrane potential after antigen stimulation. Ca2+ entry upon antigen stimulation was markedly reduced and the release of b-hexosaminidase, an indicator of mast cell degranulation, was significantly diminished (about 59%) in KCa3.1-/- BMMCs compared to wt. Finally, KCa3.1-/- mice showed a reduced IgE-mediated anaphylaxis compared to wt. Thus, our results indicate that KCa3.1 serves as positive feed-forward regulator thereby maintaining mast cell activation, which in turn is critical for anaphylactic reactions and other IgE-associated allergic disorders.

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183

Counter-regulation of paraoxonase-2 expression by endoplasmic reticulum stress and disturbance of Ca2+-homeostasis

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Various factors are implicated in the development and progression of atherosclerosis and diabetes: an imbalance of pro- and anti-oxidative enzymes, enhanced inflammatory stimuli and increased intracellular stress reactions. Together, these are involved in evident cellular apoptosis and tissue damage. The endoplasmic reticulum (ER) is centrally involved in these events, because it preserves cell function and survival and because it elicits pro-apoptotic signals under enhanced intracellular stress. We recently showed that the ER-resident enzyme paraoxonase-2 (PON2) protects vascular cells from both oxidative stress and ER stress-induced apoptosis. Examining the correlation between ER stress-inducing mechanisms and regulation of gene expression, we report that PON2 expression is linked to and depends on the cause of ER stress. ER stress induced by hindered protein modification, i.e. tunicamycin or dithiotreitol treatment increased PON2 mRNA and protein levels. PON2 overexpression protected endothelial cells from tunicamycin or dithiotreitol-induced apoptosis. In contrast, ER stress induction by disturbance of Ca2+-homeostasis, i.e. thapsigargin or Ca-ionophore A23187 treatment resulted in degradation of PON2 mRNA and protein. In line with a Ca2+-induced PON2 decay, PON2 overexpression hardly protected endothelial cells from apoptosis that resulted from disturbed Ca2+-homeostasis. Collectively, these data imply that PON2 expression is induced by ER stress, but diminished by intracellular Ca2+-dysregulation. Thus, Ca2+-mediated decay signals dominate over ER stress-induced PON2 expression. Since PON2 has anti-apoptotic functions, this finding may resemble the physiologic down-regulation of PON2 under Ca2+-mediated pro-apoptotic conditions.

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184

Cholesterol loading induces apoptosis-associated modulation of several adhesion molecules in human monocytic cells

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Objective: Adhesion and migration of monocytes play an important role in development and progression of atherosclerosis. P-selectin glycoprotein ligand-1 (PSGL-1) mediates adhesion of monocytes to endothelial cells (EC). The activated leukocyte cell adhesion molecule (ALCAM/CD166) is associated with cell migration and leukocyte invasion. The present study investigates the impact of cholesterol-enrichment on expression levels of several adhesion molecules such as PSGL-1, ALCAM, VCAM-1 (vascular cell adhesion molecule-1) and ICAM-1 (intercellular cell adhesion molecule-1) in the human monocytic cell line U937. Methods: U937 cells were enriched with cholesterol by incubation in complex with methyl-beta-cyclodextrin (MbCD). Expression of adhesion molecules was determined by flow cytometry; apoptosis by Annexin-V FITC/PI (propidium iodide) double-label cytometry. Adhesion to EC was measured in a flow chamber. Migration of calcein-AM-loaded U937 cells was quantified in 3 µm-chemotaxis chambers by fluorescence of transmigrated cells. Results: Incubation of U937 cells with cholesterol (10 - 100 µg/ml) for 18 h induced a concentration-dependent increase in early and late phase apoptosis. This was associated with reduction of PSGL-1 and ALCAM expression by >50%. In contrast, expression of VCAM-1 was strongly increased. ICAM-1 levels were not affected by cholesterol loading. Incubation with MbCD alone did not induce apoptosis. Pretreatment with the nonselective caspase/apoptosis inhibitor Q-VD-OPh (100 µM) partially prevented cholesterol-modulated alteration of adhesion molecule expression. Migration of cholesterol-enriched cells towards 10% serum was greatly diminished (by about 80%). Q-VD-OPh partially restored migration in cholesterol-enriched U937 cells. This was prevented by ALCAM-neutralizing antibodies (10 µg/ml), but not by an isotype-matched IgG. Conclusion: Cholesterol-induced apoptosis in monocytic cells is accompanied by reduced expression of ALCAM and PSGL-1 and upregulation of VCAM-1. Loss of ALCAM under conditions of cholesterol-loading and apoptosis attenuates monocyte migration, a mechanism which may expedite vascular lesion formation.

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185

Thrombin-activated endothelial cells facilitate binding and activation of human monocytes via CX3CL1

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The chemokine CCL2/monocyte chemoattractant protein-1 is a key player in the recruitment of leukocytes to the pathologic vessel wall. Expression and release of CCL2 is regulated by proinflammatory stimuli and adhesion of leukocytes to the activated endothelial cells. We have previously shown that thrombin activates expression of an adhesion molecule fractalkine/CX3CL1 in human umbilical endothelial cells (HUVEC). Fractalkine is susceptible to shedding and in its soluble form displays chemokine activity. Here we demonstrate that during the cocultivation of thrombin-activated endothelial cells and primary monocytes, the latter released CCL2 in a fractalkine dependent manner. This CCL2 release was due to the enhanced recruitment of monocytes to the thrombin-activated HUVEC, but also due to the increased expression of CCL2 by the monocytes. Both effects were dependent on fractalkine, since an antibody raised against the protein impairs both, the adhesion of monocytes to the activated HUVEC and the generation of CCL2 by monocytes. In order to delineate the molecular mechanism underlying the activation of CCL2 expression in monocytes during the cocultivation, monocytes were stimulated with increasing concentrations of the soluble form of fractalkine. No release of CCL2 was observed by monocytes upon stimulation. Together these data show that fractalkine is necessary but not sufficient for the CCL2 expression by monocytes during crosstalk with thrombin-activated HUVEC. Supported by the DFG, SFB 451.

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186

Plasmin induces chemotaxis of immature dendritic cells through the MAPK/AKT pathway

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The serine protease plasmin is ubiquitously distributed through human body in form of its precursor, plasminogen. Plasmin is generated during fibrinolysis. In addition, the proteolytic activation of plasminogen to plasmin also takes place during inflammation in the context of the so-called contact activation. Accordingly, plasmin activity is detected in chronic inflammatory conditions, such as synovial fluid of rheumatoid arthritis patients and atherosclerotic plaques. Dendritic cells (DC) play a pivotal role in chronic inflammation due to the induction of immune responses and tolerance. How their functions might be affected by plasmin is not known so far. Here we identify plasmin as a potent activator of immature DC. Stimulation of immature DC with plasmin elicited actin polymerization and concentration-dependent chemotactic responses. The efficacy of plasmin as chemoattractant was comparable to that induced by the control chemoattractant fMLP. The plasmin-induced migration of immature DC was a true chemotaxis as confirmed by checkerboard analysis. Within 5 min, stimulation of immature DC with plasmin led to a rapid activation of ERK1/2 and p38 MAP kinases, followed by phosphorylation of Akt/PKB. Activation of MAPK and Akt pathways was indispensable for the plasmin-mediated chemotaxis as shown by the use of pharmacological inhibitors of ERK, p38 and Akt kinases. Inhibition of p38 by SB203580 concomitantly blocked activation of PDK1 and Akt, indicating that plasmin-induced Akt activation is mediated by p38. In summary, we identified plasmin as a potent chemoattractant for immature DC, acting via ERK/Akt-signaling pathways. We conclude that this novel mechanism is relevant for the function of immature DC in chronic inflammation. Supported by the DFG.

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187

Cytokines and growth factors regulate the expression of the matricellular protein SMOC-1 in rat renal mesangial cells

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Cytokines like IL-1β and TNF-α are able to force c to produce and to liberate high amounts of inflammatory mediators such as nitric oxide (NO). We are interested in the cross-talk of cytokines and NO on the gene expression pattern in rat mesangial cells. Using the differential display method RAP-PCR (mRNA arbitrarily primed polymerase chain reaction) we found the matricellular glycoprotein “secreted modular calcium binding protein 1” (SMOC-1) to be downregulated by IL-1β. SMOC-1 is constitutively secreted by different cells but its biological functions remain to be investigated. We confirmed the effect of both IL-1β and TNF-α on SMOC-1 mRNA expression by Northern blotting experiments. Furthermore, we could demonstrate that this effect is in

part mediated by endogenous NO production via activation of the soluble guanylyl cyclase. Western blot and ELISA experiments using a custom-made antibody indicated that NO/IL-1β mediated SMOC-1 mRNA expression is followed by changes at the protein level. Since the highly related proteins SPARC and SMOC-2 interfere with growth factor signalling we tested the effects of TGF-β1, PDGF and bFGF on the SMOC-1 mRNA expression and found that all these factors caused a reduction of SMOC-1 mRNA and protein levels. To further investigate the role of SMOC-1 in cytokine- and growth factor signalling we established siRNA experiments to silence SMOC-1 expression. A cytokine antibody array performed with conditioned media from cells treated with SMOC-1 specific siRNA showed no difference in cytokine expression compared to controls. However, inhibition of SMOC-1 secretion by siRNA diminished mRNA expression of “connective tissue growth factor” (CTGF), a protein known to be upregulated by TGF-β in mesangial cells. Therefore, we are currently investigating the influence of SMOC-1 on TGF- β1 expression and signalling.

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188

Interleukin-22 in sepsis

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Interleukin (IL)-22 is a novel Th17 signature cytokine that is mainly produced by activated T cells and NK cells. By activating non-leukocytic cells such as hepatocytes, keratinocytes, as well as colon and lung epithelial cells, primarily via the transcription factor STAT-3, IL-22 is able to mediate important immunoregulatory functions related to infection, inflammation, and immunity. Genes that are upregulated by IL-22 include acute phase proteins, inducible nitric oxide synthase, β-defensins, and matrix metalloproteinases. Since IL-22 appears to be a novel most relevant immunoregulatory molecule we sought to investigate production of IL-22 in sepsis. Here we report that production of IL-22 is upregulated in the rat sepsis model of caecal ligation and incision (CLI). In the cell culture model of freshly isolated human peripheral blood mononuclear cells (PBMC) IL-22 was most potently induced by heat-inactivated Staphylococcus epidermidis. Application of glucocorticoids effectively suppressed production of IL-22 in both PBMC and rat sepsis, respectively. In addition, we report for the first time that IL-22 is significantly upregulated in septic patients with peritonitis. Data presented herein suggest IL-22 as a novel player in the cytokine network at work in the pathophysiology of sepsis.

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189

Protection by interleukin-22 inhalation therapy in a rat model of ventilator-induced lung injury

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Life-saving mechanical ventilation is a major pillar of intensive care medicine but nevertheless is frequently associated with severe adverse effects characterized by symptoms of acute lung injury (barotrauma) and inflammation (biotrauma). IL-22 is a newly described member of the IL-10 cytokine family that appears to target particularly hepatocytes and cells of epithelial origin. Accordingly, we observed that IL-22 mediated robust activation of human lung A549 epithelial/carcinoma cells as detected by induction of STAT3 phosphorylation and subsequent expression of SOCS3. Since IL-22 has been associated with STAT3-dependent tissue protection in murine models of acute liver inflammation, we set out to characterize immunmodulatory effects of this cytokine in a rat model of ventilator-induced lung injury (VILI). Notably, prophylactic treatment with nebulized IL-22 attenuated the course of disease in VILI. Specifically, IL-22 inhalation therapy reduced levels of the stretch-induced factors MIP-2, IL-1β, and MMP-9 in the bronchoalveolar lavage fluid of rats undergoing high-pressure ventilation. Inhalation of IL-22 mediated activation of local STAT3 and expression of the STAT3-dependent modulator SOCS3 in the lung compartment. Most interestingly, prophylactic IL-22 inhalation therapy was associated with significantly less lung damage and improved survival of the rats. Data presented herein substantiate tissue protective properties of IL-22 and furthermore indicate that local application by inhalation may be of therapeutic benefit specifically under conditions of acute lung injury and biotrauma.

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190

Genome-wide analysis displays marked induction of EBI3/IL-27B in IL-18-activated AML-derived KG1 cells: critical role of two κB binding sites in the human EBI3 promotor

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The cell line KG1 is derived from a patient with acute myeloid leukemia. Activation of KG1 cells by interleukin (IL)-18 is associated with induction of key Th1 signature parameters such as T-bet and interferon-g. Here we set out to characterize the genome-wide mRNA expression profile under the condition of short-term stimulation (4h) with IL-18 using the Affymetrix GeneChip® Array System. Besides the chemokines CXCL10, CXCL11, and CCL1 we identified Epstein-Barr virus induced gene-3 (EBI3)/IL-27B as being among those genes that are profoundly upregulated by IL-18 in KG1 cells. Thorough investigation revealed that IL-18-induced EBI3 mRNA efficiently translates into protein. Electromobility shift analysis and mutational analysis of the human EBI3 promoter identified two nuclear factor-κB binding sites as being crucial for induction by pro-inflammatory cytokines like IL-18. In addition, we demonstrate that KG1 cells express the Type A IL-27 receptor chain (WSX-1) and display STAT-1, -3 activation as well as induction of SOCS-3 under the influence of IL-27. IL-18 shows therapeutic potential in murine leukemia and is currently being evaluated in phase II clinical trials for the treatment of immunologically sensitive cancers. Since IL-27 mediates anti-cancer bioactivity in animal models, data presented herein may add a novel facet to tumorsuppressive characteristics of IL-18.

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191

Activation of smad-dependent signaling pathways by the mTOR inhibitor sirolimus in rat glomerular mesangial cells

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The mTOR kinase inhibitor Sirolimus is a drug with potent immunosuppressive and antiproliferative properties. In transplantation medicine Sirolimus is often used in combination with the calcineurin inhibitors (CNIs) ciclosporin A (CsA) and tacrolimus (FK506) in order to limit their high nephrotoxic side effects. These side effects are mainly characterized by a strong tubular atrophy accompanied by interstitial and glomerular fibrosis. We previously have shown that CNIs cause a rapid activation of the TGFb-Smad signaling cascade in rat glomerular mesangial cells thereby activating the expression of prominent profibrotic genes such as tissue inhibitor of matrix-metalloproteinase-1 (TIMP-1) and plasminogenactivator inhibitor-1 (PAI-1). In the present study, we demonstrate that Sirolimus in a dose-dependent manner, similar to CNIs, can cause a rapid activation of the Smad-signaling cascade as indicated by a rapid increase in the nuclear contents of phosphorylated Smad proteins Smad-2 and Smad-3 and accompanied by an increased DNA-binding of both transcription factors to a cognate Smad-binding promoter element (SBE). Mechanistically, the activation of R-Smads by Sirolimus depends on a rapid activation of latent TGFb and type I-and II-TGFb-receptors as demonstrated by neutralizing TGFb-strategies and by RNA interference. Interestingly, the Sirolimus-induced Smad signaling is strongly impaired by SB203580 indicating an additional involvement of the p38 MAP-kinase. Conversely, the activation of p38 by Sirolimus is affected by an inhibitor of the TGFb- receptor-I kinase thus demonstrating functional cross-talk of both different signaling pathways. Functionally, the Sirolimus-caused Smad activation leads to a significant increase in the transcriptional activity of TGFb -inducible genes CTGF and PAI-1. Finally, we found a critical involvement of reactive oxygen species (ROS) in both, p38 activation and TGFb activation since ROS scavengers strongly impaired both of these processes. Collectively, our data demonstrate that Sirolimus by release of TGFb propagates a rapid induction of the Smad signaling thereby accelerating the expression of profibrotic genes.

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192

Computer-assisted analysis of cell interactions under dynamic flow conditions

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Leukocyte migration in general and extravasation in particular is an essential mechanism in the physiology and pathophysiology of the immune system. First interactions of the extravasation process cause tethering and rolling of flowing leukocytes on the endothelial cell surface. The most widely used approaches to study this process in detail are several types of flow chamber assays. In this study we compared established evaluation methods for flow chamber assays with Cellflow® a novel computer-aided analysis software. As basis we choose two videos of one in vitro experiment and compared analysis for mean rolling fraction and mean velocity. The results for all methods correspond statistically. However for the hand counting method inter-individual differences were substantial. Unpaired t-test (n=3) documented that both computer-aided methods picked up increased mean rolling with the P values being P=0.0519 for Retrac® and 0.0015 for Cellflow®. Both methods also showed statistically significant increases regarding the mean velocity (retrac: P=0.0166; Cellflow® P=0.0111). Similarly for two additional series of experiments, the obtained velocity distributions of Cellflow® matched with those of Retrac® are almost identical. Except for the range of 6.7-20.0 mm/s Cellflow® generates significantly less velocity values relative to higher velocities. However, the ratios of absolute values compared before and after manipulation show robust similarities between both methods. To analyse the mean velocities of 100 cells, took the observers on average for 3.6±1.6 hours. The retrac-based approach did not reduce this time (4.5±1.2 hours). In contrast, Cellflow® allowed the analysis of the same experiment in less than 15 minutes. Taken together, Cellflow® was shown to be able to analyse videos documenting flow chamber assays in a reproducible manner, but in a fraction of the time needed to get the same type of data through the established methods. The functionality of this fully computer-aided software allowed user independent, consequently objective results. Moreover, the high data throughput and the saving of time highly facilitate flow chamber assays for pharmacological studies.

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193

Dendritic cells show different expression of interleukin-12 and interleukin-23 depending on genetic background and stimuli

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Dendritic cells (DC) are antigen-presenting cells and control adequate cytokine expression in defence against bacterial and viral challenge. We investigated interleukin-12 and interleukin-23 expression in immature Langerhans cells (iLC) and in bone marrow (BM)-derived GM-CSF- and Flt3L-differentiated DC. Dissimilar expression of IL-

12(p40/p35) and IL-23(p40/p19) was dependent on stimuli, growth factors and mouse genetic background. iLC exhibited a differential regulation of p40/p19 as the two IL-23 subunits were expressed sequentially on mRNA, protein and secretion level after stimulation with a TLR4 ligand. Not only p40, but also p19 was secreted as a monomer with a yet unknown function. Investigations toward IL-12 revealed a constitutive expression of p35 mRNA, but no IL-12 heterodimer secretion could be detected. Omitting GM-CSF, which has been described to down-regulate p35 mRNA, iLC cultures failed to up-regulate IL-12p70 secretion, but instead GM-CSF was absolutely necessary for IL-23 expression. To examine the role of genetics for IL-12/-23 regulation in DC we isolated BM cells from Balb/c and C57BL/6 mice. After 8 d incubation with either GM-CSF or Flt3L and stimulation with TLR4 or TLR9 ligands, both DC types from Balb/c background showed a three-fold higher molar IL-12 compared to IL-23 secretion. In contrast, in C57BL/6 mice only Flt3L-differentiated DC secreted high levels of IL-12(p40/p35), while GM-CSF-differentiated cells did not. Interestingly, both GM-CSF- and Flt3L-differentiated DC from C57BL/6 exhibited marginal expression of IL-23. Because recent investigations unravelled clearly distinguishable roles of IL-12 and IL-23 in autoimmunity, inflammation and cancerogenesis, we are now extending our studies to more detailed molecular signal identification leading to bioactive IL-12 or IL-23.

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194

Identification of the immune modulatory targets of calctriol and its low calcemic analogs during dendritic cell and T lymphocyte interaction

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A number of studies testify that calcitriol exerts strong immunomodulatory activity. We recently described in a mouse in vivo setting the inhibitory potential of calcitriol on Th1 and for the first time on Th17 effector functions in corticosteroid-treated mice with TNBS-colitis (Daniel C et al., JPET 2007), in addition we also observed strong immuneregulatory properties for the low calcemic vitamin D analog 22-ene 25-oxa-vitamin D (ZK156979) (Daniel C et al., JPET 2006). Moreover in dendritic cells (DC) we determined a differential downregulation of interleukin 12 family members as well as for IL-23p19. However the molecular targets of calcitriol and its analogs, particularly with regard to DC and T cell functions, are far from being understood in detail. Thus, the aim of the following in vitro study was to further dissect the immune modulative capacities of calcitriol and its low calcemic analogs (calcithiazol) with respect to differentiation of DC, antigen presentation as well as to direct impacts on T cells. We used the immature Langerhans cell line XS52 as APC together with an ovalbumin-specific Th1 clone. As a first readout IFN-γ production was determined using the ELISPOT assay system. Calcitriol and calcithiazol differentially affected DC differentiation, antigen presentation and T cell functions, with calcithiazol showing more pronounced effects on inhibition of IFN-γ production when applied during the antigen processing phase. Direct impact on DC differentiation and antigen processing will be further dissected using FACS analysis of MHC class II, CD80, 83 and 86 as well as by analysis of cathelicidine and cathepsins.

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195

Stimulation-dependent changes in the gene expression profile of murine Langerhans cells

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Under homeostatic conditions immature and semi-mature dendritic cells (DC) are essential for the maintenance of peripheral tolerance, but initiate strong immune responses upon full maturation. Langerhans cells (LC) at their immature state constitute the epidermal DC population. The maturation process of LC can be mimicked in vitro by short term cultivation of epidermal cell suspensions, since keratinocytes provide the required survival and stimulation factors (GM-CSF, IL-1ß and TNF-α). Here we have analysed the gene expression profiles of freshly isolated and in vitro stimulated murine LC by applying complementary methods (micro array analysis, differential display, differential screening) to monitor expression of both known and unknown genes, subsequently validated by quantitative PCR. Concerning transcriptional control, we observed upregulation of transcription factors of the NF-κB family, of distinct STAT factors, of Crip, Net and ATF4, while ATF3 and c-Fos were down-regulated. With regard to differential regulation of NF-κB and AP-1 expression in the course of LC maturation, current work is focussed on elucidating potential cross talk between both transcription factor families in DC.

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196

Induction of adaptive regulatory T cells by immature and alternatively activatd murine dendritic cells

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We have characterized the murine dendritic cell (DC) line SP37A3 which is of myeloid origin. Upon cultivation in the presence of GM-CSF and M-CSF, SP37A3 cells resemble immature DC characterized by low expression of MHC II and costimulatory molecules and accordingly a low T cell stimulatory capacity. Upon stimulation with proinflammatory cytokines, SP37A3 cells acquire a mature phenotype and potent T cell stimulatory capacity. Upon maturation in the presence of the synthetic glucocorticoid dexamethasone (DEX), termed alternative activation, cells displayed an intermediate phenotype concerning the expression of costimulatory molecules and proinflammatory cytokines, but showed upregulated mRNA expression of pro-tolerogenic factors FcgRIIB and IL-1RA. SP37A3 cells were responsive to DEX even when applied at later time points during activation suggesting functional plasticity. Both at immature state and upon alternative activation, SP37A3 cells induced de novo CD4+ and CD8+ regulatory T cells which suppressed proliferation of naïve T cells. Therefore, the myeloid DC line SP37A3 represents a suitable model to study functions of myeloid dendritic cells at tolerogenic and immunogenic state, circumventing restrictions associated with the use of primary DC and bone marrow-derived DC.

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197

The synthetic glucocorticoid dexamethasone mediates induction of tolerogenic antigen presenting cells

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Glucocorticoids (GC) exert potent antiinflammatory activity due to inhibition of NF-κB activity, and belong to the most often prescribed drugs applied in the treatment of autoimmune and allergic diseases. In this study we show that application of the synthetic GC dexamethasone (DEX) to murine bone marrow-derived DC (BM-DC) cultures decreased their allogeneic T cell stimulatory capacity in a duration- and dose-dependent manner. Long term DEX-treatment of cells during murine BM-DC or human monocyte-derived DC cultures mediated arisal of antigen presenting cells (APC) which showed reduced expression of costimulatory molecules and proinflammatory cytokines upon stimulation. Consistently, DEX-APC showed an impaired allogeneic T cell stimulatory potential. Murine DEX-APC induced de novo adaptive regulatory T cells in vitro and exerted tolerizing effects in a murine disease model of contact allergy.

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198

Glucocorticoids mediate differentiation of dendritic cell progenitors into tolerogenic antigen presenting cells characterized by a protolerogenic gene expression profile

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Glucocorticoids (GC) are produced endogenously by cells of the adrenal cortex, strongly upregulated upon inflamation as a negative feedback regulatory mechanism. Due to their strong antiinflammatory potency, synthetic GC are frequently prescribed in the treatment of severe autoimme and allergic diseases. Here we show that cultivation of murine bone marrow-derived dendritic cells (BM-DC) progenitors in the presence of the synthetic GC dexamethasone resulted in the differentiation of a population of antigen presenting cells (APC) which exerted tolerogenic effects. Gene expression profiling revealed that DEX-APC showed elevated expression of several GC responsive genes known to exert anti-inflammatory activities (FcgRIIB, IL1RA, MKP-1, Gilz). Given the therapeutic potential of genetically modified DC to enforce tolerance, we have established a suitable transduction protocol for murine BM-DC and have started to assess the T cell tolerizing potency of BM-DC transduced with antiinflammatory molecules.

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199

Characteristics of human antigen presenting cells derived from myeloid dendritic cell progenitors cultured in the presence of agents with antiinflammatory potential

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The transient character of an immune response is tightly controlled by numerous negative feedback mechanisms, and includes the production of soluble mediators with antiinflammatory function. Different tumors have been shown to secrete antiinflammatory agents as an evasion strategy. These compounds may act directly on antigen presenting cells (APC) to induce a (stable) tolerogenic state. We wanted to assess the characteristics of human APC derived from myeloid dendritic cell (DC) progenitors cultured in the presence of such factors in a comparative manner. Continuous treatment with the antiinflammatory cytokines IL-10 or TGF-ß resulted in the differentiation of APC with impaired T cell stimulatory capacity, even after optimal stimulation with a maturation cocktail (IL-1ß, TNF-α, prostaglandin E2 [PGE2]). An inhibitory effect was also noted upon long-term treatment of DC progenitors with IL-6 or PGE2, although both factors have been known to support maturation of immature DC when applied at later time points of culture. To mimick endogenous conditions, we also stimulated immature DC with either factor of the maturation cocktail. While TNF-α or IL-1ß exerted no functional maturation, PGE2 alone was sufficient to confer partial maturation. Non-stimulatory APC populations showed distinct phenotypic characteristics, and current work is focussed on analyzing their gene expression patterns to identify molecules with tolerogenic potential.

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200

Mechanisms of morphine-mediated inhibition of T cell signaling

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A large number of reports suggest immunosuppressive actions of opioids. However, up to date there is little information about molecular mechanisms underlying such immunosuppressive effects. We have earlier shown that a variety of stimuli like cytokines lead to the induction of μ-opioid receptors in T cells. In addition, the gene is induced after antigen activation of T cells. In order to get insight into functional aspects of μ-opioid receptor biology in T lymphocytes, the effect of morphine on the signaling cascade of activated primary human T lymphocytes and Jurkat T cells was tested. Morphine significanty inhibited the induction of IL-2, which is a fundamental hallmark in the activation process in T lymphocytes leading to the initiation of various subsequent immune responses. Furthermore, the activities of prominent transcription factors, which

regulate the IL-2 gene, such as AP-1, NFkappaB and NFAT were strongly inhibited by the opioid. Moreover, morphine caused a drastic inhibition of phosphorylation of adaptor proteins and kinases such as MAPK, ZAP-70 and LAT. These proteins normally are activated by phosphorylation in response to antigen activation of the cells and link the T cell receptor to the downstream transcriptional events. In addition, it was found that the crosstalk between the G-protein-coupled μ-opioid receptors and the T cell receptor cascade is mediated via cAMP and protein kinase A. We observed that incubation of Jurkat and primary human T cells with morphine for two hours induced a more than five-fold increase in cAMP formation. The cAMP-activated protein kinase A then activates the T cell protein Csk, which is known to inhibit the antigen-mediated T cell receptor signaling. These data provide a molecular explanation for immunosuppressive effects of opioids and also implicate a physiological role for endogenous μ-opioid receptor ligands in the regulation of the T cell response.
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201

Regulation of cannabinoid receptor type 1 expression in T cells
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Besides their neuronal effects, cannabinoids are potent modulators of immune functions. For example, they influence the cytokine production of T cells by inducing the production of interleukin-4 (IL-4) and inhibiting secretion of interferon-gamma. Moreover, it was shown that cannabinoids inhibit signaling events in activated T cells. Here, we investigated the expression of the cannabinoid receptor CB1 in T lymphocytes. We observed that T cells basally express low levels of CB1 mRNA. However, activation of the cells by CD3 and CD28 lead to a marked induction of the gene's transcription in primary human T cells (8-fold) and cells of the human T cell line Jurkat (29-fold). Using the decoy-oligonucleotide approach, it was demonstrated that the transcription factors AP-1, NFkappaB and NFAT were involved in this regulation. In an earlier study we showed that IL-4 induces CB1 receptor expression, as well. This upregulation was mediated by the transcription factor STAT6. A binding site for this transcription factor was identified at nt -2769 on the human CB1 gene promoter. To test, if the increase in CB1 mRNA is followed by an increase in functional receptor proteins, we investigated coupling of CB1 to cAMP. Jurkat cells pretreated with IL-4 showed a strong inhibition of forskolin-induced cAMP formation by the CB1-specific agonist R(+)-methanandamide. In contrast, no effect was observed in naive cells. The effect of R(+)-methanandamide could be blocked by the CB1 specific antagonists AM251 and AM281. Since the signaling of activated T cells can be regulated by the cAMP dependent protein kinase A, our results suggest that cannabinoids may modulate T cell signaling via a CB1-mediated regulation of cAMP formation.
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202

Effect of the HMG-CoA reductase inhibitor lovastatin on radiation-induced tumor cell-endothelial cell interactions
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Exposure of human endothelial cells (HUVEC) to ionizing radiation (IR) or inflammatory cytokines (e.g. TNFα) induces the expression of various cell adhesion molecules, including E-selectin. E-selectin gene expression is NF-κB dependent, requires Rho GTPases and promotes the adhesion of circulating tumor cells to the endothelium, thereby contributing to metastasis. HMG-CoA reductase inhibitors (i.e. statins) inhibit Rho-regulated signaling and hence influence cell adhesion (CA). In the present study we analyzed pro-adhesive effects induced by IR and TNFα on endothelial and tumor cells (TC) and, furthermore, the impact of lovastatin on tumor cell-endothelial cell interactions. To this end, human colon carcinoma cells (HT29) and HUVEC were used as experimental system. CA was determined by ELISA-based method. Additionally, we investigated the effect of lovastatin on the mRNA expression level of different CA molecules upon radiation exposure. In line with previous results we show that irradiation of HUVEC increases E-selectin mRNA and protein expression (1). In addition, we found that treatment of TC with TNFα and IR also promotes TC-HUVEC adhesion. Simultaneous irradiation of TC and HUVEC had additive effects. Lovastatin pretreatment of HUVEC attenuated TC-HUVEC interactions. In contrast, lovastatin pretreatment of TC had at best marginal effects on TC-HUVEC adhesion. Apparently, radiation-inducible adhesive factors of HUVEC are different from those of TC. To identify the type of adhesion molecules involved in IR-promoted TC-HUVEC interactions, RT-PCR-based mRNA expression of CA (e.g. E-Selectin, ICAM) was investigated. Data obtained show that basal and IR-inducible expression of CA factors differs between HUVEC and TC. Furthermore, lovastatin differentially affected IR-induced expression of CA molecules in HUVEC and TC. In summary, the data show that IR and TNFα stimulate pro-adhesive functions in both HUVEC and TC, eventually leading to an increased CA. Based on the data obtained with lovastatin, we suggest that Rho GTPases are relevant for the regulation of endothelial specifically expressed adhesion molecules only. To valuate the physiological relevance of these in vitro findings, in vivo studies are currently ongoing. Concept and tentative outcomes of these studies will be presented.1) Nübel T et al (2004) Carcinogenesis 25, 8:1335-1344
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203

Expression and function of gp130 family receptors in human peripheral monocytes
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IL-6-like cytokines regulate cells of the immune system through formation of high affinity oligomeric complexes with transmembrane receptors of the gp130 family receptors. This leads to the formation of a protein 'signaling complex' on the inner side of the cell membrane that activates JAK family of cytoplasmic tyrosine kinases, which, in turn, phosphorylate and activate the STAT transcription factors. The gp130 class of receptors currently encompasses gp130 itself, the LIF-receptor (LIF-R) and the OSM-receptor (OSM-R). Recently a novel member, the IL-31 receptor a (IL-31Rα) has been described to be expressed in a monocytic cell line. Depending on the ligand, receptors of the gp130 family can form hetero- and homodimers, whose composition dictates the identity of the intracellular signaling and biological responses. Expression and function of the IL-31Rα and most other gp130 family members in human peripheral monocytes have not been investigated yet. RT-PCR and quantitative real-time PCR demonstrated that human monocytes expressed high levels of gp130, lower levels of IL-31Rα and no LIF-R or OSM-R mRNA. Western blotting analysis confirmed expression of gp130 and IL-31Rα proteins. Immunofluorescence microscopy and flow cytometry showed that both receptors are present at the surface of monocytes. We further investigated the function of the gp130 family receptors in monocytes using IL-6 and IL-31 as ligands. In monocytes, IL-6 triggered phosphorylation of the STAT3 transcription factor and ERK1/2 MAP kinase. Differently, IL-31 activated only STAT3 but not ERK1/2. The pattern of expression of known targets of IL-6 and IL-31 in monocytes upon stimulation with either cytokine was investigated. Our data suggest that IL-31 may be an important modulator of the activation of human peripheral monocytes. Supported by the DFG, SFB 451.
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204

Histamine H4 receptor antagonism reduces hapten-induced scratching behaviour but not inflammation
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The histamine H4 receptor (H4R) is a Gi/o protein-coupled receptor that was identified and cloned in 2000. The H4R is expressed primarily on hematopoetic cells like mast cells, eosinophils, monocytes, dendritic cells and T-cells. H4R activation promotes the accumulation of inflammatory cells at sites of allergic inflammation. Thus, the H4R appears to be a novel therapeutic target for the treatment of inflammatory and immune disorders. Effects of the selective histamine H4 receptor antagonist JNJ 7777120 were tested in two murine models of allergic contact dermatitis induced by 2,4-dinitrochlorobenzene and toluene-2,4-diisocyanate, which differ in their Th1-Th2 response. Additionally to ear swelling as an endpoint for inflammation, effects of antihistamines on allergen-induced pruritus was tested. JNJ 7777120 (15 mg/kg i.p.) administered 2 h and 30 min before and 1 h after challenge did not reduce the hapten-induced ear swelling determined 24 h after challenge. This was confirmed by histological evaluation of the ear skin. A repeated administration of the haptens to the rostral part of sensitized mice resulted in a frequent scratching behaviour. A systemic administration of JNJ 7777120 (15 mg/kg i.p.) 30 min before challenge reduced this hapten-induced scratching significantly in both models of contact sensitivity. The H1 receptor antagonist cetirizine (15 mg/kg) also reduced the scratching behaviour, whereas the H2 receptor antagonist ranitidine (15 mg/kg) failed to inhibit allergen-induced pruritus. A combination of H1 and H4 receptor antagonists resulted in the strongest inhibition of scratching behaviour. These results indicate that H4 receptor antagonism fails to reduce the allergic inflammatory response but strongly inhibits allergen-induced itch.
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205

Sphingosine-1-phosphate impairs dendritic cell migration through skin and attenuates allergic contact dermatitis reaction in rats
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Sphingosine-1-phosphate (S1P) is an important lipid mediator which is involved in many biological processes. Although it was recently described that local application of S1P abrogates experimental asthma by altering dendritic cell function (Idzko et al., 2006, J Clin Invest 116:2935-2944), its role in allergic skin diseases such as atopic dermatitis remains to be elucidated. In vivo, the inhibitory action of S1P was tested in the toluene-2,4-diisocyanate (TDI) induced allergic inflammatory response. After repeated topical administration over 9 days, S1P (100 µg) inhibited the inflammatory response (ear swelling) measured 24 hours after TDI challenge. In the sensitization phase, S1P reduced the draining auricular lymph node weight, lymphocyte cell count and number of immigrated dendritic cells provoked by repetitive topical administration of TDI. Corresponding to this, the density of Langerhans cells in the epidermis was higher in S1P treated mice compared to vehicle treated. Draining lymph node cells were cultivated and stimulated with concanavalin A which results in enhanced concentrations of IL-6 and IFN-γ. The cytokine production of lymph node cells from S1P treated mice was significantly reduced. A cultivation of the ears for a skin dendritic cell migration assay resulted in a significant inhibition of dendritic cell migration by topically applied S1P, supporting that inhibition of dendritic cell migration plays a pivotal role in the anti-inflammatory action of S1P in this model of allergic dermatitis.
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206

p47phox provides a negative feedback regulation for TLR9 induced IL-12p70 in interferon-alpha producing dendritic cells independent of its NADPH oxidase activating function.
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The permanent exposure to pathogens makes decisions for tolerance or acute immune reaction absolutely mandatory. Mechanisms for the prevention of uncontrolled immune responses, leading to autoimmune reaction and chronic inflammation, are not completely clarified. We investigated the regulatory function of p47phox, an organizing protein of the NADPH oxidase, in the signaling cascade downstream of TLRs. Our initial investigations revealed an enhanced TLR9-induced (CpG2216) IL-12p70 expression in p47phox deficient compared to WT spleen cells (C57Bl/6), which -unexpectedly- was independent of reactive oxygen production. In contrast to TLR9 stimulation TLR4-induced IL-12p70 secretion was not affected, which hints to a negative feedback

regulation of IL-12p70 restricted to TLR-MyD88 signaling. In control experiments spleen cells from gp91phox deficient mice that are also not able to assemble NADPH oxidase nevertheless exhibited a feedback regulation comparable to WT cells. ROS inhibition with diphenyl iodonium had no effect on TLR9-dependent negative feedback regulation, whereas it uncovered a negative feedback now for TLR4-induced IL-12p70 secretion. With MyD88-/- and TRIF-/- spleen cells we confirmed that the p47phox feedback was restricted to the MyD88 pathway. Finally, we determined high amounts of IFN-alpha in cells exhibiting the p47phox-dependent negative feedback regulation strongly indicating that plasmacytoid dendritic cells are involved. Thus, these results suggest that independent from its role for NADPH oxidase p47phox is essential for the fine tuning of TLR/MyD88-dependent IL-12p70 response possibly preventing plasmacytoid DC from triggering autoimmunity.

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207

The CYP450 mouse model for autoimmune hepatitis: Breaking of self-tolerance in transgenic CYP2D6 and wildtype FVB-mice by viral infection

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The etiology of autoimmune hepatitis (AIH) is poorly understood although the major autoantigen, cytochrome P450 2D6 (CYP2D6), has been identified and immunodominant epitopes mapped. Therefore, we generated an animal model for human AIH using the natural autoantigen CYP2D6. We infected transgenic mice expressing human CYP2D6 in the liver (CYP-2D6 mice) with an Adenovirus-CYP2D6 vector (Ad-2D6) to break self-tolerance. Surprisingly, upon infection with Ad-2D6 not only transgenic CYP2D6 mice but also wildtype FVB mice showed several persistent features characteristic for liver damage associated with AIH. These features included massive hepatic fibrosis, 'fused' liver lobules, disorganized architecture, cellular infiltrations and focal to confluent necrosis. Further, all Ad-2D6-infected mice generated high titers of anti-CYP2D6 antibodies. Epitope mapping revealed that such anti-CYP2D6 antibodies predominantly recognized the immunodominant linear epitope recognized by LKM-1 antibodies from AIH patients. In contrast, mice infected with a control Adenovirus did neither develop liver damage nor generated anti-CYP2D6 antibodies. Interestingly the kinetics and severity of liver damage as well as antibody formation was enhanced in wildtype FVB mice compared to transgenic CYP2D6 mice. Our data indicate that the liver damage was reduced and delayed in transgenic CYP2D6 mice due to a certain degree of tolerance towards human CYP2D6 compared to wildtype FVBs. In both mouse lines injection of human recombinant CYP2D6 in complete freud's adjuvant was sufficient to induce anti-CYP2D6 antibodies but not liver damage.

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208

The role of the junctional adhesion molecule C (JAM-C) in type 1 diabetes

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Type 1 diabetes (T1D) results from the autoimmune destruction of insulin-producing beta-cells in the pancreas. Recruitment of inflammatory cells, such as T-cells, B-cells and dendritic cells is prerequisite to beta-cell-injury. Such a process includes several molecular interactions between circulating and endothelial cells. The junctional adhesion molecule (JAM) family proteins JAM-B and JAM–C, are expressed on endothelial cells of high endothelial vessels and appear to be involved in leukocyte rolling, firm adhesion and transmigration. It was recently demonstrated that after blocking of JAM-C cerulein-induced pancreatitis was efficiently attenuated in mice. Early intervention with a monoclonal antibody directed against JAM-C reduced cytokine production, leukocyte influx, and hence tissue damage. In order to investigate the influence of JAM-C on trafficking and transmigration of antigen-specific, autoaggressive T-cells we used the RIP-LCMV mice as a model system. These mice express the glycoprotein (GP) or the nucleoprotein (NP) of lymphocytic choriomeningitis virus (LCMV) as a target autoantigen specifically in the beta-cells of the islets of Langerhans and turn diabetic after LCMV infection. In such diabetic RIP-LCMV mice the expression of JAM-C is detectable around the vessels within the pancreas. Interestingly, immunohistological evaluation of pancreas sections revealed that JAM-C was expressed directly in between cellular infiltrations and beta-cells and was up regulated upon LCMV-infection. The up regulation of JAM-C expression could be confirmed by real-time RT PCR. Our data suggest that JAM-C might be involved in the final steps of trafficking and transmigration of antigen-specific autoaggressive T-cells to the islets of Langerhans and might therefore play an important role in the pathogenesis of T1D.

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209

Mutated CYLD affects the functional state of dendritic cells

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Mutations in the tumor suppressor gene CYLD are the cause for the appearance of cylindromas, bening skin appendage tumors. CYLD is a ubiquitously expressed deubiquitinating enzyme which interacts with members of the classical and alternative NF-κB signaling pathway and thereby attenuates NF-κB signaling. Recently, in transgenic mice that express a truncated CYLD protein (CYLD[ex7/8]) strong expansion of the mature B cell population due to constitutive NF-κB activation was shown (1). Interestingly, CYLD knockout (ko) mice displayed no altered B cell phenotype. Using these mouse strains, we have analyzed CYLD-associated effects on the functional state of bone marrow-derived dendritic cells (DC). DC are essential for the maintenance of peripheral tolerance under homeostatic conditions. Upon infection, DC are strongly activated in a NF-κB-dependent manner and constitute the most potent antigen presenting cells known so far.Unstimulated DC derived from wild type and CYLD ko mice displayed poor allogeneic T cell stimulatory capacity. In contrast, unstimulated CYLD(ex7/8) DC showed a significantly higher primary T cell stimulatory capacity. Gene expression profiling revealed elevated expression of NF-κB target genes in CYLD(ex7/8) DC, encoding costimulatory molecules, proinflammatory cytokines as well as accessory molecules involved in DC/T cell interaction. These findings suggest that while a lack of CYLD may be compensated by other endogenous NF-κB inhibitors, mutation-dependent expression of truncated CYLD may interfere with NF-κB inhibition and result in partial DC activation. In accordance with its role as an endogenous NF-κB inhibitor, treatment of CYLD(ex7/8) DC progenitors with glucocorticoid (dexamethasone) prevented increased expression of NF-κB target genes and the acquisition of strong T cell stimulatory capacity. Reference:1: Hövelmeyer N, Wunderlich FT, Massoumi R, Jakobsen CG, Song J, Wörns MA, Merkwirth C, Kovalenko A, Aumailley M, Strand D, Brüning JC, Galle PR, Wallach D, Fässler R, Waisman A. Regulation of B cell homeostasis and activation by the tumor suppressor gene CYLD. J Exp Med. 2007 Oct 29;204(11):2615-27.

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210

Anti-inflammatory properties of willow bark extract STW 33-I are comparable to those of aspirin and diclofenac

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Anti-inflammatory effects NSAIDs are well known as being mediated by inhibition of NFκ-B is a central antiinflammatory mechanism and as having anti cancer effects via cyclooxygenases (COXs) inhibition. The polyphenol- and salicin-rich willow bark extract STW 33-I (Proaktiv®, aqueous extract, 16–23:1) is successfully applied in the treatment of painful and inflammatory diseases (1). The aim of this study was to determine anti-inflammatory effects of STW 33-I and its polyphenolic fraction E (Fr. E) compared to well characterized NSAIDs such as Aspirin (ASS) and Diclofenac in a colon carcinoma cell line.The anti-proliferative effect of STW 33-I on undifferentiated HT-29 colon carcinoma cells was determined by quantification of lactate dehydrogenase (LDH) release and by Sulforhodamine B (SRB) measurement. Apoptosis was identified and quantified by YO-PRO-1 staining. Intracellular translocation of the NFκ-B p65 subunit was analyzed in THP-1 cells after differentiation by PMA, pre-treatment with the compounds, and afterward activation with LPS.Treatment with Diclofenac (0.1-0.2 mM) and ASS (2.5-5 mM) for 5 hours was found to induce apoptosis in HT-29 colon carcinoma cells significantly. Treatment with STW 33-I had a comparable antiproliferative effect. The pro-apoptotic effect was confirmed by the SRB method and by LDH release. Our results also indicate that treatment of THP-1 cells for 4 h with STW 33-I (50 µg/ml) was effective to inhibit the translocation of the subunit p65 into the cell nucleus. The inhibition of p65 translocation by STW 33-1 was similar to Diclofenac and ASS. Our results suggest that STW 33-I may have similar antiinflammatory and anti cancer properties as ASS and Diclofenac which suggest inhibitory effects via the COXs.Reference: 1. Nahrstedt A (2007) Wien. Med. Wochenschr.157:348-351

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211

Imaging the path of 123I- and 99mTc-labelled drugs in murine heart and brain by a multipinhole-SPECT-system

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Multipinhole SPECT methods are developing more and more into an alternative to the positron emission tomography as a tool for the in vivo investigation of pharmacokinetics and pharmacodynamics of small animal models. The study provides examples of imaging of the spatial distribution of known SPECT tracers in brains and hearts of mice. The measurements were performed by means of a conventional triple-headed TRIAD88 SPECT system upgraded with multipinhole collimators. The investigation of the brain involved ligands of receptors and transporters of the dopaminergic system ([123I]IBZM, [123I]-(S)-2-hydroxy-3-iodo-6-methoxy-N-(1-ethyl -2-pyrrolidinyl-9-methyl-benzamide) and [123I]FPCIT, [123I]-N-w-fluoro-propyl-2ß-carboxy-methoxy-3ß-(4-iodophenyl)-nortropane) as well as a tracer of cerebral perfusion ([99mTc]HMPAO, 99mTc-(D,L) hexa-methyl-propylene-amine-oxime). For the investigation of murine hearts (healthy and cardiomyopathic mice with overexpression of cardiac EP3 receptors) we have used the fatty acid [123I]IPPA (123I-iodophenyl pentadecanoic acid) and [99mTc]Sestamibi ([99mTc]-2-methoxy isobutyl isonitrile) as tracers of cardiac perfusion. Different data processing tools were compared (InVivoScope, Vinci) and quantification approaches are proposed using in vivo und ex vivo data acquisition. Conditions of dosage and detection of tracers with small target volume (striatum) as well as with the complete organ as target volume are communicated. 2 to 6fold activity of 123I- or 99mTc-labelled tracers had to be applied to allow quantification of tissue distribution in the latter case compared to tracers which bind selectively to smaller target volumes.The best time resolution was 17 min for 123I-labelled tracers ([123I]IBZM and [123I]IPPA)and 25 min for the 99mTc-labelled tracers ([99mTc]Sestamibi). The minimal activities detected in the striatum were in the range of 10-20 kBq. The effective resolution in defined experimental set-ups were confirmed by phantom measurements. Moreover the specific anatomic areas were identified by coregistration of SPECT with MRI .

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212

Constitutive activity of CB1 cannabinoid receptors leads to internalization and loss of somatodendritic effects

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The Gai/o protein-coupled CB1 cannabinoid receptor is widely distributed in the nervous system. Activation of CB1 receptors on axon terminals leads to inhibition of voltage-gated calcium channels and subsequent inhibition of neurotransmitter release and synaptic transmission (Szabo & Schlicker, Handb Exp Pharmacol 168: 318–56, 2005). Surprizingly, cannabinoid agonists applied to the somatodendritic region of neurons do not elicit effects expected from a Gai/o protein-coupled receptor, like activation of potassium currents and inhibition of calcium channels (Freiman et al., J Physiol 575: 789-806, 2006). The hypothesis of the present work was that somatodendritic cannabinoid effects are missing, because the constitutively active somatodendritic CB1 receptors are removed from the cell surface by internalization (Lenkei et al., J Neurosci 22; 3141-3153).Like in other G protein-coupled receptors, a tyrosine residue located in the conserved DRY motive plays an important role in the localization of CB1 receptors (position 3.46, T210). We used two T210 mutants of the CB1 receptor. Compared with the wild-type (CB1R-WT), the mutant T210I (CB1R-T210I) exhibits enhanced agonist and diminished inverse agonist affinity, consistent with a shift toward the active form and increased internalization. On the contrary, the mutant T210A (CB1R-T210A) exhibits diminished agonist and enhanced inverse agonist affinity and localizes on the cell membrane (D'Antona et al., Biochem 45: 5606-5617, 2006). Primary hippocampal neuronal cultures were transfected with CB1R-WT or the mutants CB1R-T210I and CB1R-T210A. Neurons were superfused and depolarization-evoked calcium transients were determined by microfluorometry. The cannabinoid receptor agonist WIN55212-2 (5 x 10-7 M) did not affect the calcium transients in cells transfected with CB1R-WT or CB1R-T210I. In contrast, in cells transfected with CB1R-T210A, WIN55212-2 significantly inhibited the calcium transients (by 51 ± 9 %; $P < 0.05$). The results support the hypothesis that the lack of somatodendritic effect of cannabinoids is due to the constitutive activity and subsequent internalization of CB1 receptors.

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213

Temporo-ammonic LTP is modulated by HCN2 channels in stratum oriens interneurons

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Hyperpolarization-activated cyclic nucleotide-gated channels type 1 (HCN1) in hippocampal CA1 pyramidal cells restrain long-term potentiation (LTP) in the temporo-ammonic (TA) pathway conveying inputs from entorhinal cortex to their distal dendrites in the stratum lacunosum moleculare (slm). We performed field EPSP recordings to analyze LTP in wild-type (wt) mice and mutants lacking the HCN2 channel (HCN2-/-) that is also expressed in the hippocampus. Resemb-ling HCN1 knockout mice, HCN2-/- mice displayed significantly enhanced LTP in the TA pathway. However, this effect was not due to a function of HCN2 in CA1 pyramidal cells, because mice with a deletion of this channel restricted to principal neurons (HCN2-NexCre) showed normal LTP. Interestingly, we observed residual HCN2 immunoreactivity in the stratum oriens of HCN2-NexCre mice overlapping with somatostatin-positive cells leading to the hypothesis that a function of HCN2 channels in oriens-lacunosum moleculare (O-LM) interneurons causes suppression of LTP in the TA input. Fitting well, whole-cell patch-clamp recordings demonstrated the presence of the hyperpolarization-activated currents (Ih) carried by the HCN2 channel in O-LM interneurons. HCN2-/- mice exhibit a significant reduction of the Ih current amplitude compared to the wt. As expected, half maximal activation of Ih currents was also shifted towards more positive potentials. We next examined whether GABA-ergic inhibition of distal dendrites in the slm varies between the genotypes. Picrotoxin (pic) increased the fEPSP slope in the TA pathway of wt, but not in HCN2-/- mice. In addition, pic abolished the difference in TA pathway LTP between the genotypes seen under normal conditions. We suggest that HCN2 channels modulate synaptic plasticity in the TA input onto distal dendrites of CA1 pyramidal cells indirectly by controlling the inhibitory function of O-LM interneurons.

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214

Effects of antidepressants and norepinephrine transporter knockout (NETKO) on hippocampal regulation of neuropeptides and neurotrophins in two mouse models of depression

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Chronic stress is a key factor in the development and acceleration of depression. The hippocampus has been implicated in the pathophysiology of this disorder and is particular sensitive to stress effects: depressed patients have reduced hippocampal volumes and rodents show stress-induced decreased neurogenesis and neuronal atrophy. These changes can be reversed by treatment with antidepressants (ADs). The molecular pathogenesis of depression, however, still remains elusive. Since ADs increase cell proliferation and neurogenesis in distinct brain regions such as the hippocampus, it has been hypothesized that long-term AD treatment produces adaptive changes in downstream targets involved in neuronal regeneration and plasticity, namely neurotrophins and neuropeptides. Thus, we examined in two mouse models of depression (restraint and chronic social stress) the effects of different classes of ADs (reboxetine, trimipramine, bupropion, citalopram and imipramine) as well as the knockout of the NET on gene regulation of neurotrophins like brain derived neurotrophic factor (BDNF), and the neuropeptides Galanin and corticotropin-releasing factor (CRF) and their receptors. In both stress models BDNF mRNA was significantly down-regulated (about 3-fold) in NET wildtype (WT) mice. Furthermore, restraint and chronic social stress caused a marked increase (3-4-fold) in the mRNA expression of Galanin and its receptors GalR1 and GalR3, while GalR2 mRNA expression remained unchanged. For CRF and its receptor CRFR1 a 3-fold mRNA up-regulation could be detected after restraint and chronic social stress. In contrast, CRFR2 mRNA was down-regulated by stress to a similar extent. The observed regulations in stressed NETWT mice could be prevented by chronic co-administration of the above mentioned ADs. Moreover, stressed NETKO mice showed similar gene expression profiles for BDNF, Galanin, CRF and their receptors as AD-treated stressed NETWT mice.Our findings indicate that neuropeptides and neurotrophins play an essential role in the pathophysiology of stress-related disorders like depression and in the action of various ADs, irrespective of their primary mode of action.

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215

The intensification of haloperidol-induced catalepsy upon repeated testing in rats is dependent on glutamatergic and serotonergic neurotransmission

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Catalepsy (i.e. the Parkinsonian cardinal symptoms rigor and akinesia) has been shown to intensify upon repeated daily testing.The detailed mechanism of this phenomenon is still not sufficiently understood but there is evidence that the intensification happens due to cognitive processes. The goal of our studies was to investigate the nature of the catalepsy intensification development. In the first experiment we intended to investigate whether the catalepsy intensification requires a consolidation period and whether this consolidation can be antagonised by excitatory amino acid antagonists. For this purpose we treated male Sprague-Dawley rats daily with haloperidol and tested them for catalepsy one hour after the injection. Immediately after the test, the rats were treated with either saline or the selective NMDA-receptor antagonist MK-801 (dizocilpine) or the selective AMPA-receptor antagonist GYKI 52466. In the second and third experiment we investigated the influence of serotonin on the development of the catalepsy intensification. Concomitant administration of the serotonin releaser MDMA with haloperidol accelerated the catalepsy intensification. In contrast, serotonergic lesion of the medial forebrain bundle decreased the haloperidol induced catalepsy intensification significantly. In conclusion, both intact glutamatergic and serotonergic systems are required for the development of catalepsy intensification. Supported by the DFG (SFB 550 – C14).

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216

The anticonvulsant efficacy of valproate is correlated with electrophysiological findings in the rat kindling model for temporal lobe epilepsy

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About 70 % of patients with temporal lobe epilepsy (TLE) show pharmacoresistance despite adequate pharmacotherapy. The substantia nigra pars reticulata (SNr) plays a critical role in propagation and modulation of epileptic activity with focus in the limbic system. A previous study showed that the antiepileptic drug valproate (VPA) is significantly less effective in reducing discharge rates of SNr neurons in kindled rats as a model for TLE than in non-kindled rats (Gernert et al., 2004). We now hypothesize that the reduction of SNr discharge rates by VPA is less expressed in pharmacoresistant than in pharmacosensitive kindled rats. Therefore we aimed to characterize rats as pharmacoresistant or pharmacosensitive to VPA in order to investigate the hypothesis that VPA's anticonvulsant effect in the rat kindling model on the one hand is correlated with VPA's ability to reduce the discharge rate of SNr neurons and on the other hand is correlated with discharge patterns of these neurons after systemic application of VPA. Amygdala-kindled rats with reproducible seizure thresholds received 200 mg/kg VPA i.p. once a week. Increases in seizure thresholds were compared to control thresholds with saline. 24 hours after a kindled seizure, we performed in vivo extracellular single unit recordings of SNr neurons. 100 mg/kg VPA was given i.v. after recording of basal activity. We evaluated discharge rates and patterns. It was possible to characterize good and poor responders to VPA: Although plasma levels of VPA were sufficiently high, animals showed individually different increases in seizure thresholds. Moreover, there was a correlation between this anticonvulsant effect in the rat kindling model and VPA's ability to reduce the discharge rate of SNr neurons: The more pronounced the anticonvulsant effect, the stronger was the reduction of SNr activity by VPA. Preliminary data failed to show robust correlations between VPA's anticonvulsant effect in the rat kindling model and discharge patterns of SNr neurons after application of VPA. These findings indicate that VPA's anticonvulsant effect is reflected on the level of the basal ganglia network. Supported by the DFG: GE 1103/6-1.

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217

Selective noradrenergic denervation of the hippocampus: effects on the modulation of noradrenaline (NA), serotonin (5-HT) and acetylcholine (ACh) release

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Selective cholinergic and/or serotonergic hippocampal denervation alters the modulation of neurotransmitter release in hippocampal slices (e.g. Birthelmer et al. [2002] Eur. J. Neurosci. 16: 1839; Birthelmer et al. [2003] Brain Res. Bull. 59:371). To investigate the effects of selective noradrenergic denervation on hippocampal neurotransmitter systems, rats were treated with 50 mg/kg (i.p.) of N-2-chlorethyl-N-ethyl-2-bromobenzylamin (DSP-4). DSP-4 reduced hippocampal NA by more than 90% (vs. sham-injected controls), whereas dopamine and 5-HT levels were unaffected. The accumulation and electrically evoked release (in absolute terms) of [3H]-NA in hippocampal slices were strongly reduced. The evoked NA release in % of [3H]-NA accumulation, however, was unaffected, suggesting that the NA release from residual noradrenergic terminals was unchanged. Accumulation of [3H]-5-HT was reduced in DSP-4 rats, whereas both spontaneous and electrically-evoked release of [3H]-5-HT

were significantly enhanced, probably due to a weaker effect of endogenous NA via α2-adrenoceptors on serotonergic terminals. Accordingly, the α2-agonist UK-14,304 [5-bromo-6-(2-imidazolin-2-ylamino)-quinoxaline] more potently inhibited the evoked 5-HT release in DSP-4 rats, whereas the α2-antagonist idazoxan failed to exert facilitatory effects. Most surprisingly, the accumulation of [3H]-choline, and both the basal and electrically-evoked overflow of [3H] from hippocampal slices preincubated with [3H]-choline were significantly increased in DSP-4 rats. In contrast, choline acetyltransferase activity was unchanged, suggesting the absence of cholinergic sprouting reactions. Since presynaptic α2-adrenoceptors are absent on cholinergic axon terminals in this tissue, we suggest, that NA via an unknown interneuronal loop also affects hippocampal ACh release.

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218

The modulation of striatal dopamine release correlates with water-maze performance in aged rats

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Cluster analysis of performance during acquisition of a declarative-like place-learning task in the water maze distinguished between subpopulations of aged rats (25-27 months) classified as moderately (AMI) or severely impaired (ASI) as compared to young adults (3-5 months). Using a slice-superfusion device, electrically- or nicotine-evoked release of [3H]dopamine ([3H]DA) from striatum was assessed in the presence of GR-55,562 (5-HT1B receptor antagonist), methiotepin (mixed 5-HT1/2 receptor antagonist) and/or sulpiride (D2/D3 receptor antagonist). All aged rats showed a reduced electrically-evoked release of [3H]DA (-30% in AMI and -45% in ASI rats). The main neuropharmacological results demonstrated age-related alterations in the 5-HT1B- and D2/D3-mediated modulation of electrically-evoked striatal dopamine release. Furthermore, regression analyses indicated a possible contribution of these alterations to the age-related behavioural deficits: the larger the deficit, the weaker the electrically-evoked release under 5-HT1B and D2/D3 receptor blockade. Extending our recent report on the modulation of striatal acetylcholine release in aged rats (Cassel et al., Neurobiol. Aging 28, 2007, 1270-11285), these new findings (Cassel et al., Neurobiol. Aging, 2008, in press) make dopaminergic and serotonergic functional alterations additional candidates to participate in age-related deficits in the water maze, most probably in interaction with formerly described cholinergic dysfunctions.

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219

Ethanol and/or 3,4-methylenedioxymethamphetamine (ecstasy) alter the spontaneous and evoked overflow of dopamine, serotonin and acetylcholin in striatal slices of the rat

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Ethanol (EtOH) potentiates the locomotor effects of 3,4-methylenedioxymetamphetamine (MDMA) in rats (Ben Hamida et al. [2007] Pharmacol. Biochem. & Behav. 84: 162-168). This potentiation might involve pharmacokinetic and/or pharmacodynamic mechanisms. We explored whether the latter could be local. Using a slice superfusion approach, we assessed the effects of MDMA (0.3, 3 µM) and/or EtOH (2 ‰) on the spontaneous outflow and electrically-evoked release of serotonin (5-HT), dopamine (DA) and acetylcholine (ACh) in the striatum, and for comparison, on 5-HT release in hippocampal and neocortical tissue. MDMA, much less effectively EtOH, augmented the outflow of 5-HT in all regions. The electrically-evoked 5-HT release was increased at MDMA 3 µM in striatal slices only. With nomifensine throughout, EtOH significantly potentiated the 0.3 µM MDMA-induced outflow of 5-HT, again only in striatal slices. EtOH or MDMA also enhanced the spontaneous outflow of DA, but MDMA reduced the electrically-evoked DA release. Without fluvoxamine throughout superfusion, EtOH potentiated the effect of 0.3 µM MDMA on DA outflow. With fluvoxamine throughout superfusion, EtOH facilitated the outflow at both MDMA concentrations. Finally, MDMA (3 µM) weakened the electrically-evoked release of ACh, an effect involving several receptors (D2, 5-HT2, NMDA, nicotinic, NK1), with some interactions with EtOH. For the first time, these data show a local synergistic interaction of EtOH and MDMA on the spontaneous outflow of striatal DA and 5-HT. This interaction seems relevant to the EtOH-induced potentiation of hyperlocomotion in MDMA-treated rats. Our findings, however, do not preclude the contribution of other pharmacodynamic and/or pharmacokinetic mechanisms in vivo.

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220

Effects of gabapentin and pregabalin on K+-evoked [3H]-GABA and [3H]-glutamate release from rat and human neocortical synaptosomes

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Anticonvulsant, analgesic, and anxiolytic effects have been observed both preclinically and clinically with gabapentin and more recently with pregabalin. One site of action of these drugs is the α2-δ subunit of neuronal voltage-sensitive Ca2+ channels (VSCC). This action results in inhibition of high-threshold Ca2+ currents and neurotransmitter release. We have shown previously in human neocortical slices that both drugs reduce the Ca2+-dependent release of [3H]-acetylcholine, [3H]-noradrenaline and [3H]-serotonin by 22-56 % without affecting [3H]-DA release. The present study addresses the effects of gabapentin and pregabalin on K+-evoked release of [3H]-GABA and [3H]-glutamate from rat and human neocortical synaptosomes. These amino acid neurotransmitters are released by two different mechanisms: Ca2+-dependent exocytotic release and Ca2+-independent reversal of uptake pumps. To evaluate the effects of gabapentin and pregabalin on exocytotic release only, transport inhibitors were used throughout superfusion experiments. Under these conditions, gabapentin and pregabalin (100 µM) reduced K+-evoked [3H]-GABA release from human neocortical synaptosomes by 39 and 47 %, respectively. These effects were antagonized by the α2-δ-ligand L-isoleucine (100 µM) which strongly suggests the α2-δ-subunit of terminal VSCC to be involved in the reduction of exocytotic [3H]-GABA release. Surprisingly, both drugs had no effect on exocytotic [3H]-glutamate release in human tissue. In rat synaptosomes, gabapentin and pregabalin failed to modify evoked exocytotic release of both [3H]-GABA and [3H]-glutamate. Our results show that in human tissue exocytotic release of [3H]-GABA and [3H]-glutamate is differently modulated by gabapentin and pregabalin. An inhibition of glutamate release as the main anticonvulsant effect, which was suggested by several studies, is not supported by our experiments. Moreover, the presynaptic reduction of the GABAergic neurotransmission appears to be rather pro- than anticonvulsive. It remains to be determined if gabapentin and pregabalin exert their clinically beneficial effects through modulation of reversed transport mechanisms of the main amino acid neurotransmitters

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221

Activation of purine receptors leads to endocannabinoid production in Purkinje cells of the cerebellum cortex

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The CB1 canabinoid receptor is typically localized on axon terminals and its activation leads to presynaptic inhibition of neurotransmission (Szabo & Schlicker, Handb Exp Pharmacol 168: 318–56, 2005). During the process of retrograde signaling, the presynaptic CB1 receptor is activated by endogenous cannabinoids (endocannabinoids) synthesized by postsynaptic neurons (Chevaleyre et al., Ann Rev Neurosci 29:37-75, 2006). The production of endocannabinoids in postsynaptic cells is usually triggered by an increase in calcium concentration and by activation of Gaq/11 protein-coupled receptors. The hypothesis of the present work was that activation of calcium-permeable ligand-gated ion channels can also lead to endocannabinoid production and retrograde signaling. For testing the hypothesis, we studied the role of P2X receptors in endocannabinoid production.Cerebellar slices were prepared from mouse brain and Purkinje cells were patch-clamped. Glutamatergic excitatory postsynaptic currents (EPSCs) were elicited by stimulation of parallel fibers. P2X receptors on Purkinje cells were activated by ejections of ATP from a pipette. ATP ejection elicited inward currents in Purkinje cells, which were frequently accompanied by calcium spikes. Fluorometric calcium imaging showed strong increases in intracellular calcium concentration when calcium spikes were occurring; without spikes calcium increased only negligibly. In experiments in which ATP led to calcium spikes, the subsequent EPSCs were inhibited by 92 ± 3 %; in the presence of the CB1 antagonist rimonabant (10-6 M), EPSCs were inhibited only by 42 ± 15 %. In experiments in which ATP did not lead to calcium spikes, ATP inhibited EPSCs by 56 ± 10 % and this inhibition was not sensitive to rimonabant.The results show that ATP can elicit endocannabinoid-mediated retrograde signaling between Purkinje cells and parallel fibers. Calcium entering the Purkinje cells via P2X receptor channels is probably not sufficient for triggering endocannabinoid production. Very likely, P2X receptors lead to depolarization and calcium entering the neurons via voltage-gated calcium channels is the trigger for endocannabinoid production.

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222

Localization of the G protein-coupled orphan receptor GPR55 in the nervous system

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Two Gαi/o protein-coupled receptors have been identified as targets for cannabinoids, CB1- and CB2 cannabinoid receptors, but there is evidence for the existence of additional cannabinoid receptors. The G protein-coupled orphan receptor GPR55, when artificially expressed in HEK cells, is activated by a series of plant-derived-, endogenous- and synthetic cannabinoids (Ryberg et al., Br J Pharmacol 152: 1092-1101, 2007). Thus, GPR55 is a potential new cannabinoid receptor. Recently, we could not observe GPR55-mediated electrophysiological effects in caudate-putamen neurons (Baer et al., Naunyn-Schmiedeberg´s Arch Pharmacol 375: R55, 2007). The aim of the present experiments was to determine the localization of GPR55 in the nervous system.In the first series of experiments, [35S]GTPγS autoradiography was carried out on slices prepared from brains of NMRI mice and mice lacking CB1 and CB2 receptors (CB knockout mice; generated in the laboratory of Andreas Zimmer, Bonn). The supposed GPR55 agonist O-1602 (5-methyl-4-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-1,3-benzenediol) increased [35S]GTPγS incorporation in the caudate-putamen of NMRI mice. However, the supposed GPR55 antagonist cannabidiol did not prevent this increase, and no increase was observed in the caudate-putamen of CB knockout animals. In the next series of experiments, we determined GPR55 mRNA concentration in 16 brain regions and the spinal cord of adult NMRI mice with real-time rT-PCR. GPR55 mRNA concentration was generally low in the brain. The highest concentrations were observed in the caudate-putamen, hypothalamus, olfactory bulb, pons and medulla oblongata.The results show that GPR55 is present in the central nervous system, although at a low concentration. The presence of GPR55 in the caudate-putamen, but lack of electrophysiological neuronal effects in this region may indicate that GPR55 is present in glial cells. An additional finding is that O-1602 is not selective for GPR55, but also acts on CB1 receptors

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223

Knockout of the norepinephrine transporter induces changes in the mRNA expression of serotoninergic genes

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Major depression is one of the most widely distributed psychatric disorders and a major health problem. A subfunction of the serotoninergic and/or noradrenergic system in the central nervous system (CNS) is believed to play a key role in the pathophysiology of this disorder. Both systems have been shown to maintain an intense crosstalk. The norepinephrine (NE) transporter (NET) is responsible for the rapid inactivation of released NE, and the NET is an important target for reuptake-inhibiting antidepressants (ADs). Although these ADs (e.g., reboxetine) acutely elevate the synaptic concentration of NE, their antidepressant effect is established only after some weeks, indicating adaptive CNS remodelling. Several studies have shown that NET knockout (NETKO) mice behave like antidepressant-treated wild-type (WT) animals. In the present study, we investigated NETKO-induced changes in mRNA-expression of serotonin (5-HT) receptors (1A, 1B, 1D, 1F, 2A, 2C, 3A, 4, 5A, 6, 7), tryptophan hydroxylase type-2 (TPH2), and the serotonin transporter (SERT) in several brain regions (cerebral cortex, hippocampus, hypothalamus, brainstem) by means of quantitative real-time PCR. We observed small (but significant) changes in mRNA expression of several serotoninergic genes in at least some of the examined brain regions: up-regulation of 5-HT2A-receptors in the cerebral cortex, down-regulation of 5-HT1F-receptors in the hippocampus, down-regulation of 5-HT3A-receptors and TPH2 as well as up-regulation of several 5-HT receptors (1A, 1D, 1F, 2A, 2C, 4 and 5A) in the hypothalamus, and in the brainstem up-regulation of 5-HT4-receptors and down-regulation of the SERT. These data confirm the cross-talk between the noradrenergic and serotoninergic system, and that the observed NETKO-induced changes may be involved in the behaviour of these mice. However, it still remains to be shown whether similar changes are also observed at the protein level.

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224

Facilitation of the protein-protein interaction between CREB and its co-activator TORC (transducer of regulated CREB) by lithium

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Lithium is an effective drug for the treatment of bipolar disorder. Although on the molecular level many targets have been identified, the molecular mechanism of its mood stabilising action is not completely understood. The observed delay of up to six months from treatment initiation to maximum clinical efficacy suggests changes in neuronal plasticity processes. The CRE-binding protein CREB is a transcription factor known to be involved in neuronal adaptation. Recently, we found an enhancement of cAMP-induced CREB-directed gene transcription by lithium which is most likely conferred by the CREB-coactivator TORC (transducer of regulated CREB). Therefore, in the present study we investigated the direct effect of lithium on the interaction between CREB and TORC1 which is mediated by the basic leucine zipper (bZip) of CREB (aa269-327) and aa1-44 of TORC. Immunocytochemistry experiments revealed lithium exerts no influence on the nuclear translocation of TORC. Using in vitro interaction assays lithium increased in a concentration-dependent manner specifically the interaction of GST-tagged CREB and [35S]-labeled TORC1 up to 2-fold. To investigate this effect in a cellular system we used a mammalian two-hybrid system consisting of a luciferase reporter gene driven by 5 repeats of the GAL4-DNA binding site and expression vectors for CREB-bZip fused to the viral transactivation domain VP16, and for TORC aa1-44 fused to the DNA-binding domain of GAL4. The specific interaction between CREB and TORC confirmed by this system was strongly enhanced by lithium measured by a 6-fold increased luciferase activity. Moreover, using Chromatin-Immunoprecipitation and quantitative real-time PCR we measured a facilitation of the interaction also within the promoter context with ~2-fold increased amounts of DNA precipitated via FLAG-TORC1 from cells treated with cAMP and lithium. Our data suggest that lithium enhances the CRE/CREB-directed gene transcription by facilitating directly the interaction between CREB and its coactivator TORC1, thus presenting a novel putative mechanism for the action of lithium.

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225

Apocalmodulin binds to wild type Cav1.4 L-type Ca2+ channels and modulates voltage dependent inactivation

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The retinal L-type Ca2+ channel Cav1.4 is distinguished from all other members of the high voltage-activated Ca2+ channel family by lacking Ca2+-dependent inactivation (CDI). In synaptic terminals of photoreceptors and bipolar cells, this feature is essential to translate graded membrane depolarizations into sustained Ca2+ influx and tonic glutamate release. Our previous work demonstrated that CDI of Cav1.4 is prohibited by a specific interaction of a distal C-terminal peptide, termed inhibitor of CDI (ICDI domain), with a region in the proximal C-terminus. Furthermore, the C-terminal components that form the calmodulin (CaM) binding region is also present and functional in Cav1.4. The aims of our study was to investigate, if the Ca2+ sensor CaM is bound to Cav1.4 channels and secondly, to define whether CaM binding has a functional consequence on channel gating. To this end we performed coimmunoprecipitation and FRET experiments with C-terminal fragments of Cav1.4 and wild type CaM or a calcium insensitive variant of CaM (apoCaM). The results led us to the conclusion that wild type and apoCaM bind to Cav1.4 channels. To find out the functional consequence of CaM binding for Cav1.4 channel gating, we designed mutant Cav1.4 channel variants that lack CaM binding. We found out that apoCaM modulates voltage dependent inactivation of Cav1.4 channels. In summary, our study demonstrates a novel action of CaM in the context of ion channels that is independent of Ca2+ binding.

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226

NF-κB signaling in neurons

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Inflammation is an underlying component of many neurodegenerative diseases. Prostaglandin E2 (PGE2), which is the final product of the increased activities of cytosolic phospholipase A2 (cPLA-2), cyclooxygenase2 (COX-2) and microsomal prostaglandin E synthase1 (mPGES-1), is an important mediator of inflammation in the brain, and its levels correlate directly with inflammation in a model of stroke. cPLA2, COX-2, and mPGES-1 have binding sites for the transcription factor NF-κB in their promoter. NF-κB is considered as a possible target for therapeutic intervention. In the present study, we stimulated primary cortical neurons (DIV 10) with TNF-α (10 ng/ml) for 6, 15 and 24 hours and examined the mRNA expression of cPLA2, COX-2, and mPGES-1 using real-time RT-PCR and also the production of PGE2. To investigate transcriptional regulation of cPLA2, COX-2, and mPGES-1 we constructed reporter fusion genes, in which a long (-1750 b) or a short (-800 b) part of the promoter directed luciferase expression. Cerebral ischemia was induced in mice by a distal middle cerebral artery occlusion (MCAO). RT-PCR demonstrated that neurons express cPLA2, COX2 and mPGES1 under basal conditions and that the expression was significantly increased 6 h after TNF-α stimulation. TNF-α also induced the release of PGE2 from neuronal cultures whereas no increase in cell death was detected at all time intervals tested as measured by the release of lactate dehydrogenase (LDH) in the medium. Reporter fusion gene assays also revealed an increase in the transcriptional activity for these genes. In addition, the induction of all three genes by MCAO was significantly reduced in mice expressing a dominant negative inhibitor of the kinase IKK upstream of NF-κB. In summary, our data show that the NF-κB cascade regulates the expression of the 3 major enzymes of the arachidonic acid cascade (cPLA2, COX2 and mPGES1) and their product PGE2 in neurons.

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227

The role of Rac1 and mTOR in synaptic potentiation and structural plasticity in chronic pain states

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Functional and structural plasticity of central synapses in pain pathways is responsible for the allodynia, hyperalgesia and pain memory caused by different insults like inflammation, neuropathy or trauma. However, molecular mechanisms underlying this experience dependent plasticity at different levels of pain transmission are not yet clearly understood. mTOR is an evolutionarily conserved serine-threonine kinase, acts as a nutrient sensor, regulates growth factor signalling and is shown to play a significant role in the Long Term Potentiation, Long Term Depression and in local translation of some plasticity relevant mRNAs in the Hippocampus. Rac1 is a small GTPase and is a central regulator of the actin cytoskeleton, spine enlargement, activity-dependent dendrite growth, and synaptic clustering of AMPAR during synapse maturation. We therefore addressed the potential role of mTOR and Rac1 in activity-dependent plasticity in the spinal cord in the context of chronic pain. We observed that mTOR and Rac are down regulated very rapidly after nociceptor activation presumably via the Ubiquitin-Proteasome pathway. Surprisingly, however, chronic inflammatory pain was associated with a up regulation of mTOR and Rac suggesting a role of mTOR and Rac1 in pain transmission. To investigate whether mTOR and Rac1 contribute to synaptic potentiation and structural modifications at spinal synapses following intense activation of nociceptors, we are currently assessing the effects of RNA1-mediated knock-down or over expression of mTOR and Rac1 on pain behaviours in models of chronic and neuropathic pain.

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228

Antinociceptive effects of the selective COX-2-inhibitors celecoxib and lumiracoxib assessed by rat BOLD fMRI

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Nowadays different forms of cyclooxygenase-2 (COX-2) selective non-steroidal anti-inflammatory drugs (NSAIDs) are available. Celecoxib is a highly lipophilic, non-acidic compound with a sulphonamide structure and is distributed almost equally throughout the body. Lumiracoxib is a lipophylic phenylacetic acid derivative with a carboxylic group and is weakly acidic which leads to higher accumulation in inflamed tissues. In order to investigate the analgesic effects of the two COX-2-inhibitors with their different biodistribution on the central pain processing we established a hyperalgesic inflammation rat pain model with repetitive heat stimuli.To directly assess their modulatory effects on cerebral pain processing we have performed functional magnetic resonance imaging (fMRI) experiments in anesthetized rats. Hyperalgesia was induced in the left hindpaw of rats by means of subcutaneous injection of zymosan A. The COX-2-inhibitors in different concentrations (0,5 mg/kg, 1 mg/kg and 5 mg/kg) or the vehicle were applied intravenously during the fMRI measurement. The inflamed left and the non-inflamed right hindpaw were stimulated alternately with four different heat stimuli (46-63 ºC). This noxious stimulation evoked robust increases in the blood oxygenation level dependent (BOLD) effect in several areas of the pain matrix (64 structures at 70% incidence threshold throughout thalamus, somatosensory cortex, cingulate cortex, insula, hypothalamus). Stimulation of the inflamed paw led to overall higher BOLD signals. With respect to the activation strength celecoxib and lumiracoxib both showed dose dependent analgesic effects - stronger for lumiracoxib. Moreover, lumiracoxib produced a stronger BOLD-signal reduction during stimulation of the inflamed paw (mostly pronounced in motoric output regions). Regarding the size of activated brain structures, only lumiracoxib led to a reduction (especially in limbic structures). Celecoxib led to an increase which was smaller for the non-inflamed, but even larger for the inflamed paw compared to the control. In conclusion, the acidic compound lumiracoxib provided better analgesic and especially anti-hyperalgesic properties.

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229

Vicious cycle in Alzheimer's disease: amyloid beta stimulates its own production

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Gradual changes in the steady-state levels of amyloid beta (Aß) in the brain of Alzheimer's disease patients (AD) are thought to initiate the amyloid cascade. Ab is derived from its precursor protein APP by intra-membranous b- and g-secretase processing. Herein, we tested the impact of oligomeric Ab (oligoAß) on the cleavage of APP using neuronal and non-neuronal cells transfected with human wild type APP. Low micromolar concentrations of oligoAb significantly decreased membrane fluidity in living HEK293-APP695 and SH-SY5Y-APP695 cells. Decreased plasma membrane acyl-chain flexibility determined by TMA-DPH anisotropy measurements was accompanied by elevated production of endogenous Aß. Accordingly, APP CTF-fragment C99 was significantly increased, indicating enhanced b-secretase cleavage. The novel finding that oligoAß stimulates the amyloidogenic cleavage by altering membrane properties was further evaluated in experiments using Pluronic F68 (PF68) (membrane rigidizer), Benzyl alcohol (BA) (membrane fluidizer). Our data show that amyloidogenic processing of APP strongly correlates with plasma membrane rigidity. Hence, membrane fluidity determines ectodomain shedding of APP regardless of the mode of fluidity manipulation, e.g. independent of cholesterol levels. To further evaluate Ab's effects on ectodomain shedding of APP, cells were treated with the g-secretase inhibitor DAPT. Cellular Aß production was abolished and membrane fluidity was increased by DAPT, indicating that also endogenous Aß interacts with membranes and enhances amyloidogenic APP processing in living cells. Based on our novel findings presented herein, we hypothesise that an age related reduction in brain membrane fluidity enhances the amyloidogenic processing of APP, leading to an amplified production of Aß. After oligomerization of Aß, oligoAß interacts with neuronal membranes and further accelerate the intramembranous proteolytic cleavage of APP, starting a vicious cycle in which endogenous Ab stimulates its own production.

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230

Behaviour of rats in an animal model of cephalgia

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Stimulation of the dura mater of rats with either interleukin-1ß (IL-1ß), a mediator of inflammation, or capsaicin, a vanilloid-receptor agonist, is used as animal model of migraine. Biochemical consequences are documented, but the impact on behaviour is not known. Our aim is the assessment of exploratory and consummatory behaviour following stimulation of the dura mater. The cranium of anaesthetized Sprague Dawley rats was trepanated. The intact dura was covered with a refillable chamber filled with saline. On the 2nd, 4th and 7th day post operationem the chamber was filled with capsaicin, vehicle or saline (4 min prior testing) or with IL-1β or saline (6 hrs prior testing). Sham operated and non-operated rats were used as controls. The following behavioural experiments were performed at 17:30-20:30 hrs:2nd day, open field: Rats were placed in a dimly lit non-aversive arena (1m x 1m, 10 lux). Distance travelled, times spent in the corners, rearings and grooming episodes were registered. 4th day, feeding behaviour and activity: Following fasting for 12 hrs the rats received food and water. Food and water consumption was calculated for 1 and 2 hours. Motor activity was registered simultaneously. 7th day, sweet agar consumption: Rats were adapted to highly palatable bait (cherry-flavoured agar). The amount of agar and food consumed within 1 and 2 hours was determined. Motor activity was assessed simultaneously. IL-1ß and capsaicin, but also vehicle and saline given 4 min before testing decreased exploration in the open field and food intake compared to controls. Saline given 6 hrs before had no effect. None of the treatments changed the agar consumption. Our study shows to our knowledge for the first time that meningeal stimulation leads to behavioural changes in rats. Both IL-1ß and capsaicin induce moderate „sickness behaviour". The filling-procedure of the chambers 4 min before testing induces adverse effects, overlapping the capsaicin effects, whereas anaesthesia has no impact on the behaviour. Headache reduced normal exploratory and feeding behaviour but had no impact on the consumption of highly palatable bait.

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231

Behavioral and pharmacological investigations in a transgenic mouse model of early-onset dystonia

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Early-onset torsion dystonia is an autosomal dominant movement disorder associated with the DYT1 gene defect with deletion of glutamic acid residue in the protein torsinA. Despite the gene defect, the pathophysiology is poorly understood. Well characterized animal models can help to understand the underlying mechanisms and thereby to develop new therapeutic strategies. Shashidharan et al. (2005) initially described a transgenic mouse model (DYT1 mice) with the human defect gene and overexpression of mutant torsinA, leading to hyperactivity, dystonic-like movements of limbs, abnormal shaking of the head and circling behaviour in affected animals. We further characterized DYT1 mice by behavioural phenotyping and pharmacological investigations. Nearly all transgenic mice showed dystonic-like postures of the hindlimbs as recently reported for some mice. However, the present data revealed that also wild-type mice sometimes exhibit similar movements. Preliminary footprint data indicate that DYT1 mice have a shorter mean step length and a greater distance of the overlap than wild-type controls in the footprint test. Treatment with 100 mg/kg L-3,4-dihydroxyphenylalanine methyl ester hydrochloride (L-DOPA) + 10 mg/kg S-(-)-α-hydrazino-3,4-dihydroxy-methylbenzenepropanoic acid (carbidopa) reduced the vertical and horizontal activity and the time to fall from the rotarod in transgenic mice in comparison to vehicle control. Furthermore the severity of dystonic-like movements, which were rated by a score-system, and the mean distance between the forelimbs increased. These results indicate that the dopaminergic system is affected in the DYT1 mice. Further pharmacological and histological studies have to clarify the relevance of the dopaminergic system. Shashidharan P, et al. (2005): Hum Mol Genet 14:125-133. 1.

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232

Behavioral function in distal middle cerebral artery occlusion of the mouse

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Stroke is one of the most common causes of death in humans. To investigate the pathophysiology of stroke and to develop therapeutic strategies, several mouse models of middle cerebral artery occlusion (MCAO) are in common use. The filament model of MCAO is often afflicted with a high mortality due to a large infarct volume. Therefore, we have turn to a model in which the distal middle cerebral artery is occluded by electrocoagulation resulting in smaller infarcts. Mortality is low. However, this model has the disadvantage to cause less obvious neurological deficits that cannot be evaluated by usual neuroscores. Therefore, we are testing mice before and after distal MCAO in various behavioural paradigms in order to characterize the behavioural effects of small cortical infarcts. In the corner test mice were placed in front of a 30° angle. The direction of turnings was recorded. Large infarcts induced by the filament model were associated with an increased turning rate to the ipsilateral side. However, after distal MCAO we have observed an increased turning rate to the contralateral side that persisted for 8 days after surgery. Sham surgery did not affect the turning rate. The rate of turning in the corner test correlated with the infarct size. Currently, we are exploring how results in the corner test correlate with other motor and cognitive functions in stroked mice (gate analysis, Collins test of handedness) in order to provide tools to analyze functional deficits in experimental stroke.

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233

Effects of repeated neurosteroid applications on the age-dependent time course of dystonia in the dtsz hamster

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Dystonia, a common movement disorder, is characterized by involuntary cocontractions of opposing muscles, frequently causing twisting, repetitive movements and abnormal postures. The dtsz hamster represents a unique rodent model of stress-inducible paroxysmal dystonia. In this animal model, a reduced density of striatal GABAergic interneurons seems to be of great pathophysiological importance. The dystonic syndrome is age-dependent and a spontaneous remission after the 70th day of life coincides with normalization of interneuron density during puberty of dtsz hamsters. It is known that the repeated administration of the neurosteroid allopregnanolone (3α,5α-Tetrahydroprogesterone) in rats is able to influence the postnatal development of GABAergic interneurons in healthy rodents. This prompted us to investigate the repeated administration of allopregnanolone, which exerted moderate antidystonic effects after acute treatment of dtsz hamsters. Therefore, repeated intraperitoneal applications of 50 mg/kg allopregnanolone acetate were compared with repeated applications of the vehicle. However, the areas under the curves did not differ between vehicle and neurosteroid treated animals. In comparison to the time course of dystonia observed in completely untreated animals in a previous study, there is a prolongation with remission of dystonia after the 90th day of life in both, vehicle and neurosteroid treated animals. These results argue against the hypothesis that repeated applications of allopregnanolone can shorten the age-dependent time course of dystonia in the dtsz hamster. Since we have seen a retarded remission of the dystonic syndrome also in the vehicle treated group, an influence of stress by repeated postnatal applications seems to be more important for the age-dependent time-course of dystonia. This study was supported by grants from the Forschungskommission of the Freie Universität Berlin.

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234

Glutamate is critically involved in seizure-induced overexpression of P-glycoprotein in the brain

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About 30% of patients with epilepsy do not respond adequately to drug therapy, making pharmacoresistance a major problem in the treatment of this common brain disorder. Mechanisms of intractability are not well understood, but may include limitation of antiepileptic drug access to the seizure focus by overexpression of the drug efflux transporter P-glycoprotein (Pgp) at the blood-brain barrier. Increased expression of Pgp has been determined both in epileptogenic brain tissue of patients with intractable epilepsy and in rodent models of temporal lobe epilepsy, including the pilocarpine model. The mechanisms underlying the increase of Pgp after seizures are unclear. We have recently suggested that the excitatory neurotransmitter glutamate, which is excessively released by seizures, is involved in the seizure-induced overexpression of Pgp in the brain. This hypothesis was evaluated in the present study in the pilocarpine model in rats. After 90 min of status epilepticus (SE), diazepam was administered, followed by either vehicle or the glutamate receptor antagonist MK-801 (dizocilpine). Following SE in vehicle treated rats, Pgp expression in brain capillary endothelial cells increased about twofold in the hippocampus, which was completely prevented by MK-801. Furthermore, neurodegeneration developing in the hippocampus and parahippocampal regions was reduced by the glutamate antagonist. In contrast, the Pgp inhibitor tariquidar did not affect the SE-induced overexpression of Pgp or neurodegeneration in most regions examined. The data indicate that seizure-induced glutamate release is involved in the regulation of Pgp expression, which can be blocked by MK-801. The finding that MK-801 counteracts both Pgp overexpression and neuronal damage when administered after SE may offer a clinically useful therapeutic option in patients with refractory SE.

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235

Benefits and risks of intranigral microtransplantation of immortalized GABA-producing cell lines in the kindling model of temporal lobe epilepsy

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Neural transplantation of different various cell types has been investigated experimentally and clinically in an attempt to develop new treatment options for intractable epilepsy. We now assessed the anticonvulsant efficacy and safety of allotransplantation of genetically engineered striatal GABAergic rat cell lines into the substantia nigra pars reticulata (SNr) of amygdala-kindled rats as a model of temporal lobe epilepsy.Three cell lines were transplanted bilaterally into the SNr of previously kindled rats: (1) the parent cell line, consisting of immortalized GABAergic cells (M213-2O) derived from embryonic rat striatum; (2) M213-2O cells (CL4) transfected with hGAD67 (human glutamic acid decarboxylase isoform 67) cDNA to obtain higher GABA synthesis than the parent cell line; (3) Control cells (121-1I) were also derived from embryonic rat striatum, but did not show GAD expression. A second control group of kindled rats received injections of medium alone.Comparable to fetal GABAergic cells, transplantation of M213-2O cells into the SNr of kindled rats resulted in significant but transient anticonvulsant effects. Neither control cells nor medium induced anticonvulsant effects. Transplantation of CL4 cells caused strong tissue reactions within the host brain of kindled but not of non-kindled rats, characterized by graft rejection with massive infiltration of inflammatory immune cells and gliosis.The observed anticonvulsant effect of M213-2O cells emphasizes the feasibility of local manipulations of seizure induction and propagation by intranigral transplantation of GABAergic grafts. The tissue reactions in response to CL4 grafts are likely due to kindling-induced activation of microglia in the SNr together with further immunological stimulation by the transfected CL4 cells. Thus, the host condition has urgently to be considered to estimate risks of neural transplantation in epilepsy. The research was supported in part by a grant of the German National Academic Foundation to MWN and in part by the NIDA IRP, NIH, DHHS.

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236

Agmatine and the protective activity of antiepileptic drugs against pentylenetetrazole-induced convulsions in mice

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Purpose: Agmatine is an endogenous amine which has been shown to exert some anticonvulsant activity, probably by inhibition of glutamate release and modulation of NMDA receptor function. The objective of this study was to assess the influence of agmatine upon the protection offered by a number of antiepileptic drugs (AEDs) against pentylenetetrazole-induced clonic seizures in mice. Materials and methods: Experiments were conducted on male Swiss mice (20-26 g). The following AEDs were used: clonazepam, ethosuximide, gabapentin, phenobarbital, tiagabine, valproate, and vigabatrin, given intraperitoneally. Agmatine was injected via the same route. The protective efficacy of AEDs against the clonic phase of seizures induced by subcutaneous pentylenetetrazole at its 97% convulsant dose (100 mg/kg) was expressed as their ED50s. Adverse (neurotoxic) effects produced by the combinations of agmatine with AEDs were evaluated in the chimney test (motor performance), step-through passive avoidance task (long-term memory), and grip-strength test (muscular strength). Results: Agmatine (50 and 100 mg/kg) did not affect significantly the convulsive threshold and up to 100 mg/kg did not modify the protective activity of all AEDs tested, except for vigabatrin. Interestingly, the anticonvulsant action of vigabatrin was considerably reduced by agmatine at 50 and 100 mg/kg. In addition, agmatine (100 mg/kg) alone or combined with AEDs, at doses providing a 50% protection against the clonic phase, was without influence on in the chimney and grip-strength test. On the other hand, long-term memory was impaired when agmatine (100 mg/kg) was combined with gabapentin, vigabatrin, and valproate. Conclusion: From a preclinical point of view, agmatine does not seem a promising anticonvulsant agent in combinations with antiepileptic drugs.

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237

Evaluation of the rat free exploratory paradigm as an animal test for trait anxiety in rats

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The free exploratory paradigm is regarded as a reliable test for trait anxiety in mice (Griebel et al., Behav Pharmacol 1993, 637-644) but it may be useful in the research for the neurobiological basis of anxiety in rats, too. Previously we could show that rat strains differ in their desire for non-forced exploration of novel areas, like the surroundings of their familiar cage when the grid-covers of the cage were removed (Rex et al., Pharmacol Biochem Behav 1996, 107-11). In the present study this test was validated behaviourally using naïve Sprague Dawley and Wistar rats obtained from different sources. Sprague Dawley rats were tested at the age of 30, 55, 78 and 110 days. Behavioural validation also included assessment of seasonal variation, sex-specific impact and habituation to the test. Additionally, the test was evaluated pharmacologically using the known “anxiolytic” and “anxiogenic” drugs, diazepam (1, 2.5 mg/kg IP) and caffeine (50 mg/kg IP), respectively. Parameters measured were: The number of rats starting to explore the outside within 10 min, the percentage of rats of a group exploring the outside, the latency to the first escape, the number and duration of the escapes. Latency to the first escape was the most reliable and sensitive parameter. Seasonal variability of the latency to the first escape was low. Rat strains and sexes differ in the latency too, while age-related differences have less impact. Diazepam (2.5 mg/kg) decreased and caffeine (50mg/kg) increased the latency to explore the outside of the cage. We conclude that the free exploratory paradigm can be used to study anxiety-related behaviour also in rats.

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238

Antistress effects of St. John's wort in a pharmacological model

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It has been shown that antidepressants like St. John´s wort extract (SJW), but also fluoxetine, significantly increased markers of anti-oxidative capacity, e.g. superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in brain homogenates as well as erythrocyte lysates in unstressed mice [1]. The aim of the present study was to evaluate the mechanisms of action of SJW extract (STW 3-VI, Laif® 900) in a chronic restraint stress (CRS) model using male Sprague-Dawley rats. Since there is a strong connection between stress and selected neurodegenerative as well as mental disorders such as depression and anxiety [2], the effects of STW 3-VI extract on CRS induced neuroendocrine, neuroimmunological and behavioral changes, on the production of free radicals and antioxidant enzyme activities in the hippocampus and hypothalamus as well as on the protection of immune organs of stressed and non-stressed rats were evaluated. Markers of antioxidant capacity, such as SOD, GPx and catalase (CAT) in the hippocampus and hypothalamus, and on plasma hormone levels (ACTH, corticosterone), as well as on inflammatory markers such as IL-6, TNF-α and C-reactive protein were determined in addition to behavioral changes.Results show that CRS (1h for 21 consecutive days) significantly decreased thymus and spleen indices in CRS treated controls (control non-stress: 1.64 ± 0.07; 2.41 ± 0.09 vs. control stress: 1.21 ± 0.03; 2.10 ± 0.06, $p < 0.01$). This result indicates that the immune function was diminished when the animals were exposed to CRS. The thymus and spleen indices were unchanged for the unstressed animals receiving fluoxetine (10 mg/kg), or SJW extract (125, 250, 500 and 750 mg/kg, respectively). However, treating stressed rats with fluoxetine or SJW extract produced a significant and dose dependent increase in both thymus and spleen indices. These data provide new insight into the effects of stress on neuroimmunological and antioxidative parameters and point to an anti stress effect of St. John’s wort. References: [1] Michalski, C. et al. (2007) Planta Med 73:991, [2] Herken, H. et al. (2007) Arch Med Res 38:247;

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239

Chronic overexpression of cardiac β2-subunits in transgenic mice leads to ventricular alterations typical of human heart-failure

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In human heart-failure activity of single ventricular L-type Ca2+-channels and expression of stimulatory β2-subunits are increased. Mice constitutively overexpressing the human cardiac L-type Ca2+-channel pore Cav1.2 (tg Cav1.2) show normal cardiac function at 4-5 months. At an age of ≥9 months severe heart-failure develops and activity of single ventricular Ca2+ channels and ventricular expression of auxiliary β2-subunits is significantly increased, thus resembling features of human heart-failure. Recently we showed that acute (48h) induction of cardiac overexpression of a rat β2a-subunit (tgind β2a) causes a preterm “heart-failure phenotype” of enhanced single-channel gating in young (4-5 months) tg Cav1.2 (Hullin et al., PLoS 2007;2:e292). We now investigated the effect of chronic overexpression of β2a in hearts of tgind β2a or double-transgenic mice (tg Cav1.2 x tgind β2a) at an age of 3-4 months, i.e. when tg Cav1.2 hearts are still non-failing. Wildtype, tg Cav1.2, tgind β2a and tg Cav1.2 x tgind β2a mice were treated with the β2a-inducing drug tebufenozide by osmotic mini-pumps for 4 weeks. In both tgind β2a and tg Cav1.2 x tgind β2a, overexpression of β2a-subunits significantly increased slow inactivation time-constant and shifted the steady-state inactivation curve to the left by 7mV. Peak current density was slightly changed in tgind β2a (-12±1 pA/pF vs. -10±1 pA/pF in wildtypes) or tg Cav1.2 x tgind β2a (-18±1 pA/pF vs. -16±1 pA/pF in tg Cav1.2). In contrast single-channel activity was significantly enhanced (Ipeak, wildtype: -30±7 fA vs. tgind β2a: -72±21 fA; tg Cav1.2: -20±4 fA vs. tg Cav1.2 x tgind β2a: -40±4 fA). While contractility and blood-pressure were unaffected by β2-overexpression histological analysis revealed interstitial fibrosis and hypertrophy of cardiomyocytes in hearts of double transgenic animals.In conclusion we developed a mouse model of cardiac hypertrophy that by cardiac overexpression of Ca2+-channel β2-subunits resembles ventricular Ca2+ current alterations typical of human heart-failure.

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240

Effects of inducible and myocyte-specific inhibitor-1 overexpression on contractility in in the murine heart

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Inhibitor-1 (I-1) acts as an amplifier of cardiac β-adrenergic signaling by inhibiting type-1 phosphatases (PP1) only in its PKA-phosphorylated form at Thr35. In contrast, phosphorylation at Ser67 by PKCa attenuates I-1´s inhibitory activity. I-1 is markedly downregulated in failing human hearts.To define the long-term in vivo effects of I-1, transgenic mice (TG) with cardiac specific I-1 overexpression (aMHC-promoter) were generated. I-1 TG developed cardiac hypertrophy and dysfunction, but an increase in PP1 abundance and activity (~3-fold) indicated compensatory changes. We therefore generated a double TG mouse model (dTG) with cardiac-specific and temporally regulated expression (aMHC-Tet-Off system) of a mutated form of I-1 (I-1*). In I-1* Ser67 was replaced by non-phosphorylatable Ala to avoid I-1 deactivation by PKCa. Functionality of recombinant PKA-phosphorylated I-1* against PP1 was confirmed by phosphatase assays (IC50: 16±2 nM). To explore I-1 cause-effect-relationship in a bidirectional manner, I-1*dTGs and the transactivator single TGs, which served as

controls, were crossed with I-1 KO mice until they were on a complete I-1 null background (dTG/KO). Western blotting revealed that doxycycline (Dox)-free water feeding was sufficient to elicit transgene expression (~8-fold) in the adult heart, whereas transgene expression in dTG/KO fed with Dox-water (0.2 mg/ml) in utero and after birth was immunologically not detectable. Importantly, Dox-free water feeding up to 20 weeks was not associated with changes in PP1. Moreover, in vivo analysis by cardiac catheterization revealed hypercontractility in dTGs/KO in the "ON-state" (12300±300 vs. 10600±600 mmHg/sec, n=6, $p<0.05$). This is consistent with the proposed function of I-1* and was accompanied by higher phospholamban phosphorylation (~2-fold, $p<0.05$).Our results indicate that the aMHC-Tet-Off-dTG/KO approach allows careful determination of I-1 cause-effect-relationships including reversibility-studies. Moreover, it will help to define the potential therapeutic value of changing I-1 protein levels after the onset of cardiac remodelling, either during the compensated or decompensated states of hypertrophy. These questions are currently under investigation.

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241

Genome-wide transcriptional profiling of two mouse strains with differential susceptibility to doxorubicin-induced cardiotoxicity

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Heart failure following treatments with anthracyclines remains an unresolved medical problem. We identified two mouse strains, BALB/c and C57BL/6, which are insensitive and sensitive, respectively, to the cardiac side-effects of a chronic doxorubicin (DOX) treatment. The heart failure in C57BL/6 mice was evidenced by gradually diminishing left ventricular function and enlarged cardiac cavity. Cardiac accumulation of DOX did not significantly differ between the two strains. Genome-wide expression profiling of untreated animals revealed differences in the expression of several hundred genes. However, these differences were statistically insignificant, since all genes represented particularly large GO (gene ontology) categories. Statistically significant DOX-induced gene expression changes were more numerous in C57BL/6 mice and involved DNA repair genes. Both strains exhibited altered expression of genes implicated in organ development, indicating activation of tissue regeneration. In conclusion, genetic makeup is a strong determinant of the DOX-induced cardiotoxicity in the mouse. Higher sensitivity of C57BL/6 mice to DOX may be related to DNA damage. Variants of genes involved in the response to DOX-induced DNA damage may modulate cardiac responses to DOX chemotherapy.

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242

Promotion of agonist-independent constitutive IK,ACh channel activity by atrial tachycardia remodeling in canine atrial cardiomyocytes

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Background: Although atrial tachycardia (AT) appears to promote agonist-independent constitutively-active IK,ACh that increase susceptibility to atrial fibrillation (AF), the underlying changes in IK,ACh-channel function are unknown. Here, we studied the effects of AT on single IK,ACh channel activity in dog atria. Methods: In order to study effects of high frequency atrial activity in otherwise healthy hearts we have investigated constitutively active IK,ACh in a dog model of atrial tachypacing. IK,ACh was measured with cell-attached voltage-clamp in isolated left atrial myocytes of control (CTL) and AT (7 days, 400 min-1) dogs. Results: AT prolonged inducible duration of AF episodes from 44±22 to 413±167 s (N=9/gp, $P<0.001$). In the absence of cholinergic stimulation, single-channel openings with typical IK,ACh conductance and rectification properties were sparse under control conditions. AT induced prominent agonist-independent IK,ACh activity due to increased opening-frequency (fo) and open-probability (Po; ~7- and 10 fold respectively versus control), but unaltered open time-constant and single-channel conductance. With maximum IK,ACh activation (10 µM carbachol, CCh), channel open-probability was enhanced ~42-fold in control cells, but only ~5-fold in AT-remodeled myocytes. The selective Kir3-blocker tertiapin (100 nmol/L) reduced fo and Po by 48% and 51% respectively ($P<0.05$ for each) without altering other channel properties, confirming the identity of IK,ACh. AT had no significant effect on mRNA or protein abundance of Kir3.1 and Kir3.4 channel subunits underlying atrial IK,ACh.Conclusions: Increased constitutive activity of IK,ACh in AF is proposed to be the result of high sustained atrial rate per se rather than a consequence of underlying clinical conditions. These results suggest an important role for constitutively active IK,ACh channels in AT-remodeling and support their interest as a potential novel AF-therapy target.

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243

Overexpression of the serotonin 5-HT4a receptor: a mouse model for cardiac arrhythmias

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Serotonin (5-HT) exerts various effects in the human cardiovascular system. In the human heart, the 5-HT receptor isoform 5-HT4 is expressed in atrium and ventricle. Moreover, 5-HT is thought to play a proarrhythmic role in human atrium, mediated by 5-HT4 receptors. To study these receptors in more detail, we generated transgenic mice (TG) with heart specific overexpression of the human 5HT4a receptor. Northern blotting and immunohistochemistry revealed overexpression of the receptor on the RNA and protein levels. Protein expression of important cardiac regulatory proteins like SERCA, phospholamban, and calsequestrin remained unchanged. Also, the heart weight remained unchanged compared to littermate controls. During perfusion of isolated work performing heart preparations, only TG but not wild type (WT) hearts displayed arrhythmias under basal conditions. β-adrenergic stimulation of perfused hearts by isoproterenol (1µM) increased phospholamban phosphorylation on serine 16 and threonine 17 whereas 5-HT increased these phosphorylations only in TG but not in WT hearts. 10µM 5-HT increased contractility in isolated perfused TG hearts but not in WT. Moreover, 5-HT effects could be blocked completely by the 5-HT4 receptor specific antagonist GR125487 (10nM). Intravenous infusion of 5-HT increased left ventricular pressure and its first derivative in TG but not in WT mice measured by left ventricular catheterization. The effects were still present after pretreatment of TG mice with reserpine (5mg/kg). The positive inotropic effect of 5-HT in TG was accompanied by an increase in $Ca2+$ transients and L-type $Ca2+$ channel current (by 101%±17%, $p<0.05$ vs. Ctr). In intact animals, echocardiography revealed an inotropic and chronotropic effect of subcutaneously injected 5-HT (1mg/kg) in TG but not in WT. These findings demonstrate functional expression of 5-HT4 receptors in the heart of TG mice. Therefore, 5-HT4a receptor-overexpressing mice might be a useful model to investigate the influence of 5-HT in the development of cardiac arrhythmias and heart failure.

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244

Arrhythmogenic right ventricular cardiomyopathy by plakoglobin-deficiency is prevented by preload reduction

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Introduction: Heterozygous plakoglobin deficiency (Pg+/–) in mice causes right ventricular enlargement, right ventricular dysfunction, and ventricular tachycardias (VTs) of right ventricular origin, compatible with human arrhythmogenic right ventricular cardiomyopathy (ARVC). Lack of right ventricular histological changes and accelerated development of the phenotype by endurance training suggest a functional desmosome associated defect in this model. We tested whether preload-reducing therapy prevents ARVC in Pg+/– mice. Methods and results: Fourteen littermate pairs of 3-months-old Pg+/– mice and 5 pairs of matched wildtype (WT) mice underwent 7 weeks of endurance training by swimming (increasing from 10 to 90 min/day). Half of the mice received preload-reducing therapy (furosemide, nitrates, and molsidomine). All functional measurements were performed by investigators blinded to genotype and therapy. Right ventricular size was increased in untreated Pg+/– mice after training, but not in treated Pg+/– mice (echocardiographic RV area untreated 58±6 vs. treated Pg+/– 37±3 mm^2; $p < 0.001$; WT 35±3 mm^2; mean±SD; 15–50 Mhz transducers). Preload-reduction lowered the inducibility of VT (> 1 s duration) by a single right ventricular extra stimulus in isolated, Langendorff-perfused hearts (1/6 treated vs. 10/14 untreated Pg+/– mice; $p < 0.05$). High density epicardial mapping showed activation patterns consistent with re-entrant VT in Pg+/– mice. Right and left ventricular effective refractory periods (ERP) were not different between groups. Right ventricular longitudinal and transversal conduction velocities (Vl and Vt) were reduced in Pg+/– mice following premature stimulation (ERP + 10ms; WT Vl 62±10, Vt 37±7 vs. Pg+/– Vl 51±8, Vt 30±7 cm/sec; mean±SD; p=0.015/0.058), but were not affected by preload-reducing therapy. Conclusions: Heterozygous plakoglobin deficiency leads to right ventricular enlargement in response to increased preload and directly affects myocardial conduction velocity. Preload-reducing therapy prevents right ventricular enlargement and lowers the inducibility of re-entrant VT in trained Pg+/– mice.

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245

Inhibition of the interactions between protein kinase A and A kinase anchoring proteins in cardiac myocytes

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Stimulation of the β-adrenergic signalling pathway in cardiac myocytes results in local activation of cAMP-dependent protein kinase (PKA) and phosphorylation of several PKA substrates. These include the L-type calcium channel, phospholamban and adenylyl cyclases V/VI. The localized actions of PKA are mediated by A kinase anchoring proteins (AKAPs). Screening of 20,000 small molecules revealed a compound, FMP-API-1, that disrupts the interaction between AKAP18d and PKA regulatory subunit (RII). FMP-API-1 specifically inhibits the AKAP18d and RIIa interaction while it does not affect AKAP18d and RIIb interaction. In neonatal cardiac myocytes low concentrations of FMP-API-1 inhibit isoproterenol-induced (β-adrenoreceptor-mediated) increases of L-type calcium channel currents. This effect is most likely due to the inhibition of interaction between RII and AKAP18a that anchors PKA to L-type calcium channels. FMP-API-1 also increases the isoproterenol-mediated cAMP formation presumably by inhibiting the interaction between AKAP150 and RII that regulates the activity of adenylyl cyclases V/VI. In higher concentrations, FMP-API-1 activates PKA cAMP-independently in vitro and in cell lysates of neonatal cardiac myocytes and causes the oligomerization of RII subunits and AKAPs. The increased PKA activity results in enhanced phospholamban phosphorylation. Phospholamban is a regulator of the calcium reuptake into the sarcoplasmic reticulum and an important therapeutical target in heart failure. FMP-API-1 provides a new tool for studying compartmentalized cAMP signalling pathways and may lead to a new therapeutic strategy for the treatment of cardiovascular diseases.

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246

An important role of pentamer formation for phospholamban phosphorylation

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Phospholamban (PLN) is present in the cardiomyocyte both as a monomer and a pentamer. It regulates sarcoplasmic reticulum (SR) Ca2+ uptake by inhibiting the SR Ca2+-ATPase SERCA2. This inhibitory effect is relieved by phosphorylation of PLN. We previously described a PLN missense mutation (PLNR9C) that inhibits phosphorylation of co-expressed wild-type PLN (PLNwt) leading to constitutive PLN activation, dilated cardiomyopathy and terminal heart failure. Here we show that PLNR9C is dependent on the formation of pentamers to exhibit these detrimental effects. Cardiac morphology and function were compared in transgenic mice with heart-specific overexpression of either PLNR9C (TgPLNR9C) or a monomeric PLNR9C mutant (TgAFA-PLNR9C) that cannot participate in the assembly of pentamers. 20-week-old TgPLNR9C mice revealed severe ventricular dilation, myocyte enlargement and massive interstitial fibrosis leading to terminal heart failure at the age of 21±6 weeks. Conversely, strain- and age-matched TgAFA-PLNR9C mice were morphologically indistinguishable from wild-type mice and lived for at least one year. To explore the consequences of monomeric and pentameric PLNR9C on cardiac function, 8- to 10-week-old mice were investigated by left ventricular catheterization and echocardiography. At this age, no signs of heart failure were found in either mouse line; left ventricular diameters and contractile function were not different among hearts. Importantly, only TgPLNR9C mice demonstrated impaired cardiac relaxation and an attenuated lusitropic response to dobutamine, consistent with strong inhibition of SERCA2 by PLN and impaired phosphorylation of PLN. Two dimensional gel electrophoresis using mouse heart lysates confirmed that dobutamine induced phosphorylation of PLN was weak in TgPLNR9C mice, but intact in TgAFA-PLNR9C mice. The data show that in vivo PLNR9C blocks phosphorylation of PLNwt only if it participates in pentamer formation. Since impaired PLNwt phosphorylation within the pentamer is sufficient to bring down its overall phosphorylation state, the pentamer seems to play an important role in PLN phosphorylation.

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247

Functional and structural profiling of the human thrombopoietin gene promoter

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Human thrombopoietin (TPO) is involved in cardiovascular disease (CVD) as it regulates megakaryocyte development and enhances platelet adhesion/aggregation. As THPO promoter structure is still controversial, using RT-PCR, we evidenced that THPO transcription is cell line-dependently initiated at two alternative promoters, which we newly designated P1a and P1. We subsequently electrophoretically scanned and resequenced these portions in 95 and 46 patients with CVD, respectively, and identified eight variants (-1450/del58bp, C-920T, A-622G, C-413T, C+5A, C+102A, G+115A, and C+135T). After subcloning of 1032 bp fragments of THPO P1 in pGL3-basic vectors, five molecular haplotypes (MolHaps1-5) were, respectively, observed: [A-622-C-413-C+5-G+115; wildtype (wt)], [A-622-T-413-C+5-G+115], [G-622-T-413-C+5-G+115], [A-622-C-413-A+5-G+115], [A-622-C-413-C+5-A+115], and analysed in reporter gene assays in HEK293T and HepG2 cells. While MolHaps 2, 4, 5 were significantly more active than wt (all P-values ≤0.01), MolHap3 exerted a substantial loss of promoter activity (P<0.0001 in HEK293T; P=0.001 in HepG2, compared to wt). EMSAs revealed that A-622G and C-413T in single assays differed from MolHaps in their DNA:protein interaction patterns and Supershift assays identified C/EBPδ as binding protein exclusively for the -622A allelic portion. We herein redefined the transcriptional organisation of THPO and conclude that the P1 promoter is differentially regulated by complex genetic constellations.

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248

Transcriptional activity of the human biglycan promoter is altered by single nucleotide polymorphisms

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Introduction: Biglycan (BGN), a small leucine rich proteoglycan (SLRP) and component of the extracellular matrix (ECM), interacts with type I collagen, regulates collagen fibril formation and has been suggested to be involved in the pathophysiology of cardiovascular disease. The aim of our study was to identify and to functionally characterize genetic variants within the BGN gene promoter.Material and Methods: We scanned 95 high-risk patients with myocardial infarction (MI) from the ECTIM study for genetic variants. Five promoter SNPs were introduced into respective PCR amplicons (180 bp) by site-directed mutagenesis, subcloned into reporter gene vector pGL3-promoter and transfected into HEK293T and EA-Hy926 endothelial cells. Cells were kept under basal conditions or stimulated with 10-8M PMA or 0.5 mM 8-Br-cAMP, respectively for 12 hrs.Results: We identified four SNPs in the 5'-regulatory region (G-849A, A-578G*, C-501A, G-151A), one in the 5'UTR (G+94T**), two synonymous SNPs in exons 2 (Ser47Ser) and 4 (Ser206Ser), and five in introns. BGN promoter activity was cell type-dependent and allele-specific. In unstimulated HEK293T cells, A-578G was generally inactive (P=0.8 compared to shuttle vector), but strongly activated by cAMP (4-fold over shuttle vector) exclusively when carrying the A allele (P=0.002). The highest activity was seen in G-151A, yet with significantly less activity of the A allele (P=0.017), this effect was nearly egalized by cAMP (P=0.033). The 5'UTR was transcriptionally active exclusively when carrying the -151G allele (P=0.002) irrespective of the stimulation regime None of these effects were seen in EA-Hy926, where the 5'UTR was consistently silent, and cAMP had an activating effect only on 151A.Discussion: Our results show, that genetic variants reside in enhancer regions of the BGN promoter and cause functional alterations both cell type- and allele-specifically. We currently investigate whether these effects sum up to altered tissue-specific BGN gene expression in the context of molecular haplotypes of the entire BGN promoter. * rs11796997, ** rs 5945197

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249

Action potential prolongation in cardiomyocytes from mice with inactivation of transcription factor ATF-1

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Chronic stimulation of the β1-adrenoceptor and subsequent activation of the cAMP-dependent signaling transduction pathway by elevated plasma catecholamines plays a central role in the pathogenesis of heart failure. The transcriptional regulation by transcription factors of the CREB/CREM/ATF-1 family represents a fundamental mechanism of a gene control in response to cAMP which might contribute to β1-adrenoceptor-mediated detrimental cardiac changes. The functional role of the ubiquitously expressed activating transcription factor 1 (ATF-1) in the heart is currently not known. Here, we studied the consequences of a functional inactivation of ATF-1 in single isolated ventricular cardiomyocytes of ATF-1-knockout mice (KO) and corresponding wild-type littermates (WT) on action potential parameters and L-type calcium channels in order to address the influence of ATF-1 on cardiac ion channels. Both groups showed no significant difference in cell size (membrane capacity).Action potentials (APs) were recorded via whole cell current clamp using standard intracellular and extracellular solutions (KO, n=8 vs. WT, n=5). Only cardiomyocytes with resting potentials <-70 mV were observed. Action potential duration parameters APD50 and APD90 were significantly increased in KO mice (APD50 in ms. KO: 7.9±1.1*; WT: 3.9±0.3. APD90 in ms. KO: 78±14*; WT: 27±7. *P<0.05 vs. WT). L-type calcium channel currents were measured in whole cell voltage clamp configuration under potassium channel blockade (via CsCl) and functional sodium current elimination (preclamp to -40 mV). No significant alteration in basal current amplitude (peak current in pA/pF, KO: -2.6±0.2, WT: -3.1±0.3), shape and position of I-V relationship (peak at 5-10 mV), steady-state-activation (V50 for half maximal activation in mV, KO: -9.2±0.4, WT: -9.4±0.5) or steady-state-inactivation (V50 for half maximal inactivation in mV, KO: -24.8±0.3, WT: -22.7±0.4) were observed (n=8). Taken together, ATF-1 knockout mice show a prolongation of action potential duration which cannot be explained by alterations of L-type calcium channels. In conclusion, our data suggest ATF-1 as an important regulator of cardiomyocyte function possibly implicated in the regulation of cardiac ion channels (Supported by the DFG).

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250

The effects of cariporide on intracellular pH regulation in isolated rat ventricular myocytes measured by multichannel fluorescence detectors using two excitation wavelengths

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Cariporide (Hoe 642) is a Na+/H+ exchange (NHE) inhibitor exhibiting cardioprotective properties during ischaemia and reperfuion in animal hearts. Conventionally, the effect of NHE-blockade is measured by fluorescence microscopy of a single myocyte using intracellular fluoroprobes as indicators of the intracellular pH (pHi) and an overflow equipment for the manipulation of pHi. Experimental results of superior quality can only be elaborated if ratio methods are used. Fluorescence microscopy of single heart cells and the need for ratio methods hinder the multiplication of samples, though assaying a manifold of tests in parallel and just in time is thought to be an necessary prerequisite to determine concentration-effect curves (CEC) or to compare different drugs, especially taking into account that isolated heart cells rapidly change their physiological characteristics under storage conditions. That is why we developed 12-channel fluorescence detectors, each channel completely equipped with two different wavelengths (505 and 435 nm) for excitation, with silicon photodetectors and operational amplifiers, the digitized signals of which are sent to a conventional personal computer to be displayed and stored. The excitation and detection units match the 96-well formatted 12-well strips (Greiner 701071), which are used for the fixation of myocytes, 10000 per well, using Poly-L-Lysin (Sigma P4707). Enzymatically isolated heart cells were prepared according to Haworth et al. (1982, Cell Calcium 10, 57) and preloaded with 2 µM BCECF acetoxy methyl ester (Molecular Probes, B1150). Acid load of heart cells was achieved by a 10 min exposure of cells to a salt solution supplemented with 10 mM NH4Cl for 10 min and the subsequent exchange by standard physiological salt solution buffered with 20 mM HEPES in the absence of bicarbonate (Loh et al., 1996, Br J Pharmacol 118, 1905) using an overflow apparatus. Cariporide significantly inhibited the recovery of pHi to control levels. CEC for cariporide were determined resulting in an pEC50 of 6.7 +/- 0.1 (n=12) matching the EC50-values of 0.08 µM ofr hNHE1 reported by Kleemann and Weichert (Idrugs 2: 1009, 1999).

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251

Engineered skeletal muscle: A simple model to identify statin-myotoxicity

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A well known adverse effect of statin treatment is skeletal myotoxicity. We aimed at bioengineering skeletal muscle as an in vitro test-bed to identify myotoxic statin effects. Methods: Engineered skeletal muscles (ESMs) were generated from adult Wistar rat skeletal myoblasts (1.25x106/ESM) with or without rat fibroblasts (0.4x106/ESM), matrigel (10%) and collagen (0.6 mg/ESM). Reconstitution-mixtures were poured into circular moulds yielding ring-shaped constructs after 5 days. ESMs were cultured for another 10 days on phasic stretch devices and treated with increasing concentrations of cerivastatin, atorvastatin, and pravastatin or DMSO (0.02%) as control for the last 5 culture days. Force of contraction was measured under isometric conditions in Tyrode's solution (1.8 mM calcium, 37 °C). Constructs were weighed and prepared for immune fluorescence (IF)-, hematoxylin&eosin (HE)-staining or Western blotting (WB). Results: ESMs made solely from skeletal myoblasts were soft and developed little force. Addition of fibroblasts into the ESM-reconstitution mixture yielded constructs with higher rigidity (Young's modulus: 4.4±0.5 vs. 1.0±0.03 kPa; n=5; p<0.05), appreciable force of contraction (0.66±0.1 vs. 0.13±0.04 mN at 2.5 Hz; n=5; p<0.01), and differentiated muscle bundles (actin-IF and HE). High stimulation frequencies elicited tetanic contractions with maximal force at 40 Hz (1.3±0.2 mN; n=5). Carbachol (1 µM) induced a reversible block of muscle contraction indicating the presence and functionality of nicotinergic acetylcholine receptors. Statin treatment caused a concentration dependent decrease in force of contraction which was most pronounced in cerivastatin (EC50 = 8 nM) and pravastatin (EC50 = 17 nM) but less obvious in atorvastatin (EC50 = 400 nM) treated ESMs (n=4/group and concentration). Interestingly, only cerivastatin increased ESM-weight 2-fold (p<0.05) indicating "tissue oedema". Contractile failure was paralleled by sarcomere breakdown (IF and WB: sarcomeric actin). Conclusion: We developed ESM as a test-bed to screen for statin-induced myotoxicity. Importantly, we found that cerivastatin and pravastatin induce a myotoxic effect on ESM at clinically relevant drug concentrations suggesting that ESM may be an appropriate model to study the mechanism of myotoxicity.

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252

Heart failure and fibrosis in mice with cardiac-specific overexpression of methyl-CpG- binding protein 2 (MeCP2)

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Regulation of gene expression by histone acetylation/deacetylation plays a basic role in the development and progression of cardiac hypertrophy and failure. Gene transcription can be silenced by histone deacetylation and binding of methyl-CpG-binding proteins to methylated promoter areas. In the present study, we analyzed expression of MeCP2 in the heart during the development of hypertrophy and failure and investigated its cardiac function in a transgenic mouse model. Expression of MeCP2 was determined in wild-type (WT) and α2ABC-adrenoceptor-deficient (α2ABCKO) mice after induction of cardiac hypertrophy by transverse aortic constriction (TAC). In WT mice, TAC increased ventricle/body weight ratio by 154±12% as compared to sham-operated mice after 8 weeks. In WT TAC mice, cardiac MeCP2 mRNA levels were decreased by 92±5% as compared to the sham group (p<0.01). Chronic elevation of circulating catecholamines in α2ABCKO mice caused a similar decrease in cardiac MeCP2 mRNA levels to 27±15 % of WT controls (+27% ventricle/body weight ratio). In order to test if MeCP2 expression affects cardiac myocyte growth, rat neonatal cardiac myocytes were cotransfected with GFP and MeCP2 or empty vector. Cardiac myocyte areas increased from 467±26 µm2 at baseline to 637±44 µm2 after phenylephrine treatment and to 755±40 µm2 after phenylephrine plus MeCP2 transfection (n=59-69 myocytes per group, p<0.05). To further investigate the cardiac function of MeCP2, transgenic mice expressing MeCP2 under control of the α-MHC gene promoter were generated. Unexpectedly, all MeCP2 transgenic founder mice were lost at 6-8 weeks of age due to severe cardiomyopathy. These mice were characterized by cardiac myocyte hypertrophy, severe fibrosis, pulmonary edema and pleural effusion. These results demonstrate that expression of MeCP2 is downregulated during heart failure and cardiac-specific expression in transgenic mice induces a severe postnatal cardiomyopathy. Thus, in addition to histone deacetylases, MeCP2 may play an important role in the control of gene expression during the development of cardiac hypertrophy and failure.

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253

A novel modification of ERK1/2 results in hypertrophy specific signaling

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The extracellular regulated kinases ERK1/2 play an important role in cardiac hypertrophy. In the heart, only Gq-mediated ERK1/2 activation results in cardiomyocyte hypertrophy even though the G-proteins Gq and Gi both activate ERK1/2 via their Gβγ subunits. The underlying mechanism of this differential ERK1/2-activation is unknown.Here we show a novel type of ERK1/2-activation, which mediates cardiac hypertrophy. A novel modification of ERK2 is initiated by direct interaction with Gβγ subunits when Gβγ subunits are released from Gq (but not Gi). This modification caused hypertrophy of isolated cardiomyocytes and converted non-hypertrophic into hypertrophic stimuli. The mechanism was also observed in hypertrophied murine and failing human hearts. Taken together, this so far unknown modification of ERK2 seems to result in hypertrophy specific signaling of ERK1/2.

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254

Expression of ABCG1 and ABCA1 in patients with dilative cardiomyopathie

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Transport proteins of the ABC-type are involved in a wide range of biological processes. While members like P-gp or BCRP have been shown to play important roles in physiological barriers, proteins like ABCA1 or ABCG1 are involved in the transport of lipids, especially for cholesterol. In this context, both proteins are involved within the pathogenesis of atherosclerosis, and polymorphisms inside the ABCA1 gene have been identified as risk factors for the development of ischemic heart disease. However, less much is known about the expression of ABCA1 and ABCG1 in other heart diseases like dilative cardiomyopathy. We therefore studied the cardiac expression of both transporters in an animal model of enterovirus CVB3 induced inflammatory cardiomyopathy. In addition we compared the cardiac ABCG1 and A1 expression in patients suffering from dilative cardiomyopathy (DCM) in dependence on the enterovirus diagnostic.Using real-time PCR, we could measure cardiac expression of both proteins and found a reduced expression 4d after CVB3 infection (time of max. virus load) for ABCG1. After 8 (max. inflammation), 12 and 28d however, the expression of ABCA1 (and ABCG1) was significantly enhanced to 168% (261%), 195% (275%) and 167% (282%) compared to uninfected animals. Similar results were obtained in human heart samples, here we studied the ABCA1 and ABCG1 mRNA expression in DCM patients, which are positive (n=15) or negative (n=13) for enterovirus and we could detect a significantly enhanced ABCG1 and ABCA1 expression in virus-positive patients.Taken together this data indicate a disease dependent regulation of cardiac ABCA1 and ABCG1 expression. Moreover, processes in response to viral infections (e.g. inflammation) also affect the expression of these transporters.

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255

Overexpression of the cardiac Na+/Ca2+ exchanger causes ventricular arrhythmia in transgenic mice

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Systolic Ca2+ removal via the Na+/Ca2+ Exchanger (NCX) generates an inward current (INCX). It has been suggested that INCX could trigger afterdepolarizations resulting in ventricular arrhythmias (VT). In this case, pharmacological inhibition of NCX could offer a new strategy in the treatment of VT. To study NCX mediated proarrhythmia, we used mice with heart-directed threefold NCX expression and increased INCX current. NCX-overexpressor mice (Tg; 12 weeks) exhibited mild left ventricular (LV) hypertrophy with normal echocardiographic cardiac function (LV mass: Tg 98±6, WT 91±5 mg; n=5 per group). Monophasic APs recorded in Langendorff perfused whole hearts subjected to AV nodal block, were prolonged at 70% and 90% repolarization in Tg (n=8) vs. WT (n=3) during pacing at 100 and 150ms cycle lengths (90% repolarization at 100 ms cycle length: Tg: 32±5ms; WT 17±2ms; p<0.05). 8/10 Tg hearts developed VT (n=7) and/or late phase III afterdepolarizations (n=1). None of three WT hearts developed arrhythmias, consistent with the usual incidence of spontaneous VT in wildtype mice (0-20%, Kirchhof et al Circulation 2006). Using the patch clamp method, APs and ionic currents were measured in myocytes isolated from Tg and WT hearts. APs were prolonged in Tg (APD90%: Tg: 160±19ms, n=20; WT: 88±10ms, n=19; p<0.05) while resting membrane potential (Tg: 69.0±0.8mV, WT: 68.1±0.8mV) and AP amplitude (Tg: 107.6±2.6mV, WT: 111.9±2.9mV) were similar. Since a prolongation of AP duration and proneness to arrhythmia can also result from reduced K+ carried transient outward current (Ito) or altered L-Type Ca2+ current (ICa), we measured Ito and ICa in Tg vs. WT. No differences in ICa amplitude were observed between (Tg: 10.8±0.8pA/pF, n=17) and WT (9.7±0.7pA/pF, n=14). Peak Ito was increased in Tg (at +60 mV: Tg: 40.5±6.0pA/pF, n=20; WT: 25.4±3.8pA/pF; n=20; p<0.05). We conclude that enhanced activity of NCX prolongs the ventricular action potential, induces afterdepolariztaions and promotes VT. This would be especially relevant in the setting of heart failure, where an increased expression of NCX has been described. Pharmacological NCX antagonists may be a promising tool to prevent and treat VT.

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256

Tonic inhibition of cardiac L-type calcium channel activity by the inhibitory G protein Giα3

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The classical, acute cholinergic inhibiton of cardiac L-type calcium channel activity is mediated by Giα2 (Nagata et al., Circ Res. 2000;87:903-9). In contrast, we found evidence that chronic inhibition of L- type calcium current seems not to depend on Giα2, but on another pertussis toxin (PTX)-sensitive G protein, such as Giα3. (Foerster et al., Proc Natl Acad Sci U S A. 2003;100:14475-80). Here, we directly examine this hypothesis, by investigating mice lacking Giα3 using whole-cell and single-channel electrophysiology in isolated cardiomycytes.The peak whole-cell calcium current density was enhanced in Giα3 knockout mice (-13.4±0.6 pA/pF, n= 15), compared with C57Bl6 wildtype controls (-10.1±0.8 pA/pF, n=17, p < 0.05). A slight leftward shift by -3mV in the voltage-dependence of steady-state inactivation hints at structural or regulatory events mediating this change, rather than a simple increase in channel number. Confirmingly, single-channel activity is significantly enhanced in myocytes from Giα3 knockout mice. This translates into peak ensemble average currents of -54.7±7.8fA/channel (n=13) in Giα3 knockout, and -48.1±7.7fA/channel (n=12) in wildtype controls, respectively (barium currents, measured at + 20 mV). As expected, PTX pretreatment (1.5µg/ml, 3 h, 37°C) abolished the difference between wildtype (n=7) and knockout (n=9) calcium

current density. Taken together, the inhibitory G protein Gi3 exerts a slight but significant reduction of basal cardiac calcium channel activity. This subtype-selective pathway likely explains the chronic suppression of calcium currents observed in mice with transgenic overexpression of the β2-adrenergic receptor. (Heubach et al., Brit J Pharmacol 2001;133:73-82, Foerster et al., Proc Natl Acad Sci U S A. 2003;100:14475-80.).
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257

Mechanisms underlying cardiac L-type Ca2+ channel dysfunction and expression in diabetic mouse heart
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Diabetic cardiomyopathy is regarded as an entity characterized by impaired diastolic function and little or absent ventricular hypertrophy. In murine hearts of a model of obese type 2 diabetes (db/db mice showing a leptin-receptor dysfunction) we found decreased ventricular expression of the L-type Ca2+ channel pore protein Cav1.2, reduced whole-cell Ca2+ current density, but unchanged single-channel activity (Pereira et al., Diabetes 2006;55:608-15). We now test whether our findings in the db/db model are typical and general features of diabetes mellitus. Furthermore we aim to elucidate the underlying metabolic mechanisms. Thus we have screened ventricular Cav1.2 protein expression by Western-blot analysis in mouse models showing metabolic phenotypes typically observed in diabetes. In lean, hyperglycemic mice lacking insulin receptor substrate 2 (IRS-2-/-) Cav1.2 expression was unchanged (106±20%, n=7, n.s.). Wildtype mice on high fat diet (obese and hyperglycemic) revealed a trend towards Cav1.2 decrease (87±34%, n=6, n.s.). A marked decrease of ventricular Cav1.2 was found in normoglycemic, dyslipidemic female mice lacking the insulin receptor in skeletal muscle (MIRKO) (58±12%, n=4, p<0.05) and in another mouse model of obese type 2 diabetes expressing a dysfunctional leptin (ob/ob), respectively (60±22%, n=8, p<0.05). In cardiomyocytes of these ob/ob mice, the reduced Cav1.2 expression is numerically consistent with a slight decrease of Ca2+ current density, and a small increase of single L-type Ca2+ channel activity. In conclusion Ca2+ channel dysregulation is found in some but not all mouse models resembling features of type 2 diabetes. Increased levels of plasma triglycerides, but not hyperglycemia or obesity, is the factor common for all mouse models with cardiac Ca2+ channel remodeling identified so far.
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258

Cardiac-specific overexpression of mutated calsequestrin leads to impaired Ca2+ handling
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Calsequestrin (CSQ) is the main Ca2+ binding protein in the lumen of the junctional sarcoplasmic reticulum (SR). It binds Ca2+ with high capacity and may play an important role in regulating the Ca2+ release of the SR. Mutations in the cardiac CSQ gene (CASQ2) have been linked to catecholaminergic polymorphic ventricular tachycardia (CPVT). The CASQ2 mutation at amino acid residue 307 (CSQD307H) seems to be involved in sudden cardiac death in young patients. To investigate whether this mutation leads to changes in cellular Ca2+ handling, we generated transgenic mice with heart-directed overexpression of either mutated (CSQD307H) or native CSQ (CSQNT) and studied L-type Ca2+ currents and Ca2+ transients in isolated cardiac myocytes. L-type Ca2+ currents were measured by voltage clamp and Ca2+ transients by field stimulation on different cardiac myocytes. The basal ICa density was decreased in CSQD307H (5.1±0.7 pA/pF, n=15) and CSQNT (5.3±0.3 pA/pF, n=15 compared to wild type cardiac myocytes (12.9±1.8 pA/pF, n=9, p<0.05). Additionally, the inactivation of ICa was slowed in CSQD307H (12.6±1.3 ms) and CSQNT (13.5±1 ms) compared to wild type littermates (7.1±0.2 ms, p<0.05). The peak amplitude of Ca2+ transients was increased by 71% in CSQD307H (n=35) but reduced by 62% in CSQNT (n=23) compared to wild type cells (n=35, p<0.05). Moreover, CSQD307H (n=35) showed a reduced tinactivation of Ca2+ transients (by 21%) compared to wild type myocytes (p<0.05). In contrast, tinactivation was increased by 117% in CSQNT compared to wild type cells (n=35, p<0.05 vs. WT, p<0.05 vs. CSQD307H). The diastolic Ca2+ level was increased by 16% in CSQD307H compared to wild type (p<0.05). In contrast, the diastolic Ca2+ level was not different between CSQNT and wild type cells. This study suggests that altered L-type Ca2+ currents and Ca2+ transients in CSQD307H may contribute to a cytosolic Ca2+ overload leading to triggered arrhythmias in patients with the CPVT phenotype.
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259

HCN3 contributes to the ventricular action potential waveform in the mouse heart
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The hyperpolarization-activated cation current Ih plays a key role in cardiac pacemaking and the spontaneous beating of the heart. Four genes (HCN1 to 4) encode the channels underlying this current. In the heart, many details are known for the HCN subtypes 1, 2 and 4. By contrast, almost nothing is known about cardiac HCN3. To characterize the functional role of HCN3 in the murine heart we generated HCN3 deficient mice (HCN3-KO) and screened them by telemetric ECG recordings. These experiments revealed a prolongation of the T-wave in HCN3-KO mice by 16%, suggesting a change in ventricular repolarization. In all mammals, ventricular repolarization proceeds in a synchronized wave advancing from the base of the heart to its apex and from epicardial to endocardial myocardium. For the orderly sequence of repolarization to occur, endocardial myocytes must have longer action potential durations than epicardial myocytes. Thus, the T-wave is generated when endocardial and epicardial action potentials start to separate from each other during repolarization. To clarify the underlying mechanism of the prolonged T-wave, we isolated epicardial and endocardial murine cardiomyocytes from wild type and HCN3 deficient mice and recorded action potentials by the whole cell patch clamp method. These experiments showed that the epicardial action potential duration (APD) of HCN3-KO mice (APD90: 19.0 ± 1.9 ms; n=28) was consistently shorter than the APD of wild type mice (APD90: 31.5 ± 4.5 ms; n=22). The epicardial action potentials of WT and HCN3-KO mice separate in the late repolarization phase during a membrane potential window between the reversal potential of Ih and the resting membrane potential. These data suggest that HCN3 significantly contributes to the ventricular action potential waveform by generating a depolarizing inward current during repolarization. Given the importance of a coordinated repolarization process for the arrhythmia-free heart, future experiments will show, if changes in HCN3 expression levels will lead to cardiac disease.
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260

Conditional deletion of potential pacemaker channels in the mouse heart via the Cre/loxP system using a SAN/AV node specific Cre mouse
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Various ion channels are thought to underlay the pacemaker activity in the sinoatrial node (SAN) of the mammalian heart. Some of them, like HCN4, are almost exclusively expressed in the cardiac conduction system, strongly suggesting a sinoatrial specific function. Others, such as the L-type calcium channel CaV1.2 and the intracellular calcium release channel Ryr2 are found in all cardiac cells where they are known to be involved in regulating calcium homeostasis and in the excitation-contraction coupling process but may also participate in pacemaking. Cardiac-specific knockout mice using the Cre/loxP system are a useful tool to elucidate the roles of those channels in the heart. However, their function in the adult sinoatrial node has been elusive to date because (i) both global and heart-specific knockout resulted in embryonal lethality and (ii) a heart-specific knockout using various inducible Cre mice lead to deletion of the genes in cells of both the working myocardium and the cardiac conduction system. This resulted, e.g. in the case of Ryr2 deletion, in a fulminant atrial-/ventricular phenotype possibly obscuring the effect of sinoatrial Ryr2-deletion. We now generated three mouse lines in which we used the SAN/AV-node specific, tamoxifen-inducible HCN4-KiT mouse to delete HCN4, Cav1.2 or Ryr2 specifically in the cardiac conduction system. Since all three lines survive the conditional knockout of their targeted gene, this approach now allows us to assess the physiological role of HCN4, Cav1.2 and Ryr2 in the sinoatrial and AV-node.
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261

The role of PP5 in cardiac contractility
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Protein phosphatase 5 (PP5), a Ser/Thr phosphatase, is expressed in all mammalian tissues examined, including the heart, but its (patho)physiological role is unknown. Transgenic (TG) mice with heart-specific overexpression of PP5 were generated, and cardiac contractility of wildtype (WT) and TG mice was analyzed with the in vitro technique of the isolated work performing heart preparation (working heart). Parameters of myocardial function were analyzed under basal conditions and under β-adrenergic stimulation by isoproterenol (10-6 M). At the end of the experiment, hearts were shock frozen in liquid nitrogen and prepared for Western blotting. Under basal conditions, hearts of TG mice developed increased diastolic (WT: 6±0.9; TG: 10±1.7 mmHg) and decreased systolic pressure (WT: 86±2.6; TG: 71±3.0 mmHg) in the left ventricle (p<0.05). Moreover, relaxation times were prolonged (TF 90%: WT: 46±2.2; TG: 60±3.3 msec) and heart rate was lower in hearts of TG mice (WT: 330±15.0; TG: 286±7.6 BPM) compared to WT mice (p<0.05). Maximum and minimum of the first derivative of left ventricular pressure were lower in hearts of TG mice (-dp/dt: WT: -2134±135; TG: -1448±144 mmHg/sec; +dp/dt: WT: 3453±188; TG: 2210±223 mmHg/sec; p<0.05). After stimulation with isoproterenol, the differences between WT and TG mice in relaxation time (TF 90%: WT: 31±1.5; TG: 45±3.0 msec), heart rate (WT: 411±9.0; TG: 362±12.1 BPM) and first derivative of the pressure of left ventricle (-dp/dt: WT: -4511±385; TG: -3102±334 mmHg/sec; +dp/dt: WT: 7909±591; TG: 5898±489 mmHg/sec) were preserved (p<0.05). Western blot analyses revealed a decreased phosphorylation of phospholamban (PLB) at Ser16 in hearts from TG mice compared to WT mice under basal conditions (p<0.05), but not after β-adrenergic stimulation. Therefore, PP5 may be involved in the regulation of basal cardiac contractility.
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262

In vivo effects of dimethyl sulfoxide on infarct size in hearts of male Wistar rats
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Dimethyl sulfoxide (DMSO) was first synthesized 1867 and is the most common solvent used for application of water insoluble substances in pharmacological research. The substance serves further medical purposes as an enhancer of skin permeability in topical applications, as a preservative in (tissue) cryoprotection, and as an antiphlogistic agent. As early as in the Sixties of the last century, direct cardiac effects of DMSO such as alterations of myocardial contractility have been described. To test the hypothesis that DMSO may induce ‘pharmacological preconditioning’, we examined the effects of various doses of DMSO on myocardial infarct size in an in vivo rat model. DMSO was

applied at the beginning of experimental procedures dosed at 1, 10 or 100 µl/kg in 1 ml/kg i.v. injections, followed by a 15 min waiting period (n=5-6 per group). Myocardial infarction was induced by 30 min occlusion of the left coronary artery and subsequent 180 min of reperfusion. Infarct size was determined based on 2,3,5-triphenyltetrazolium chloride (TTC). The obtained infarct sizes amounted to 57.7±3.3 % of the area at risk (AAR) in the control group, DMSO treated animals revealed averaged infarct sizes of 48.2±9.4 % (1 µl/kg), 28.0±4.2 % (10 µl/kg) and 31.1±2.3 % (100 µl/kg), respectively. Notably, application of DMSO in a dose of 10 µl/kg prior to the reperfusion phase resulted in significantly increased infarct sizes (55.5±1.2 % in control vs 64.8±2.1 % after application of DMSO). Thus, in our in vivo model DMSO was capable of inducing ‘pharmacolocial preconditioning’ with a striking potency. This action may frequently constitute a confounding factor in cardiovascular research.

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263

Enhanced cardiac function and calcium cycling alterations in ATF1 knockout mice

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The transcriptional regulation by the CREB/CREM/ATF1 family factors represents a fundamental mechanism of a cAMP-dependent gene control possibly involved in the pathogenesis of heart failure. Activating transcription factor 1 (ATF1) is expressed ubiquitously, however, his role in the heart remains unknown. To elucidate the cardiac consequences of ATF1 ablation, we studied cardiac function in adult mice with a global inactivation of ATF1 (ATF-/-) in comparison to wild-type controls (ATF+/+) at an age of 14-18 weeks. Measurements of calcium transients on isolated adult cardiomyocytes were performed. The cell shortening of ATF1-/- cells was increased (in %, mean±SEM. ATF1-/-, 4.02±0.7*; ATF1+/+, 1.64±0.3; n=15; *P<0.05 vs. ATF1+/+), and the calcium transient (CaT) amplitude was significantly higher (ATF-/-, 0.120±0.006*;ATF+/+, 0.076±0.018; n=15). The time to 90% CaT decay was prolonged (CaTT90% in ms. ATF1-/-, 224±19*; ATF1+/+, 145±8; n=15) as well as the time to 90% relaxation (TtR90% in ms. ATF1-/-, 458±16*; ATF1+/+, 404±18; n=15). Furthermore, we assessed left ventricular (LV) function through LV catheterization. Analysis of obtained pressure-volume loops showed increased maximal LV pressure (LVPmax), ejection fraction (EF), maximal speed of contraction and relxation (dP/dtmax, dP/dtmin) and cardiac output (CO) of ATF1-/- mice.

	ATF1+/+ (n=17)	ATF1-/- (n=17)
Heart rate [bpm]	366±13.8	381±17.7
LVPmax [mmHg]	79.9±1.8	91.7±2.9*
EF [%]	49.7±1.1	64.2±2.9*
dP/dtmax [mmHg/s]	4980±200	6053±336*
dP/dtmin [mmHg/s]	-5423±230	-6306±310*
CO [µl/min]	1551±144	2203±239*

Hence, the presence of ATF1 is essential for a regular cardiomyocyte function, and its ablation leads to increased cardiac contractility and altered calcium cycling in the cardiomyocytes.

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264

Probing cardiomyocyte transcription factor interactions in living cells in real-time

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Cardiomyocyte hypertrophy is a key event that promotes the induction and progression of heart failure. A wide range of signaling pathways has been described to mediate cardiomyocyte hypertrophy through activation of pro-hypertrophic cardiomyocyte transcription factors. Interestingly, the various hypertrophic signaling pathways converge on a very limited number of transcrition factors, still being able to induce different hypertrophic phenotypes. Thus, our hypothesis is that specific transcription factor complexes form upon distinct stimuli. In addition, different stimuli may account for specific kinetic patterns of complex formation. We therefore aim to systematically screen for the formation of potential transcription factor complexes and to analyse the time-course of these events in living cardiomyocytes. To this end we make use of the FRET-technique (fluorescence resonance energy transfer), allowing to measure interactions between two proteins in real-time. To measure intermolecular FRET, constructs containing either a cyan or yellow fluorescent protein at the C-terminus of each transcription factor were generated. To systematically screen for specific transcription factor interactions, we are currently studying a set of cardiomyocyte transcription factors and repressors including Nfatc2, Nfatc3, Nfatc4, Mef2c, Egr-1, Nab1, Gata4, Srf and Creb. Ultimately, this study aims to decipher stimulus-specific activation and complex formation of pro-hypertrophic transcription factors in living cardiomyocytes in real time.

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265

Increased expression of PP2A protects against lipopolysaccharide-induced stress

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Heart-specific overexpression of the catalytic subunit of the protein phosphatase 2A (PP2A) in transgenic mice under control of the α-myosin heavy chain promotor causes hypertrophy and impaired contractility. The hearts of these mice also suffer from ventricular dilatation and a diminished response to β-adrenergic stimulation. The time of decay of the Ca2+-transient and time of relaxation of the cardiac myocytes were prolonged compared to cardiac myocytes from wild type mice. To further investigate the importance of PP2A in the heart, transgenic (n=10) and wild type (n=10) mice were exposed to lipopolysaccharide (LPS) -induced stress. These mice were intraperitoneally injected at day one with 30 µg of LPS per g body weight. After three days, successful induction of LPS-induced sepsis was indicated by presence of greatly enhanced interleukine-6 in the plasma. Moreover, Northern blots revealed LPS-induced expression of tumor necrosis factor alpha. The hearts were isolated and perfused in a work-performing modus. We noted a decrease in systolic left ventricular pressure in the hearts of LPS treated wild type mice compared to the hearts of untreated wild type mice (72.5 ± 2.3 mmHg vs. 43.2 ± 3.8 mmHg), whereas the hearts of transgenic mice showed no decrease in systolic pressure after LPS-treatment (68.4 ± 4.2 mmHg vs. 61.0 ± 2.1 mmHg). Additionally, maximum rate of left ventricular pressure development was reduced in LPS treated wild type mice (2453 ± 256 mmHg/s in untreated wild type vs. 1076 ± 269 mmHg/s in LPS-treated wild type) but not in transgenic mice (2111 ± 279 mmHg/s in untreated mice vs. 1806 ± 280 mmHg/s in LPS treated mice). Likewise, maximum rate of pressure decline was reduced in LPS treated wild type mice (-1387 ± 163 mmHg/s in untreated mice vs. -576 ± 128 mmHg/s in LPS treated mice) but not in transgenic mice (-1105 ± 175 mmHg/s in untreated mice vs. -1024 ± 182 mmHg/s in LPS treated mice). These data indicate that the increased phosphatase activity was accompanied by impaired contractility, but protected against LPS-induced stress.

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266

AAV-9-mediated shRNA-delivery to knockdown phosphatase-inhibitor-1 in the murine heart

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Inhibitor-1 (I-1) acts as a conditional amplifier of β-adrenergic signalling downstream of PKA by inhibiting type-1 phosphatases (PP1). I-1 is like β1-adrenoceptors (-AR) downregulated in failing hearts and presumably contributes to protection against excessive catecholamine levels usually occurring in patients with heart failure. Genetic ablation of I-1 in mice reduced the vulnerability to acute and chronic pathological b-adrenergic signaling. The long term aim of this project is to study the impact of recombinant adeno-associated viral (AAV)-mediated RNA interference inhibition of I-1 and β1-AR (as a “positive control”) in the setting of preexisting experimental heart failure. The AAV serotype 9 has been chosen for its proven selectivity for the myocardium after intravenous injection.In a pilot experiment, AAV-9 encoding EGFP under control of a cardiac MLC2-promoter fused to a CMV-enhancer was delivered into mice via tail vein injection (3*1011 viral genomes/mouse). Two weeks after injection, transduction efficiency (monitored by EGFP-epifluorescence with confocal microscopy) amounted to ~30% of cardiomyocytes in the left ventricle with homogeneous transmural distribution. Other tissues including skeletal muscle and liver were GFP-negative.For the generation of specific shRNA-AAV-9, a 23-nt oligonucleotide specific for I-1 or b1-AR (palindromic sequences forming double-stranded molecules including a loop) and corresponding scrambled sequences were first introduced into a vector containing the PolIII-H1-RNA promoter. The H1-I-1/β1-AR-shRNA cassette was subcloned into the final pAAV-CMV-GFP plasmid. The AAV-H1-I-1/b1-AR-shRNA constructs were tested in stable cell-lines overexpressing either I-1 or b1-AR. 48 h after liposome-based transfection EGFP-epifluorescence was detected in >80% of the cells. RT-PCR and Western blotting revealed ~70% reduction in I-1/β1-AR mRNA (after 48 h) and ~50% reduction in I-1 protein abundance (after 72 h) compared to the corresponding scrambled controls. From these 4 constructs large-scale viral productions were successfully generated by cotransfection with the helper plasmid pDF2 and purified using iodixanol gradients. Replicative titers were determined by ELISA-based measurement of capsid numbers. These viruses are currently tested in isolated cardiomyocytes and in vivo in mice for potential toxicity and efficiency.

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267

Signaling pathways involved in thromboxane A2-receptor mediated tonic contraction of porcine coronary smooth muscle

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Previously, we could demonstrate M3-receptor-mediated tonic contraction of porcine coronary smooth muscle (CSM) in the absence of L-VOCC-dependent Ca2+-influx: store-operated Ca2+entry (SOCE) was the main source of activator Ca2+. Moreover, SOCE obviously stimulated arachidonic acid (AA) signalling pathways. Here, we studied by means of specific inhibitors the signal transduction pathways underlying the thromboxane A2-receptor-mediated (10 µM U-46619) tonic contraction of endothelium denuded CSM strips in the absence of L-VOCC-dependent Ca2+influx (1 µM verapamil). In contrast to M3-receptor-dependent contraction, inhibitors of AA metabolism, i. e. 4-BPB (PLA2 inhibition), RHC-80267 (DAG-lipase inhibition), and indomethacin (COX inhibition) had no significant effects on the TXA2-receptor-mediated contractile response. Likewise, inhibition of SOCE by SKF 96365 reduced contraction only to 74±6% of control (p<0.05). However, Y-27635 (5 µM), an inhibitor of Rho associated kinase, abolished the response and GF109203X (10 µM), an inhibitor of PKC, reduced the response to 30±8% (p<0.001). Conclusion: 1) store-operated Ca2+entry makes only a small contribution to TXA2-receptor-mediated tonic contraction of CSM and, hence, PLA2 or other AA signaling pathways are not involved significantly. 2) activation of PKC and, in particular, activation of Rho-associated kinase are essential signaling steps, eventually leading to sensitization of the contractile machinery to small the amount of activator Ca2+delivered by Ca2+-release from SR.

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268

Effects of estradiol and medroxyprogesterone acetate on atherosclerosis and thrombosis in ApoE-/- mice

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The role of estradiol in initiation and progression of atherosclerosis is still a matter of debate. Therefore the aim of this study was to elucidate the effects of endogenous and exogenously substituted estradiol on atherosclerosis, endothelial function and risk for thrombosis as well as the effects of medroxyprogesterone acetate (MPA) alone or in combination with 17-β-estradiol (17-β-E) in ApoE-/- mice. For this purpose ApoE-/- mice were bilaterally ovariectomized (OVX) and substituted with placebo, 17-β-E (1.1 µg/d), MPA (27.7 µg/d) or a combination of 17-β-E and MPA. In addition these animals were fed a high-cholesterol diet. Plaque-scores in whole aortas were significantly reduced by 30 % in ovariectomized animals compared with control animals. The analysis of endothelial function of thoracic aortic segments from ovariectomized animals showed a significantly improved acetylcholine (Ach)-induced vasorelaxation (EC50-values Ach: OVX, 34.75 nM ± 7.3 nM vs. control, 71.61 nM ± 16.0 nM, n = 5 - 7). The effect on plaque-score was reversed by treatment with 17-β-E. Compared to placebo-treated animals MPA showed a significant reduction of plaque-scores whereas 17-β-E + MPA were similar to placebo-animals. To determine the risk for thrombosis the time to stable occlusion of the right carotid artery was measured in the model of photochemically induced thrombosis. In ovariectomized animals compared with control animals as well as in placebo- and 17-β-E-substituted animals no differences occurred. In contrast MPA-treated animals and those with a combined substitution of 17-β-E and MPA showed significantly shortened times to occlusion. These data support the conclusion that endogenous estradiol mediates pro-atherogenic effects without any influence on thrombosis in ApoE-/- mice fed a high-cholesterol diet while MPA has anti-atherogenic but pro-thrombotic effects in these animals.

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269

Release of 5-hytroxytryptamine from heart and isolated adult mice ventricular cardiomyocytes

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5-Hydroxytryptaymine (5-HT) exerts many effects in the cardiovascular system. At least 14 different 5-HT4 receptors subtypes are known. Usually platelets are regarded as the main source of 5-HT in the heart. Recent studies revealed that tryptophan hydroxylase and L-amino acid decarboxylase mRNA are expressed in cardaic tissues and cardiomyocytes (CM) of several species. These findings give a hunch that 5-HT secreted from CM could be implicated in the cardiac pathophysiology of the 5-HT. Here, we determined the level of 5-HT and its precursor tryptophan (TPH) in heart tissue as well as in isolated adult mice ventricular CM and tissue from an neuroendocrine tumor. 5-HT and TPH were detected by high performance liquid chromatography with fluorescence detection. The hearts were perfused using a saline buffer for 15 minutes to remove remaining blood, homogenized in liquid nitrogen and subsequently the level of 5-HT and TPH determined. The adult mice heart tissue contained 4.8 ± 1.1 ng 5-HT respectively 149.8 ± 31.5 ng tryptophan per gram wet weight (n=12). For comparison, the plasma level of 5-HT was determined as 292.2 ± 70.0 ng per ml. Immediately after isolation of the adult mice cardiomyocytes the content of 5-HT in the sedimented CM pellet amounted to 1.4 ± 1.0 ng (n=3), meanwhile the content of TPH amounted to 587.2 ± 25.6 ng per mg protein. (n=3). Following resuspension of the pellets the CM were incubated 10 min at 37°C. After this, the CM pellet contained barely 0.2 ± 0.2 ng 5-HT respectively 43.8 ± 3.9 ng TPH per mg protein (n=6). By contrast, in the supernatant 5-HT was amounted to 5.5 ± 1.3 ng and TPH to 412.5 ± 106.0 ng per mg protein (n=6). Using immunohistochemistry, no specific signal for 5-HT could be detected. However, this is apparently due to sensitivity differences in the methods employed: immunohistochemistry revealed clear signals for 5-HT in tumor tissue and in the very same tissue 5-HT levels a hundred fold larger than in the heart were detected by HPLC. Thus, the cardiomyocytes provide a local source of 5-HT in the heart. The amount of cardiomyocyte-derived 5-HT may mediate several cardiac affects by serotonin 5-HT4 receptors.

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270

Biochemical characterization of ventricular cardiomyocytes from A2A adenosine receptor overexpressing mice

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Adenosine is a protective metabolite that is generated during stress responses in the heart. A2A-adenosine receptor (A2A-AR) up-regulation has been found in failing human hearts, whereas A1-AR and A3-AR expression remained unchanged. To elucidate the (patho)physiological role of increased A2A-AR expression, transgenic mice with cardiac specific overexpression of A2A-AR (TG) were generated. The aim of this study was to investigate the function of overexpressed A2A-AR in the ventricles. The A2A-AR is positively coupled to adenylyl cyclase (AC) via Gs. Thus, we comparatively measured the cAMP content and the phosphorylation of phospholamban at serine 16 (PLB-S16) in isolated ventricular cardiomyocytes from TG and wildtype mice (WT) after the specific stimulation of A2A-AR with CGS21680 in the presence of the A1-AR antagonist DPCPX (1 µM). The maximum effect on cAMP accumulation was reached at 1 µM of the A2A-AR agonist CGS21680 and amounted to 163.9 ± 10.1 % of the control (n = 5). The phosphorylation of PLB-S16 induced by 1 µM CGS21680 was comparable with the effect of 1 µM isoprenaline. The effects on cAMP as well as on the phosphorylation of PLB-S16 were completely abolished by the specific A2A-AR antagonist ZM241385. On the other hand, no effect of A2A-AR stimulation on cAMP content was observed in WT, whereas the phosphorylation of PLB-S16 was slightly increased at 1 µM CGS21680, the highest concentration studied. In contrast, the effect of the β-adrenoceptor agonist isoprenaline (1 µM) on cAMP content as well as on the phosphorylation of PLB-S16 was attenuated in TG.In summary, our results show that overexpressed A2A-AR are functionally coupled to AC. Moreover, the attenuated effects of isoprenaline indicate possible heterologous desensitization of the β-adrenergic system by overexpressed A2A-AR. This might be of relevance also in failing human hearts. (Supported by the BfArM)

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271

Functional role of thrombin on embryonic stem cell-derived cardiomyocytes

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Under appropriate culture conditions, embryonic stem cells are capable of differentiating into different somatic cell tissues. We used the transgenic ES cell line CGR8, expressing enhanced green fluorescent protein (EGFP) under the control of the a-myosin heavy chain (a-MHC) promoter, to investigate the effects of thrombin on cardiomyogenesis. First, we analysed by RT-PCR the mRNA expression levels of all three thrombin receptors (protease activated receptor, PAR)-1, PAR-3 and PAR-4 at different stages of EB development. The highest expression level of PAR1 was observed at differentiation day 6 with a slow reduction up to day 10. Therefore, these cells were stimulated with thrombin (1 U/ml) at different time points and beating frequency of EBs was measured between days 9 to 14. Thrombin induced an increase of beating frequency in comparison with control EBs, indicating a functional role of thrombin during cardiomyocyte development. However, fluorescence analysis of EGFP expression in both, thrombin-stimulated and control cells, revealed an equal amount of cardiac cells. It is concluded that thrombin is an important functional regulator of early cardiomyocytes differentiated from ES cells without influencing cardiomyogenesis.

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272

Keyrole of hyaluronan syntase-3 in control of vascular smooth muscle cell phenotype

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Atherosclerosis is characterized by the thickening of aterial intima due to the accumulation of cells and extracellular matrix (ECM), including hyaluronan. HA is a large polysaccharide, which is synthesized at the plasma membrane by three HA-synthase isoformes (Has1-3). However, the functional significance of the individual HAS-isoenzymes is unknown. Therefore the aim of the present study was to investigate function and regulation of HAS3 in human arterial smooth muscle cells (VSMC). VSMC were derived from coronary arteries and used between passage 4-9. VSMCs were transduced with lentiviral constructs encoding human has-3, to investigate the impact of HAS3 dependent HA synthesis VSMC phenotype. In addition, HAS3-si RNA was employed to decrease endogenous HAS3 expression and HA secretion. HAS3 overexpression induced a 3-4 fold increase in HA-secretion, a shift to lower molecular weight HA, enlarged pericellular HA coat formation, induced microvillus-like cell surface protrusions and increased proliferation of VSMC. HAS3-siRNA reduced HAS3-mRNA levels to 50 ± 22%, reduced FCS induced migration and proliferation of SMC. IL-1β and TNFα, classical proinflammatory and also pro-atherogenic cytokines, induced HA secretion, while interleukin 10, considered anti-inflammatory cytokine, had no effect on HA secretion. IL-1β increased pericellular formation of HA coats and HA secretion into the cell culture medium in VSMCs. However only HAS3-mRNA is specifically induced by IL-1β and TNFα. Dexamethasone and the NFkB inhibitor Bay11-7082 prevented HA synthesis and HAS3-mRNA induction by IL-1β, suggesting IL-1β induces HA and HAS3 via NFkB activation in VSMC. Furthermore activated U937 cells augment HAS3mRNA in SMCs. This HAS3 induction, is significantly reduced by a combination of neutralising antibody against IL-1β and TNFα. These results suggest that HAS3 dependent HA-synthesis promotes human VSMC proliferation and migration and that HA synthesis by HAS3 occurs in response to pro-atherogenic cytokines, which could be of relevance for neointimal hyperplasia during vascular pathologies such as atherosclerosis and stent restenosis.

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273

MicroRNA-21 controls activity of cardiac fibroblasts through regulation of the ERK/MAPkinase

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MicroRNAs are short, endogenous, single-stranded RNA molecules, which post-transcriptionally regulate gene expression. Specific binding to messenger-RNAs leads to their degradation or to inhibition of their translation. By now, more than 500 human genes, encoding for microRNAs have been identified.Using microRNA-arrays we identified several microRNAs that are differentially expressed in heart failure. The deregulation of these microRNAs was validated by Northern blot analysis in different stages of heart failure, in mouse models as well as in human myocardium. miR-21 displayed a notably early and strong up-regulation , which was three-fold ($p < 0.01$) higher in modest and six-fold higher ($p < 0.01$) in manifest heart failure. By computational analysis of the miR-21 promoter region and luciferase based reporter assays of miR-21 promoter fragments we were able to identify two essential transcription factor binding-sites that are involved in the transcription of miR-21 in the heart. We could show that CREB and SRF seem to be responsible for the strong up-regulation of miR-21 during cardiac hypertrophy and heart failure. Blocking miR-21

expression by antisense “morpholinos” in zebrafish led to a severe cardiac phenotype with pericardial edema and massive impairment of cardiac function.When we analyzed different fractions of primary cardiac cells, we found miR-21 to be mainly expressed in non-myocytes. We then transfected primary cardiomyocytes and cardiac fibroblasts with synthetic miR-21 precursors or inhibitors. While we observed no phenotype in transfected cardiomyocytes, over-expression of miR-21 led to an activation of cardiac fibroblasts as determined by isoleucin incorporation. On the other hand we observed strongly enhanced apoptosis of cardiac fibroblasts (100%, $p<0.05$) after inhibition of endogenous miR-21 with synthetic inhibitors. As one target of miR-21 we could identify an inhibitor of the MAPkinase signaling pathway. Over-expression of miR-21 induced activation of the ERK-MAPkinase pathway.These data are the first to suggest regulation of the MAPkinase signaling pathway by a microRNA. This mechanism seems to take place mainly in cardiac fibroblasts and could be a critical factor of cardiac fibrosis.

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274

Development of an in vitro assay system for screening for protein-phosphatase-inhibitor-1 inhibitory compounds

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Inhibitor-1 (I-1) acts as a conditional amplifier of β-adrenergic signalling downstream of PKA by inhibiting type-1 phosphatases (PP1) only in its PKA-phosphorylated form. I-1 is like β1-adrenoceptors downregulated in failing hearts and presumably contributes to protection against excessive catecholamine levels in heart failure. Disruption of the I-1 gene in mice resulted in partial protection from catecholamine induced lethal arrhythmias and hypertrophy. This suggests that pharmacological I-1 blockade may represent an attractive new therapeutic strategy. Here, we aimed to develop a reliable cost-efficient in vitro assay system to screen a library for chemical compounds that have inhibitory effects on I-1 activity. Therefore, we established a colorimetric protein phosphatase inhibition system (recombinant PP1 mediated dephosphorylation of the chromogenic substrate p-nitrophenyl phosphate [pNPP]) in a single assay in a multi-well-format. This assay was first characterized with the chemical phosphatase inhibitor cantharidin and the PP1-protein-inhibitor-2 (I-2), which is in contrast to I-1 constitutively active. Cantharidin and I-2 concentration-dependently inhibitet PP1 activity with expected IC50 of 550 nM and 2.8 nM, respectively, emphasizing the principle feasibility of this system. However, PKA-phosphorylated (activated) recombinant I-1 (up to 3 µM) exerted no significant phosphatase inhibition. Western blotting with phospho-specific antibodies showed that I-1 phosphorylation completely disappeared during 5 min incubation with the PP1, indicating dephosphorylation and thus deactivation of I-1. This issue was solved by using thiophosphorylation of I-1 with ATP-g-S. Thiophosphorylated I-1 inhibited PP1 activity with an IC50 of ~400 nM. Thus an assay has been established that consists of PP1, thiophosphorylated I-1 and pNPP and should allow the identification of compounds that inhibit I-1´s inhibitory effect on PP1.

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275

Anti-inflammatory and antiatherogenic effects of the NF-κB inhibitor acetyl-11-keto-β-boswellic acid in LPS-challenged apoE-/- mice

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The classical risk factors for atherosclerosis do not fully explain the incidence of the disease and there is increasing recognition of an important link between inflammation and atherosclerosis. We have now studied the effects of acetyl-11-keto-β-boswellic acid (AKβBA), a natural inhibitor of the proinflammatory transcription factor NF-κB, on the development of atherosclerotic lesions in apolipoprotein E-deficient (apoE-/-) mice. Atherosclerotic lesions were induced by weekly i.p. injection of lipopolysaccharide (LPS, 50 µg) in apoE-/- mice. LPS alone increased atherosclerotic lesion size by two-fold and treatment with AKβBA significantly reduced it by ≈50 %. Moreover, the activity of NF-κB was also reduced in the atherosclerotic plaques of LPS-injected apoE-/-mice treated with AKβBA. As a consequence, AKβBA treatment led to a significant down-regulation of several NF-κB-dependent genes such as MCP-1, MCP-3, IL-1α, MIP-2, VEGF and TF. By contrast, AKβBA did not affect the plasma concentrations of triglycerides, total cholesterol, anti-oxidized LDL antibodies and various subsets of lymphocyte-derived cytokines. Moreover, AKβBA potently inhibited the IκB kinase (IKK) activity immunoprecipitated from LPS-stimulated mouse macrophages and mononuclear cells leading to decreased phosphorylation of IκBα and inhibition of p65/NF-κB activation. Comparable AKβBA-mediated inhibition was also observed in LPS-stimulated human macrophages. Thus, the inhibition of NF-κB activity by distinct ingredients of plant resins from species of the Boswellia family might represent an alternative for classical medicine treatments for chronic inflammatory diseases such as atherosclerosis. Supported by the DFG, SFB 451.

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276

Heme oxygenase-1 and simvastatin: molecular mechanisms and in vivo effects

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The antioxidant and cytoprotective enzyme heme oxygenase-1 (HO-1) is a target of the HMG CoA reductase inhibitor, simvastatin. Previous studies have shown that simvastatin induces HO-1 protein and mRNA levels in different cell types. Previously, we explored the underlying mechanisms by studying the effect of different kinase inhibitors on simvastatin-induced HO-1 expression. We found that the p38 mitogen-activated protein kinase (MAPK) inhibitor, SB 203580, and the phosphatidylinositol 3-kinase (PI3K) inhibitor, LY 294002, antagonized the stimulating actions of simvastatin on HO-1 mRNA production and promoter activity in cultured endothelial cells as measured by Northern blot and reporter gene activity, respectively. In addition to transcriptional activation, simvastatin prolonged the half-life of HO-1 mRNA as detected by Northern blot. Simvastatin-dependent mRNA stabilization was reduced in the presence of LY 29004. In contrast, SB 203580 showed no effect under these conditions. Therefore, to determine if these effect occur in vivo, transgenic mice containing the full-length HO-1 promoter driving expression of the reporter gene luciferase, received a single bolus of simvastatin (100 mg/kg body weight) by oral gavage, and HO-1 promoter activity was measured by in vivo bioluminescence imaging (BLI). We found an increase in HO-1 promoter activity in the upper abdomen and the thorax, confirmed by elevated HO-1 mRNA levels in lung and heart tissues. In addition, increases in lung, liver and heart HO activities were also observed. In summary, we demonstrate that HO-1 induction by simvastatin occurs via transcriptional activation and mRNA stabilization, and leads to subsequent increases in HO activity. This pathway of HO-1 induction may explain, at least in part, the antioxidant, anti-inflammatory and anti-atherogenic benefits of simvastatin observed in patients with cardiovascular diseases.

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277

Heparin-nitroxide - a novel substance for antioxidative protection of vascular extracellular space and EPR/MR imaging

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Extracellular oxidative stress plays a pathophysiological role in ischemia/reperfusion and inflammation-related cardiovascular diseases. Nitroxides (NR) are stable radicals known for its excellent antioxidants and paramagnetic properties and used as cytoprotectors and EPR/MR imaging probes. Among the limitations of NR are short in vivo half-life time (minutes) and interference with intracellular metabolism. Heparin is a widely used anticoagulant, and it is known to have binding sites on the endothelial cell surface and vascular extracellular matrix. For targeted delivery of NR to these strategically important sites, we have synthesized a number of heparin-NR conjugates (Hep-NRs). Results: Hep-NRs exert antioxidant and paramagnetic properties comparable to the reference compound, TEMPOL. Hep-NRs bind with high affinity to the vascular tissue, competing for the same binding sites with non-modified heparin and myeloperoxidase. In contrast to TEMPOL, our Hep-NRs do not penetrate cells and persist in vascular tissue for hours (in vivo L-band EPR evidence). Conclusion: Novel Hep-NRs might be useful for the selective protection of vascular extracellular space in ischemia/reperfusion, as well as, for EPR imaging of blood vessels.

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278

Function of cAMP response element modulator (CREM) in aortic smooth muscle cells

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Development of vascular proliferative diseases is associated with modifications in the physiology of vascular smooth muscle cells (VSMCs). Transcription factors CREB/CREM (cAMP responsive-element binding protein and modulator) are supposed to play an important role in the regulation of VSMCs cell differentiation, proliferation and apoptosis via the binding to a cAMP-response element (CRE) of respective target genes. Here we studied mice with a global inactivation of CREM (KO) and wild-type controls (WT) to elucidate the role of CREM in VSMCs.Transfection of VSMCs with a CRE controlled luciferase reporter construct showed a higher activation of CRE-controlled luciferase activity in KO vs. WT VSMCs under control conditions (mean±SEM; in % of WT. KO, n=18, 180±25*; WT, n=11, 100±28. *$P<0.05$ vs. WT) and after stimulation with 10µM forskolin, an activator of the adenylyl cyclase (in % of non-stimulated WT control. KO, n=8, 1260±220*; WT, n=8, 670±127. *$P<0.05$ vs. WT). Interestingly, this difference in CRE-mediated transcriptional activity was not accompanied by changes in aortic contractility. Furthermore, detailed histochemical analysis of VSMCs in aortic sections revealed no significant difference in the basal proliferation rate (number of Ki-67 positive cells per 10000 cells. KO, n=3, 5.2±2.8; WT, n=3, 6.7±1.2) as well as in the apoptosis rate (number of TUNEL-positive cells per 10000 cells. KO, n=3, 2.0±2.0; WT, n=3, 2.9±1.8). In conclusion, elevated CRE-mediated transcriptional activity in KO mice is coherent with the general view of CREM as a repressor of transcriptional function. Hence, the increased transcriptional activation did not alter contractility of aortic vessels as well as the apoptosis and proliferation rate of VSMCs, CREM mediated gene expression does not seem to play a major role under normal physiological conditions. Further investigations are necessary to clarify the role of CREM in the vasculature particularly in the regulation of gene expression under long term stimulation and in pathological state. (Supported by the IZKF Münster).

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279

Protection against cold preservation injury by catecholamines is determined by redox activity and lipophilicity

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During previous studies, catecholamines and related compounds proved to be effective against cold preservation injury. Based on the differences in potency of protection amongst these substances, we hypothesized that variation of the molecular structure may yield more efficacious compounds. Modification of dopamine by an alkanoyl group greatly improved efficacy of protection. This was associated with increased cellular uptake and loss of hemodynamic action. Further variation revealed that only compounds bearing two hydroxy groups in ortho or para position at the benzene nucleus, i.e. strong reductants, were protective and efficiency correlated with logP value. Although a strong reducing capacity was required for protection, general antioxidants, i.e. N-acetyl cysteine and ascorbate, did not efficiently prevent cold preservation injury. Similarly, inhibition of NADPH oxidase had no effect on cell survival during hypothermia. Although dopamine increases heme oxygenase 1 levels, its protective effect is not mediated by this enzyme as shown by knockdown experiments. We demonstrate that protection against preservation injury by catecholamines is exclusively mediated by two structural entities, i.e. strong reducing capacity and sufficient lipophilicity.

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280

Glucose utilization, body weight and blood pressure are differentially regulated in various rat strains after high caloric feeding regimes

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The Metabolic Syndrome has become a problem of epidemic proportions in Western countries and is accompanied by an increased incidence of cardiovascular mortality. Thus, we aimed to generate a rat model, that indeed mimics the symptoms hypertension, diabetes and obesity, but whose insulin resistance, hyperphagia and hyperleptinemia is not the result of genetic modifications in the leptin receptor as seen in other rat strains (ZDF-rat, Koletsky SHR-OB). The leptin and insulin resistance of ZDF rats or SHR-OB is only partially comparable to that of patients featuring the Metabolic Syndrome. This derogates the value of the mentioned rats strains for experimental investigations. Sprague Dawley rats (SDR) or SHR were fed for 12 weeks with a formula (16.4 kJ/g) or a cafeteria diet (19.7±0.4 kJ/g). Controls received standard diet (11.7 kJ/g). Compared to standard feeding, the bodyweight was increased in SDR and SHR only when rats were fed with the cafeteria diet. The increase in bodyweight could be attributed to an enhanced energy intake and less to an impairment of their physical activity (measured with the InfraMot System, TSE). In parallel to bodyweight, plasma leptin and adiponectin were heightened, confirming the increase of the fat mass. In cafeteria diet fed SHR, baseline glucose (13%), insulin (67%), C-peptid (19%) and HOMA-Index (50%) were increased, whereas glucagon (27%) was reduced. This prediabetic condition was confirmed by an oral glucose tolerance test (1g glucose/kg bodyweight), since plasma insulin was markedly enhanced (60%) for maintaining plasma glucose within the control range. Such an impaired glucose homeostasis was partially observed in cafeteria-diet fed SDR, and by no means in SDR after feeding them with the formula diet. Selectively in cafeteria diet fed SHR, blood pressure (15 mmHg) and heart rate (50 bpm) were higher than in appropriate controls. In summary, when SHR were fed with a high caloric cafeteria diet they develop hypertension, obesity and an impaired glucose homeostasis. Thus enables us for further pharmacological and molecular studies using an animal model that features the Metabolic Syndrome similar to the clinical situation.

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281

Cardiovascular response to norepinephrine transporter inhibition depends on menstrual cycle phase

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Objective. A previous study suggested that cardiac norepinephrine transporter (NET) function may be reduced in women compared with men. The gender difference may be mediated through female sex hormones. Design and Methods. We studied 16 healthy eumenorrhoeic women (25±1 yrs). They underwent testing on four occasions during two consecutive menstrual cycles in the early follicular (EF, day 5±0 after onset of menstruation) and mid-luteal phase (ML, day 22±0). In a randomized, cross-over, and double blind fashion, subjects ingested 8 mg of the selective NET inhibitor reboxetine or matching placebo. We monitored heart rate, finger blood pressure, brachial blood pressure, and thoracic bioimpedance. Results. Venous estradiol concentrations were 150±10 pmol/l in EF and 450±33 pmol/l in MF (p<.001); venous progesterone concentrations were 2±0 nmol/l in EF and 36±3 nmol/l in MF (p<.001). With NET inhibition, supine blood pressure increased 14±0.4/9.8±0.3 mmHg in EF and 12±0.4/7.5±0.2 mmHg in MF (p<.01 / <.001). In contrast, the tachycardic response to NET inhibition was reduced in EF compared with ML (2.0±0.5 vs. 9.9±0.6 bpm, p<.001). During EF, NET inhibition increased cardiac output 14±1 % while peripheral resistance remained unchanged. In ML, cardiac output increased 19±1 % and peripheral resistance decreased 9±1 % (p<.01 for both compared to EF). Conclusions. Female sex hormones appear to alter the hemodynamic response to systemic NET inhibition in human subjects. The phenomenon may be explained by an effect of female sex hormones on NET function, on compensatory cardiovascular responses, or both mechanisms combined.

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282

Comparative pharmacology of chemically distinct NADPH oxidase inhibitors

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Excessive reactive oxygen species (ROS) may contribute to several pathologies including cardiovascular diseases. With respect to molecular sources of such oxidative stress, NADPH oxidases are prime candidates as they represent the only known enzyme family that has ROS as its sole product. However, the level of evidence for a role of NADPH oxidases in oxidative stress and disease is low and solely confined to genetic mouse models and a range of enzyme inhibitors. Here we identify the triazolo pyrimidine backbone, represented by VAS3947, as a pharmacophore fulfilling all the key criteria of specificity and efficacy in different cells, tissues and ROS assays. None of the tested cells (CaCo-2, HL60, A7r5) were specific for one of the catalytic subunits, NOX1-4, of NADPH oxidases. Diphenylene iodonium (DPI) inhibited also other flavoproteins, such as xanthine oxidase (XOD) and nitric oxide synthase (NOS) making it an inappropriate tool to define the role of NADPH oxidases in a given system. Apocynin interfered with L-012 based chemiluminescence and varied considerably in efficacy and potency, similar to (4-2-amino-ethyl)-benzolsulfonyl-fluoride hydrochloride (AEBSF). In contrast, the triazolo pyrimidine, VAS3947, consistently inhibited NADPH oxidase activity in cell-free as well as cell- and tissue-based assays with low micromolar potency and no direct interference, neither with ROS nor with other ROS forming or flavin containing enzymes. Applying this tool to a rat model of spontaneous hypertension, dihydroethidium (DHE) detectable ROS formation appeared to be largely due to NADPH oxidase-derived and superoxide dismutase quenchable superoxide but not due to XOD or NOS activity. These data caution on the wide use of apocynin, DPI and AEBSF in proof-of-concept studies on the role of NADPH oxidases in oxidative signaling and stress and suggest that triazolo pyrimidines are the first compounds to fill this experimental and potential therapeutic gap.

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283

Human respiratory epithelial models for pulmonary drug delivery and toxicity studies

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The respiratory tract is currently considered as an alternative to gastrointestinal or dermal drug delivery systems, due to the fast absorption and the absence of first pass metabolism characteristic of this tissue's physiology. For the drug development process, scarcity of good and reliable in vitro models (as an alternative for animal testing) remains a long standing problemFor this purpose Calu-3 culture conditions, such as culture medium, trypsinization, freezing/thawing and transport buffer selection were standardized in our laboratory. Effect of a popular solvent, dimethylsulfoxide (DMSO), and pH (6.6-8.0), were also analysed. We also performed preliminary transport experiments with the marker compounds Fluorescein, Mannitol, Rhodamine123, Digoxin and Propranolol. In addition, already inhalative administered compounds were also analysed for their transport across the monolayer. The obtained results indicate that the Calu-3 cell line is a potential model for drug absorption studies, high throughput screening of toxic compounds, drugs and pre-formulations in the upper respiratory tract.pAEpC have been previously isolated and characterized in our lab, and have also proven to be a good model. They consist of a mixture of type I and type II pneumocyte-like cells, and exhibit good barrier properties Improvement of the model is currently taking place, regarding time of duration of the cell culture, cryopreservation or extent of cell differentiation, to set up a system that could be routinely used for drug transport studies. A list of compounds (such as local drugs delivered to the lungs, consumer products, chemicals, efflux substrates, small APIs, etc) is going to be tested with both cell types in terms of permeability across this barrier, in order to obtain experimental data that would allow the comparison of these results with in vivo data and with other epithelial cell models, and thus to check the validity of these systems as a drug permeability test model. Financial support by EU Project-No.: MRTN-CT-2004-512229 "Pathogenesis of pulmonary disease" PULMONET is gratefully acknowledged.

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284

Modulation of cytokine release by LPS and drugs in human lung epithelial cell lines

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Lung epithelial cells have been shown to release pro- or anti-inflammatory cytokines which possibly interact with β2-adrenergic receptors and thus may determine the severity of airway disorders. In the tracheobronchial epithelial cell lines 16HBE14o--, Calu-3 and also in a type-II-pneumocyte cell line, termed A549, we have investigated the release pattern of certain cytokines (IL-1β, IL-8, TNF-α) in response to different stimuli. Cells were treated with lipopolysaccharide (LPS: E.coli), either in combination with different sera (fetal calf serum [FCS], newborn calf serum [KS] and normal human serum [NHS]), or the cytokines IL-1β, IFN-γ and TNF-α or drugs such as β2-agonists (e.g. isoproterenol, clenbuterol) or dexamethasone. The levels of IL-1β, IL-8 and TNF-α release were determined using ELISA. The IL-8 secretion was increased concentration-dependently upon LPS exposure in media supplemented with each serum in alveolar A549-cells, whereas the cytokine level remained at basal value in Calu-3- and 16HBE14o- cells. In all three cell lines, the release of IL-1β and TNF-α could be stimulated by LPS only in the presence of NHS. After stimulation with TNF-α, IL-8 production increased markedly in all three cell types (A549: IL-1β [1000x], TNF-α [250x]; Calu-3: IL-1β [11x], TNF-α [10x]; 16HBE14o-: IL-1β [7x], TNF-α [22x]). IFN-γ had no effect on the secretion of IL-1β, IL-8 and TNF-α. The IL-8-secretion of both bronchial epithelial cell lines Calu-3 and 16HBE14o- increased in response to isoproterenol and clenbuterol, whereas the IL-8-level of the alveolar cell line A549 remained at basal values. Dexamethasone reduced the IL-8- and IL-1β-production in all three cell lines. TNF-α release was not affected by either β2-agonists or dexamethasone. This study

indicates that LPS in the presence of different sera as well as bronchodilators similarly enhance cytokine release in lung epithelial cells.
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285

Upregulation of the HO-1 gene by proton pump inhibitors - molecular mechanisms and functinal consequences
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Increasing evidence suggests the involvement of reactive oxygen species (ROS) in the pathogenesis of gastro-duodenal diseases. Proton pump inhibitors (PPIs) are potent anti-secretory drugs. In addition to acid suppression, PPIs provide mucosal protection by mechanisms not yet fully understood. The aim of this study was to investigate whether induction of the anti-oxidant enzyme, heme oxygenase-1 (HO-1), accounts for the pleiotropic effects of PPIs. Lansoprazole and omeprazole enhanced the activity of the HO-1 promoter in stably transfected NIH3T3-HO-1-luc cells. In addition, HO-1 mRNA induction in endothelial cells was abrogated in the presence of actinomycin D and cycloheximide suggesting transcriptional regulation and involvement of protein de novo synthesis. These observed increases HO-1 gene expression at both the transcriptional and translational levels by lansoprazole and omeprazole occurred also in macrophages and were associated with a stimulation of HO enzyme activity. Pretreatment with superoxide dismutase (SOD) did not affect PPI-mediated HO-1 induction, precluding a mediator role of ROS. Preincubation with the phosphatidylinositol-3-kinase (PI3K) inhibitor LY294002 resulted in decreased HO-1 promoter activity and HO-1 mRNA levels. HO-1 induction by PPIs was associated with a reduction of NADPH-dependent free radical formation in macrophages, which are considered to be crucially involved in inflammatory processes leading to gastric injury. Inhibition of free radical formation was abolished in the presence of the HO inhibitor, chromium mesoporphyrin (CrMP), suggesting a causal link between PPI-mediated HO-1 induction and their anti-oxidant actions. In summary, the present results demonstrate that HO-1 is a target of PPIs and mediator of antioxidant actions. Our observations may explain the cytoprotective and anti-inflammatory actions of PPIs that, in addition to acid suppression, contribute to the beneficial effects of these drugs in patients with gastro-duodenal disease.
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286

Effects of the hyaluronsynthase Inhibitor 4-Methylumbelliferone on esophageal squameous cancer cells
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Abundant production of hyaluronic acid (HA) in the vicinity of gastrointestinal cancer cells is a hallmark of tumor development. Hyaluronan accounts for a plethora of malignant properties of cancerous phenotype such as invasive potential, proliferation, metastatic spread and adhesion to stroma tissue. Esophageal cancer is a rare but severe kind of gastrointestinal cancer which is differentiated in adenocarcinoma and squameous cell carcinoma of the esophagus. 4-methylumbelliferone (4MU) is an inhibitor of the hyaluronan synthases (HAS1-3) and is thought to have promising properties as an anticancer agent. We used a squameous cancer cell line (OSC1) expressing HAS3>HAS2>>>HAS1 to evaluate the specific effects of 4-MU on the phenotype of OSC1 and the underlying pathways. OSC1 exhibit a unique and strong expression of microvillus-like structures, which are associated with the hyaluronan matrix. These structures were shown to be involved in cancer cell invasion. 4MU induced resolution of these structures and caused a distinct morphological change in OSC1 which was at least partly mediated by calpain-mediated cleavage of focal adhesion kinase (FAK). These actions subsequently led to a resolution of focal adhesions and a collapse of the cytoskeleton. Moreover, treatment with 4-MU resulted in decreased phosphorylation of ERK1/2 and AKT, two downstream targets of FAK. The effects of 4-MU were not CD44 dependent as shown by use of CD44-blocking antibody. Lentiviral delivered knockdown of HAS3 expression mimicked all effects of 4-MU treatment, showing that the observed effect were dependent on hyaluronan depletion. As a consequence, 4MU inhibited proliferation (58.6 % ± 8.5 %, mean ± SEM, n=5, *p < 0.05) and migration (45 % ± 4 %, mean ± SEM, n=3, *p<0.05) of the cancer cells. In addition, 4MU diminished invasive growth of the cancer cells under co-culture conditions. We conclude that 4-MU inhibits malignant phenotype of OSC1 cells by interfering with cell-HA interactions which might hold potential for clinical application.
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287

Beta-adrenoceptor mediated regulation of the calcium-dependent BK current in detrusor muscle
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Opening of large-conductance Ca2+-activated K+ channels (BKCa) causes hyperpolarization and reduces detrusor muscle tone. Stimulation of β-adrenoceptors (β-AR) was reported to activate BKCa, and block of the channels attenuated β-AR-mediated relaxation in guinea-pig and rat detrusor. However, little is known about the relevance of this putative interaction in other species. Therefore we have studied the effects of BKCa modulators on detrusor relaxation in response to (-)-isoproterenol (Iso) in mouse, pig and man, and directly examined BKCa currents. Pre-contracted mouse and pig detrusor strips (40mM KCl) relaxed in response to Iso, murine muscle being more sensitive than porcine (–logIC50 [M] 8.9±0.1, n=10, versus 8.5±0.1, n=12; p<0.05). Blocking BKCa with iberiotoxin (0.1 µM) or paxilline (10 µM) did not affect the Iso-concentration response curves suggesting that activation of these channels may not contribute to the Iso-induced relaxation of KCl contractions in these species. Next we studied putative Iso effects on BKCa by measuring current (IBK,Ca) in response to clamp steps from –10 to +50 mV in isolated cells. Mean current densities were 89±7, 118±21 and 71±10 pA/pF in pig, man and mouse, respectively. The BKCa activator NS1619 (30 µM) enhanced current by 145%, 129% and 81% in pig, mouse and man, respectively, whereas paxilline (1µM) completely blocked IBK,Ca in all cells. Iso (1µM) increased in IBK,Ca from 60±11 to 75±14 pA/pF, i.e. by 28% (n=12) in mouse, and from 94±18 to 109±12 pA/pF (38%; n=12) in man with not further increase by 10 µM, indicating that maximum BKCa activation in response to β-AR stimulation is less than with NS1619. The Iso effects were inconsistent in porcine cells. The β2-AR-selective blocker ICI118,551 (50nM) did not influence the Iso-induced IBK,Ca increase in man, impaired the Iso response in mouse and completely abolished the paradoxic effect in pig. In conclusion, we could not confirm that block of BKCa channels attenuates β-AR mediated detrusor relaxation in the species studied. Nevertheless we provide evidence, that b-AR stimulation can modulate BKCa channels and that this effect is mediated via β2-AR in pig and mouse.
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288

Muscarinic receptor expression and receptor-mediated contraction in juvenile and adult detrusor
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Urinary bladder function is known to mature during fetal and postnatal development, including changes in neurotransmitter regulation of detrusor contraction. However, only few experimental data are available about muscarinic receptor antagonist function in the urinary bladder from young animals. Here we compare muscarinic receptor-mediated contractions in juvenile and adult porcine detrusor.Urinary bladders from young (8-12 weeks; 12-35kg body weight) and mature pigs (>40 weeks; >100kg) were used. Muscarinic receptor expression was determined by real time PCR and radioligand binding. Detrusor contraction was measured with a force transducer, L-type Ca2+ currents (ICa,L) of detrusor myocytes were recorded with standard voltage clamp technique.The expression of mRNA was similar in juvenile and adult porcine detrusor for M2 (18±5 vs. 16±7 fg/ng, n=8-10) and M3 receptors (39±16 vs. 32±12 fg/ng, n=10-12). The number of [3H]QNB binding sites (Bmax 36±5 vs. 48±6 fmol/mg) and their affinity for the radioligand (KD 7.3±2.3 vs. 8.0±1.9 pmol/l, n=10 each) were not significantly different in juvenile and adult pigs. Carbachol was slightly less potent in juvenile than in adult tissue: pEC50 [M] 5.5±0.1 (n=54/14) versus 5.8±0.1 (n=52/14; p<0.01). Although pEC50 values for carbachol were different, the actual rightward shifts of the concentration-response curves (CRC) in the presence of the M3 antagonist DAU 5884 (1-10nM) were similar resulting in pKB of 9.1±0.2 (juvenile) and 8.6±0.1 (adult). The spasmolytic drug propiverine (0.1-100µM) also shifted the CRC for carbachol to the right and additionally reduced maximum contractions. Potency and efficacy of propiverine were similar in both juvenile and adult tissue. ICa,L amplitudes in were not significantly different: 0.8±0.1 vs. 1.0±0.2 (n=14/6). Propiverine reduced ICa,L in detrusor myocytes with similar potency in both: pIC50 [M] 4.6±0.3 (n=7/3; juvenile) vs. 4.8±0.2 (n=7/3; adult).In conclusion, these data suggest that expression and function of M2 and M3 receptors are similar in detrusor of juvenile and adult pigs. Therefore, similar responses to antimuscarinic compounds could be expected in young and adult patients.
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289

Pharmacological profile of STW 5 opens new options for the treatment of functional gastrointestinal diseases
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Most studies of the mechanisms of action of STW 5 (Iberogast®) conducted until now were focussed on functional dyspepsia. We could show a region specific inhibition of muscular tone in gastric fundus and corpus (1) and an activation of gastric antrum by this herbal fixed combination in the organ bath as well as in clinical studies (2). The aim of the actual studies was to identify further treatment options by assessing the effect of STW 5 in other regions of the gastro-intestinal tract. In vitro studies in human colon preparations and in the human epithelial cell line T84 showed a pro-secretory effect of STW 5. This effect was mediated through epithelial activation of cAMP and Ca-dependent chlorid channels, as well as by the activation of secretomotoneurons of the enteric nerval system. In part this pro-secretory effect was also prostaglandin dependent. Studies of the muscular activity of human colon preparations in vitro showed a significant inhibition of basal tone and of phasic activity by STW 5. This effect was TTX insensitive and therefore myogen. The inhibitory effect was shown in longitudinal as well as in circular muscle. In vitro registration of the lower esophageal shincter of the guinea pig showed a significant tonicising effect of STW 5. Interestingly STW 5 did not influence the pyloric tone. All observed effects were dose dependent (0.256-2.056 mg/ml STW 5) and reversible. These effects of STW 5 open options for the treatment of a number of functional gastro-intestinal disturbances, as intestinal hyposecretion, hyperactive resp. spastic intestine, and atonic oesophageal sphincter. This is of special relevance in motility-related gastro-intestinal diseases, as are irritable bowel syndrome and gastroesophageal reflux.References: 1. Schemann M (2006) Phytomed 13 SV:90-99; 2. Pilichiewicz A (2007) Am J Gastroenterol 102:1-8
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290

Obesity and cancer: possible linkage by bacterial agmatine production

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Obesity may be linked with its well-known associated increased risk of developing colorectal malignancy by the bacteria predominant in the gut and their ability to produce and to release the antiproliferative L-arginine metabolite agmatine. Therefore, ten different bacteria species representing the spectrum of the predominant intestinal bacteria species, were studied for differen-ces in agmatine production and release. Furthermore, we investigated whether human neoplastic colon cancer tissue and the adjacent macroscopically normal tissue differ in agma-tine content and the expression of enzymes involved in agmatine homeostasis. The bacteria studied strongly differ in their agmatine production. Strong producers were Bacter-oides vulgatus, Lactobacillus reuteri, Lactobacillus acidophilus and Veillonella dispar. A medium agmatine synthesis was found in Bacteroides fragilis, Bacteroides theta-iota-omi-kron, Bifidobacterium bifidum and E. coli Nissle. Eggerthella lenta and Enterococcus faecalis produced no or only very low amounts of agmatine. Agmatine content in human colon cancer tissue amounted to about 50 % of that found in adja-cent macroscopically normal tissue. Expression of mRNA encoding arginine decarboxylase and diamine oxidase in neoplastic tissue specimens was by 74% and 50%, respectively, lower than in the adjacent macroscopically normal tissue. Expression of mRNA for ornithine decarb-oxylase was increased by 143%, whereas no significant difference in the expression of agmatinase was observed. In conclusion, the present data provide evidence for an involvement of agmatine homeostasis in regulation of cell proliferation and transformation. Hence, modification of the intestinal micro-biota by dietary intake of bacteria producing high amounts of agmatine or by supporting the living conditions of such bacteria in the gut might be useful in the prevention of tumor-ge-ne-sis. Moreover, taking into account the ability of intestinal tissue and liver to accumulate agmatine after oral administration, intestinal and liver cancer may be treated by oral admi-nistra-tion of agmatine.

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291

Development and pre-validation of an in-vitro method for the predicition of drug-drug interactions and intestinal absorption as a tool for drug candidate selection

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Advances in the combinatorial chemistry and high throughput screening of the compound libraries for pharmacological activity resulted in the increase of the new chemical entities (NCEs). Determination of the biopharmaceutical properties such as intestinal permeability of these NCEs is of sure of crucial importance at the drug development phase. In addition to lack of appropriate biopharmaceutical properties the drug candidates can fail the late clinical studies due to the drug-drug interactions. Most of the drug-drug interactions along with the limitations in intestinal absorption are caused by the efflux system protein such as P-glycoprotein.In this study our aim is to establish and pre-validate an in-vitro bi-directional cell culture model using MDCKII, MDCKII-P-gp and Caco-2. In the pre-validation study propranolol, atenolol and mannitol were used for the investigation of the appropriateness of the Caco-2 cell line in a range of high to low permeability respectively and and apparent permeability coefficient (Papp) clearly decreased with the increasing molecular weight of the markers. Functional expression of the P-gp in this cell line was shown with the bidirectional transport experiments done with Rhodamine 123 which resulted in the asymmetric transport of the substance with a efflux ratio of 16.5. In the experiments with Digoxin (1 µM), the efflux ratio was found to be 21.7 which is decreased to 1.24 and 1.07 in the presence of Cyclosporine A (12 µM) and Verapamil (200 µM) respectively.Substrates of P-gp like; Fexofenadine, Saquinavir, Paclitaxel, Loperamide, Quinidine, Talinolol and Celiprolol along with the specific inhibitor LY-335979 will be used as reference substances in the further experiments in compliance with recently relieved draft FDA Guideline on in-vitro testing of drug-drug interactions. Leading drug candidate selection based on the biopharmaceutical properties and drug-drug interactions using epithelial cell line systems will be facilitated by the results of this study.Financial support from the EU project MEMTRANS is gratefully acknowledged.

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292

Induction of epithelial-mesenchymal transition (EMT) in 5/6 nephrectomised (NX) rats treated with uranylnitrate (UN)

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EMT is a candidate mechanism in progression of fibrotic disorders. It includes disintegration of cell-cell contacts, loss of polarity, and basement membrane degradation. There is only limited in vivo evidence to support EMT in chronic fibrotic kidney disease.Previously we could show that NX induces mainly glomerular sclerosis whereas single UN administration was followed by disseminated interstitial fibrosis. The combination of NX and UN resulted in a stronger damage after 20 weeks. Long term studies should give an inside into the mechanism of renal fibrosis.In 60-day-old rats at a two step operation for NX was done. Three weeks later 0.5 mg UN/100 g b. wt. was administered. Experimental groups: sham operated controls, NX, UN, NX-UN. Renal function and morphology were investigated after 1, 6, and 12 months.There was no chronic effect of UN on renal function, but blood pressure was significantly enhanced 6 month after UN, NX and NX-UN. Body weight and renal functional parameters were seriously impaired by NX. The combination with UN deteriorated distinctly NX induced functional damage.Electron microscopic analysis showed that UN caused significant losses of cell-cell contacts and appearance of intercellular clefts in epithelium barrier of proximal as well as distal tubules. Furthermore in dilated proximal tubules protruded epithelium with focal restricted loss of brush border could be observed. The dilation of basal invaginations and diminution of epithelial contacts to basement membranes is remarkable. More pronounced are changes after combination of NX and UN. The highlight is progressive glomerular reorganization which implicates significantly enhanced thickness of basement membranes and diminution of filtration narrows in the Bowman capsule, respectively. Furthermore affected tubules rimmed by flattened epithelia clearly demonstrated partial losses of basement membranes with concomitant accumulation of extracellular fibres. Although brush border of proximal tubules seems to be unaffected, epithelial cells exhibited remarkable labelling of vesicles with α-SMA moving towards to the outer plasmalemma as a sign of an early stage in EMT of renal tubules.

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293

The intermediate conductance Ca2+-activated K+ channel KCa3.1 contributes to the diuretic and natriuretic effects of furosemide: insights from knockout mice

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In the thick ascending limb of Henle's loop (TALH), K+ channels are important in generating cell membrane potential, regulating cell volume, apical K+ recycling coupled to K+ entry via Na+-K+-2Cl- co-transporters and basolateral K+ recycling coupled to Na+-K+-ATPase activity. The diuretic drug furosemide is widely used to treat patients with acute and chronic heart failure. However, some edematous patients rapidly encounter furosemide resistance, in which diuretic response is diminished or lost. The mechanisms of diurectic resistance are not fully understood. To address whether the intermediate conductance Ca2+-activated K+ channel KCa3.1 is associated with furosemide resistance, wild-type (wt) and KCa3.1-deficient (KCa3.1-/-) mice were subjected to renal clearance and urinary flow experiments. Na+ excretion was determined before and 30 minutes after i.v. furosemide administration (2mg/kg body weight). Baseline urinary flow rate, glomerular filtration rate (GFR), absolute and fractional urinary Na+ or K+ excretion were similar in KCa3.1-/- and wt mice. In both genotypes, furosemide did not significantly affect blood pressure nor GFR, but produced significantly greater diuresis and natriuresis in wt than in KCa3.1-/- mice (7.9±0.8 vs. 4.9±0.9 µl/min; 717±58 vs. 419±99 nmol/min, respectively). Furthermore, significantly lower fractional urinary Na+ excretion was observed in KCa3.1-/- compared to wt mice after furosemide administration (1.7±0.3 vs. 3.2±0.5%, respectively). Thus, our results suggest that KCa3.1 loss-of-function is associated with furosemide resistance, maybe due to alterations of tubular transport rather than renal hemodynamic changes.

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294

Pyrrolidine dithiocarbamate attenuates lipopolysaccharide-induced acute renal failure in mice

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Inhibition of nuclear factor (NF)-kappaB activation has been suggested to provide a useful strategy for the treatment of septic shock. However, the impact of NF-kappaB inhibition on lipopolysacchride (LPS)-induced acute renal failure (ARF) is unclear. ARF is a severe complication of sepsis leading to fluid metabolism alterations despite unchanged or increased plasma vasopressin (AVP) levels. Therefore, we examined the effect of the NF-kappaB inhibitor pyrrolidine dithiocarbamate (PDTC) on LPS-induced downregulation of vasopressin V2 receptors and aquaporine (AQP)-2 channels.LPS injection caused a time- and dose-dependent decrease in V2 receptor and AQP-2 channel mRNA abundance without alterations in plasma AVP levels in mice. The LPS-induced increase in renal TNF-α and IL-1β was attenuated by pretreatment with PDTC. Further, PDTC prevented the LPS-induced downregulation of V2 and AQP-2 expression. Moreover, PDTC attenuated the LPS-induced decrease in glomerular filtration rate (GFR), urine output and urine osmolality. Injection of TNF-α or IL-1β decreased V2 and AQP-2 expression. LPS-induced downregulation of AQP-2 and V2 expression was not affected in mice with deficiencies for TNF-α or IL-1 receptor-1. In addition, LPS decreased adenylyl cyclase (AC) isoform type 5 and 6 mRNA expression, which was attenuated by PDTC. In vitro, in cortical collecting duct cells, cytokines decreased the expression of V2 and AC 6 mRNA abundance and attenuated dDAVP-stimulated cAMP generation. In conclusion, PDTC treatment prevents LPS-induced downregulation of V2, AQP-2, AC 5 and AC 6 expression in the kidney possibly through inhibition of LPS-induced cytokines. In addition, PDTC attenuates LPS-induced acute renal failure.

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295

Lidocaine protein binding alterations after clonidine co-administration to rat tissues. Possible pharmacodynamic implications in heart function measured by echocardiography

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Lidocaine is a membrane stabilisator with amide type local anaesthetic properties even in low doses (4,4-5mg/Kg), which is useful in microsurgical procedure. Clonidine is an α-adrenergic agonist, with more affinity for α2- receptors, indicated for the hypertension treatment, by regulating the production of catecholamines (epinephrine and norepinephrine). Moreover, clonidine is added to local anesthetic infusions, with the intention of improving postoperative analgesia. The aim of the study was to investigate the influence of clonidine co-administration on the binding extent of 14C lidocaine to tissues and its pharmacodynamic effect in heart as well.20 Wistar rats divided into 4 subgroups (n=5) were used; Group I and Group II received lidocaine I.M in the masseter. Groups III and IV received lidocaine as above and clonidine 1mcg/kg. 15 min after the initial treatment ultrasound examination of heart function (heart rate, diameter

of left ventricle in systole and diastole, Ejection fraction, ejection time and posterior wall velocity) was performed in animals of Group I and III. In group II and group IV heart examination took place 30 min after the initial injection. After the echocardiographic examination the animal of all Groups were sacrificed. Specimens were obtained and lidocaine free fraction in serum and tissues estimated via ultrafiltration with beta counter. Statistic analysis was performed via t-test.In conclusion the above results show that the kinetics of lidocaine were altered by clonidine co-administration. Mechanisms related to protein binding alterations may be involved. However, this pharmacokinetic interaction is not followed by changes in pharmacodynamic parameters including those of heart function estimated by echocardiography. Table I : Free fraction of lidocaine in serum and tissues

Drugs	Serum µg/ml	Masseterµg/g (weight of tissue)	Mandible µg/g (weight of tissue)	Heart µg/g (weight of tissue)
I. Lidoc 15min	$(1,506\pm0,237)\cdot10^{-3}$	1,702±0,305	0,535±0,077	1,305±0,206
II. Lidoc 30min	$(1,919\pm0,284)\cdot10^{-3}$	1,142±0,35	0,674±0,196	1,17±0,173
III. Lidoc+clon 15min	$(2,413\pm0,304)\cdot10^{-3}$	7,974±2,59	1,489±0,196	1,544±0,157
IV. Lidoc+clon 30min	$(3,401\pm0,463)\cdot10^{-3}$	18,707±7,593	3,744±0,249	2,034±0,382

Serum I/III and II/IV $p<0,05$ Mandible I/III $p=0,01$ and II/IV $p<0,01$

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296

Intestinal absorption of baclofen: In-situ perfusion studies with its enantiomers in rats

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rac-Baclofen is a centrally acting gamma-aminobutyric acid agonist used against spasticity of spinal origin, and mainly the R-(-)-enantiomer is responsible for the observed effect. It is administered perorally or intrathecally and is mainly excreted unchanged by the kidneys. Studies on blood-brain barrier transport rates yielded no difference between the enantiomers. However, previous studies in man had shown slightly higher plasma levels for the R-(-)-enantiomer after p.o., but not i.v. dosage. The aim of the studies in rats was an elucidation of the intestinal permeability of the two enantiomers and of the absorptive transport inhibition by taurine. Intestinal perfusions were performed in White Wistar rats (n=6 for each compound), and ileum and jejunum were perfused (single-pass perfusion), with an initial perfusate concentration of 0.05 mM for both baclofen enantiomers. Talinolol, gabapentin, rac-baclofen and the fluoro analogon of baclofen (as racemate) were included for comparison or as control. The average effective Peff's in jejunum [and ileum] amounted to 13.11 + 1.12 [13.29 + 1.08] x 10-5 cm/s for the R-(-)-enantiomer and 11.87 + 0.98 [11.2 + 1.33] x 10-5 cm/s for the S-(+)-enantiomer. I.e., no significant difference was detected between the enantiomers of baclofen, while permeability Peff of the fluoro analogon was approximately 25 % higher and Peff of gabapentin was only 50 % of baclofen Peff. Taurine reduced Peff significantly for both baclofen enantiomers, for the fluoro derivative, and gabapentin. Hence, this experimental model does not provide evidence that stereoselectivity in baclofen absorption is the cause for the observed Cmax differences.

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297

Brain penetration of ivermectin in MDR1 and Bcrp knockout mice, and in Mrp2-deficient TR-rats

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Penetration of macrocyclic lactones (ML) such as ivermectin across the blood-brain barrier is highly restricted by the ATP-driven efflux transporter P-glycoprotein (P-gp), coded by the MDR1 gene, and so ML-sensitive receptors in the central nervous system normally are not exposed to high drug concentrations. However, in P-gp lacking mdr1-/- knockout mice and dogs with inherited MDR1-/- mutation, ivermectin brain penetration is highly increased provoking severe neurotoxicosis characterized by apparent depression, ataxia, somnolence, mydriasis, salivation, and tremor. Besides P-gp, other drug efflux carriers, such as the breast cancer resistance protein Bcrp and the multidrug resistance-associated protein Mrp2, are also expressed at the blood-brain barrier. As it was recently shown, that ivermectin in vitro also interacts with those drug efflux carriers we investigated in vivo if in addition to P-gp, Bcrp and Mrp2 also restrict the entry of ivermectin into the brain. We applied radiolabeled ivermectin to mdr1-/- and bcrp-/- knockout mice and wild-type mice as well as to Mrp2-deficient TR- rats and normal Wistar rats, and then analyzed brain penetration and organ distribution. In the mdr1-/- knockout mice we found 36-60 fold higher ivermectin brain concentrations compared to the wild-type mice. In contrast, bcrp-/- knockout mice and TR- rats showed no differences in brain concentrations or organ distribution of ivermectin compared to wild-type mice and normal Wistar rats, respectively. This indicates that P-gp is the only relevant efflux carrier for ivermectin in vivo at the blood-brain barrier.

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298

Circadian changes in intestinal secretion: Drug-drug interactions with the P-glycoprotein (P-gp) substrate talinolol

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P-Glycoprotein (P-gp) is an ATP-dependent efflux pump and particularly responsible for, e.g., intestinal secretion of some drugs. Recently, it was shown that day-vs.-night differences of intestinal secretion in situ in rats exist for the β-adrenoceptor antagonist talinolol (TAL), a P-gp substrate. It was claimed that P-gp may play a role in this process. The aim in the present study was to test the relevance of the in-situ results for in-vivo conditions in studies in Wistar albino rats. TAL (20 mg/kg) was administered to 12h-fasted rats during the day and during the night perorally, either alone (controls) or together with vinblastine (VBL; 0.1 mg/kg i.v.). Areas under the curve (AUC) were calculated via the linear trapezoidal rule (0-8h). TAL AUC values in day experiments (1.70 ±0.94 µg.h/ml) were similar as in night experiments (1.65 ±0.87 µg.h/ml). Vinblastine increased TAL AUCs significantly during the day (2.39 ±1.45 µg.h/ml), however, it decreases TAL AUCs during the night (1.25 ±0.53 µg.h/ml). As opposed to the in-situ studies (Peff: day>night), no day-vs.-night difference in oral availability of TAL was detected in vivo. Moreover, the coadministration of VBL enhanced TAL AUC in vivo during the day, but reduced it at night. We hypothesize that VBL may affect absorptive TAL transport, or that disposition (including intestinal concentrations) differs considerably between day and night.

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299

The role of topoisomerase II alpha and oxidative stress in dexrazoxane- and doxorubicin-induced apoptosis

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The clinical use of the biodioxopiperazine dexrazoxane (DRZ) to prevent anthracycline-induced cardiotoxicity in cancer patients has been limited, in part due to the fears of reduced tumor response. DRZ and anthracyclines may interact via their common target topoisomerase II alpha (TOPO2A). We investigated the effects of DRZ and of the anthracycline doxorubicin (DOX) in a tumor cell line with conditionally regulated expression of TOPO2A. At clinically relevant concentrations of 0.1 µM and 1 µM (DOX) and 50-200µM (DRZ), either drug induced apoptosis accompanied by double-stranded DNA breaks and mediated by the mitochondrial p53 pathway. DOX-induced apoptosis was fully TOPO2A-dependent at 0.1 µM DOX, but only partly dependent at 1 µM DOX. DRZ-induced apoptosis was mostly (>80%) TOPO2A-independent, despite the unexpected finding of a total TOPO2A depletion by DRZ. On the other hand, most apoptosis induced by either drug was inhibited by N-Acetylcysteine (NAC). In conclusion, both DOX and DRZ induce apoptosis largely mediated by oxidative stress and the mitochondrial p53 pathway. The depletion of TOPO2A by DRZ provides an explanation for the previously reported strong effect of addition-sequence and exposure-times on cell killing by combinations of biodioxopiperazines and anthracyclines.

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300

Contribution of cathepsin B to doxorubicin induced apoptosis

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The anthracycline doxorubicin (dox) is one of the most important anticancer agents. A variety of mechanisms are responsible for its antitumoral effect including intercalation into DNA, oxidative stress, inhibition of topoisomerase II with DNA strand breaks as well as activation of the apoptotic cascade. Increasingly, a potential role of proteases of the cathepsin family, especially cathepsin B (cat B), in apoptotic processes is discussed. Dox itself is able to induce the expression of cat B. Thus, it seems possible that this protease is involved in dox induced apoptosis. To test this hypothesis we used the siRNA technology to knock down cat B expression in the cervix carcinoma cell line HeLa by retroviral transfection. After several passages under selection with hygromycin, we analysed cell clones transfected with the retroviral vector including the cat B specific siRNA or with control retroviral vector. By using Real-Time-PCR, immunoblot and ZAMC-assay we could identify a cell clone in which cat B expression was down-regulated to nearly 10%. Expression of other apoptosis relevant cathepsins, namely cathepsin D and L, was not affected. We selected this cell clone to further investigate an involvement of cat B in dox induced apoptosis and determined caspase 3 activity, expression of apoptosis relevant proteins, the mitochondrial membrane potential with TMRE and the cell viability by Alamar Blue. Incubation with 1 µM doxorubicin resulted in a significantly elevation of caspase 3 activity in control transfected cells which was significantly inhibited in cat B suppressed cells. Experiments with the cell permeable cat B inhibitor CA-074Me showed also a decreased caspase 3 activation upon dox treatment. Expression of the pro-apoptotic protein Bax was slightly elevated in control transfectants but dimished in cat B suppressed cells whereas Bcl-2 expression showed no significant regulation. The dox induced loss of mitochondrial membrane potential and cell viability was significantly stronger in control compared to cat B siRNA transfected cells. Furthermore, cleavage of Bid into truncated BID and release of cytochrome C from mitochondria to cytosol after doxorubicin incubation was strongly more appearant in control transfectants than in cat B suppressed cells. In summary, our results indicate a role of cat B in apoptotic cell death induced by dox and helps to understand the apoptotic machinery activated upon dox treatment.

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301

Antiproliferative effect of a novel semisynthetic compound, 3-cinnamoyl-11-keto-β-boswellic acid, in human prostate cancer cells

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Prostate cancer is the most frequently diagnosed malignancy in males in the United States and most other industrialized Western countries. It proceeds from a localized, androgen-dependent stage to an advanced, invasive and metastatic disease associated with the loss of androgen dependence. A variety of antitumor agents have been tested in patients with androgen-independent prostate cancer, yet without any significant prognostic benefit. We have previously shown that the pentacyclic triterpenoid acetyl-11-keto-β-boswellic acid (AKβBA) induces apoptosis in prostate cancer cell lines in vitro and in vivo. In comparison to AKβBA, the deacetylated derivative 11-keto-β-boswellic acid (KβBA) showed a decreased efficacy, pointing to the importance of the ester group

at carbon number 3 of ring A. The aim of the present study was to synthesize a new ester derivative of KβBA with enhanced proapoptotic activity. We have linked cinnamic acid to the hydroxy group at carbon 3 of ring A of KβBA to produce 3-cinnamoyl-11-keto-β-boswellic acid. It is known that cinnamic acid has only a low cytotoxicity in glioblastoma, melanoma and lung carcinoma (IC50 = 1 to 4.5 mM). Consistently, cinnamic acid showed nearly no cytotoxic effect on the androgen-independent prostate cancer cell line PC--3, whereas 3-cinnamoyl-11-keto-β-boswellic acid was highly toxic, potentiating the cytotoxic activity of KβBA by about sevenfold (IC50 = 3.6 μM). Cell cycle analysis of the PC-3 cells treated with 3-cinnamoyl-11-keto-β-boswellic acid revealed that the cells accumulated in G0/G1. This indicates that 3-cinnamoyl-11-keto-β-boswellic acid could serve as a lead compound in the development of novel antitumor therapeutics. Supported by the Deutsche Krebshilfe.
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302

Influence of epidermal morphology and lipid consumption on in vitro percutaneous permeation rates
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In vitro permeation experiments are used to study transdermal drug transport in both pharmacological and toxicological research. Although these experiments take place under reproducible conditions, the results show considerable inter- and intraspecies variances caused by different morphological and biochemical skin characteristics. We investigated different morphological parameters and epidermal lipid composition of four skin types (cattle: udder skin, pig and dog: lateral abdominal skin, rat: abdominal and back skin), which are accepted in vitro permeation models. To detect influences of epidermal morphology and lipid composition on transdermal drug transport, the observed skin characteristics were compared with results of in vitro permeation experiments. Morphological skin parameters were obtained in vertical and horizontal cryostate slices (haematoxilin-eosin stained). Epidermal lipids were detected quantitatively and qualitatively on HPTLC-plates with a special sequence of three solvent systems after chloroform-methanol extraction of heat-separated epidermis. Furthermore, percutaneous permeation experiments took place in Franz-type diffusion cells with flufenamic acid, ibuprofen, indomethacin, and salicylic acid. The present study demonstrates a general ranking for permeation rates (pig < dog < cattle < rat), while each species shows the same ranking for drug permeability (salicylic acid < indomethacin < ibuprofen < flufenamic acid). In addition to reports in the literature, the transdermal drug transport correlates positively with the lipophilicity and negatively with the melting point of the drug. Comparison of the permeation rates with skin morphology and epidermal lipid composition shows a positive correlation of permeation rates with total epidermal lipid content and an inverse correlation with stratum corneum thickness. Contrary to former reports, no direct influence of several epidermal lipid fractions on transdermal drug transport could be detected.
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303

The role of mechanosensitive G-protein-coupled receptors in vascular smooth muscle cells
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Since the nature of the mechanosensors in the signalling cascade leading to myogenic constriction in vascular smooth muscle cells are still elusive, the mechanosensitivity of individual steps in the signalling cascade was examined. In this study we identified Gq-coupled receptors as the mechanosensors in vascular smooth muscle cells. These receptors initiate the classical signalling cascade leading to PLC-dependent activation of the DAG-sensitive TRPC3 and 6 channels. Receptor activation and the subsequent signalling cascade that results in channel activation could be blocked at different levels. The PLC inhibitor U73122 (10 μM) and the non-hydrolysable GTP analogue GDP-β-S (2 mM) in the pipette solution completely abolished the effect of TRPC6 activation by membrane stretch. Notably, antagonists or inverse agonists such as diphenhydramine (100 μM), atropine (100 nM to 1 μM), candesartan (100 nM), losartan (1 μM) and darusentan (10 μM) suppressed activation of corresponding heterologously expressed Gq-coupled receptors such as H1, M5, AT1 and ETA receptors by membrane stretch. Using bioluminescence resonance energy transfer (BRET), β-arrestin was shown to be recruited to the receptor by cell swelling similar to agonist stimulation. Additional expression of AT1 receptor in mechanically unresponsive A7r5 vascular smooth muscle cells from rat thoracic aorta conferred mechanosensitivity on the cells, indicating that this property depends on receptor densitiy. Isolated smooth muscle cells from myogenic renal arteries had a high endogenous AT1 receptor densitiy sufficient for receptor activation in response to membrane stretch. In isolated cerebral and in mouse renal arteries, myogenic tone was profoundly diminished after incubation with the inverse AT1 receptor agonist losartan without involvement of angiotensin II secretion. Simultanous agonist and mechanical stimulation led to a leftward shift of the angiotensin II-concentration response curve. Thus, Gq-coupled receptors may be important components of the mechanosensory complex in vascular smooth muscle cells.
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304

Activated factor X (FXa) alters NADPH oxidase subunit expression in human vascular smooth muscle cells
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Objective: Cardiovascular diseases such as atherosclerosis and diabetes are associated with disturbed coagulation and increased reactive oxygen species (ROS). Thrombin-mediated cleavage of protease-activated receptor-1 (PAR-1) enhances vessel wall thrombogenicity in part via NADPH oxidase-dependent ROS formation. The redox-regulatory effects of PAR-2, which is barely expressed in healthy vessels but is upregulated by vascular injury, have not been defined. We have examined the influence of the PAR-1/PAR-2 ligand FXa on expression of NADPH oxidase subunits (NOX1, NOX4, p47phox) in human vascular smooth muscle cells (VSMC). Methods: VSMC from human saphenous vein (passge 4-8) were synchronised by serum-deprivation (72h) prior to stimulation with FXa (30 nM). Total RNA was extracted, reverse transcribed and mRNA expression determined by quantitative realtime PCR. Results: FXa induced a rapid and transient increase in NOX1 mRNA expression that was maximal at 1h (to 414±105% of control, P<0.05) and restored to control levels by 6h (all n=4). Modest suppression of NOX4 and increases in p47phox expression were observed at 3-6h but were not statistically significant (n=5). Preliminary data indicate that transcriptional changes in NADPH oxidase expression precede maximal FXa-stimulated intracellular ROS generation (dihydroethidium fluorescence) seen at 6h. Given that PAR-1 may be regulated by oxidative stress, we also determined potential positive feedback effects on FXa receptor expression. No change in PAR-1 mRNA was observed but PAR-2 was increased to 163±23% of control at 24h (n=6, P<0.05). Conclusions: FXa may contribute to vascular oxidative stress through modulation of NADPH oxidase expression and ROS generation, followed by late induction of PAR-2. Whether this serves to promote the thrombogenic cycle in reponse to FXa or as a counterregulatory mechanism to limit the mitogenic effects of PAR-1 is currently under investigation.
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305

Behavioural analysis of two stocks of Sprague-Dawley rats obtained from different breeders
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Introduction. Sprague-Dawley is common an out-bred rat strain often used as a background strain for genetic manipulations. Since it is known that stock differences of laboratory animals can result in contradictory outcomes of similarly conducted behavioural tests, we investigated the behaviour of two stocks of Sprague-Dawley rats derived from different breeders (Janvier, France = RjHan:SD, and DIMED Schönwalde, Germany = Shoe:SPRD). Methods. All animals were housed under the same conditions for at least two weeks before the behavioural experiments started. Rats were tested for their anxiety-related behaviour and motor activity in the elevated plus-maze (EPM) and the open field (OF) test. Exploration and habituation learning was investigated in the hole board (HB) task conducted on two consecutive days. Furthermore, food and water intake as well as motor activity were recorded in feeding boxes over one hour. Results. RjHan:SD showed less C-returns in the EPM and an increased number of entries into the centre of the OF indicating a less anxious behaviour. Both stocks habituated to the HB in a comparable manner. Feeding and drinking behaviour was slightly, but not significantly, increased in RjHan:SD, and they showed a higher activity in the feeding boxes than Shoe:SPRD. Conclusions. RjHan:SD and Shoe:SPRD displayed different behaviours in various tests which could be caused either by systemic inbreeding or by different housing conditions before they arrived at our institute. The results point out that rat stocks, even though they are from the same strain, should not be exchanged during an ongoing experimental study.
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306

Calcium/calmodulin kinase II, a binding partner of the multi PDZ domain protein MUPP1 in mammalian spermatozoa, regulates calcium-induced acrosomal exocytosis
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The success of acrosomal exocytosis, a multistage reaction process, which sperm can undergo only once in their life, strongly relies on the coordinated interaction of participating signaling molecules. Recently, the Multi PDZ domain protein MUPP1, comprised of 13 distinct binding motifs, has been demonstrated to be involved in the acrosomal secretion in mammalian sperm cells. Using an inhibitory anti-MUPP1 antibody and a photosensitive calcium chelator we now report that MUPP1 appears to be not involved in the final step of SNARE-promoted membrane fusion but seems to recruite regulatory elements of the pre-fusion steps of acrosomal tethering and docking. Since molecular components mediating the accurate primary physical link between the outer acrosomal membrane and sperm plasma membrane have not yet been identified in sperm cells, attempts were made to explore whether Calcium Calmodulin Kinase II (CaMKII), recently found to interact with MUPP1 in the postsynaptic density, is also an active binding partner of MUPP1 in mammalian spermatozoa. Using solubilized sperm cells and a panel of GST fusion proteins containing different non-overlapping MUPP1 protein fragments, we found that CaMKII, which shows a striking co-localization with MUPP1 in the acrosomal region, strongly interacts with the PDZ domains 1-3. Additional functional experiments in which CaMK II was blocked by KN93 revealed that inhibition of CaMK II in mammalian spermatozoa led to a significant potentiation of acrosomal secretion in mammalian spermatozoa. Furthermore, antagonizing Calcium Calmodulin dependent activation of CaMKII by KN93 also prevented interaction of CaMKII with MUPP1. These results not only led to the first identification of a MUPP1 binding partner in mammalian spermatozoa but in addition give an indication that CaMKII might be involved in preventing spontaneous acrosomal secretion.
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307

Possible role of NEP in food consumption and fat accumulation

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Via its great variety of substrates the metalloenzyme neutral endopeptidase (NEP) influences several metabolic pathways e.g. in circulation with the degradation of angiotensin, bradykinin and natriuretic peptides; in pain and mood-regulation with the degradation of opioids; and Alzheimer's disease with the catabolism of amyloid peptides. Moreover, NEP seems to play a role in regulation of food intake and in fat accumulation. We compared peptidase activities of HBM-mice (high body mass), especially bred for high growth, with appropriate CON-mice (control). Thereby it arose that the HBM-line envinced a significant lower NEP-activity. We tried to answer the question whether the NEP-activity could be altered by fetal programming via special diets in pregnancy and lactation. When supplied with food with high leucine content we found an increase of NEP-activity in the CNS. In accordance to former experiments on NEP-deficient mice it arose that "normalization" of NEP-activity led to a clear decrease of abdominal fat. These investigations were completed by quantification of orexigene peptides like glucagon-like peptide 1 and galanin which are known substrates of the NEP.

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308

Chemoprotective effect of Hypericum perforatum extract in chemically induced skin carcinogenesis

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Hypericum perforatum (St. John's Wort) extracts (HPE) demonstrate various biological actions and are commonly used in traditional medicine (natural antidepressants, topical healing of wounds or burns, etc.) There are limited studies that aim to determine the chemopreventive effect of HPE in skin carcinogenesis and their conclusions are controversial. Aim of the present study was to investigate the chemoprotective effect of Hypericum perforatum olive oil extract in chemically induced skin carcinogenesis in Swiss Albino mice. For the above experiment a two-stage skin carcinogenesis model in Swiss Albino mice was used. In this model pre- and malignant lesions and tumors are induced by 7,12-dimethylbenz[a]anthracene (DMBA) and promoted by croton oil. The duration of the experiment was 25 weeks. During the first week two topical applications of DMBA (sol 0,24% in acetone) were at the dorsal skin of the mice. For the following 24 weeks, the animals received two topical applications of croton oil every week until the end of the experiment. The above method resulted in 100% carcinogenesis in mouse skin (papillomas and squamous cell carcinomas). Topical application of HPE 30 min prior every croton oil application, resulted in significant reduction in the number of tumors appeared per mice, the number of tumor-bearing animals and the transformation of papillomas to squamous cell carcinomas. Furthermore, in the animal groups applied HPE retention of tumor formation and proliferation was observed. Our results were verified histologically.

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309

Analysis of microRNA effects on MSC differentiation to adipocytes

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MicroRNAs (miRNAs) are a recently discovered class of small (18-25 nt), endogenous, evolutionarily conserved, noncoding RNA molecules that posttranscriptionally regulate gene expression. They have been shown to play a prominent role in regulation of differentiation and metabolism. Here, we analyzed the effect of miRNAs on differentiation of mesenchymal stem cells (MSCs). The ability of MSCs to differentiate into a variety of cells, including fat cells, osteoblasts and vascular endothelial cells, makes them an attractive therapeutic tool for regenerative medicine. Initially, we focused on miRNA-143, which has been shown to be expressed during adipogenic differentiation. First, we analyzed different vector configurations for their expression levels of miRNA-143 in MSCs. A cytomegalovirus (CMV) promoter driven lentivector was designed carrying (i) pre-miRNA-143, including upstream and downstream flanking sequence (CMV-miR143), (ii) miRNA-143, cloned 5´ of the GFP coding sequence (CMV-miR143GFP), and (iii) miRNA-143, cloned 3´ of the GFP coding sequence (CMV-GFPmiR143). We transduced MSCs with different doses of each miRNA-lentivector and analyzed the levels of mature miRNA-143 three days after transduction by Real-Time PCR. The CMV-GFPmiR143 construct induced the most efficient production of mature miRNA143 in MSCs (up to ~15 fold increase of miRNA-143 as compared to wild-type cells). Next, we analyzed the effect of miRNA-143 expression on the differentiation of MSCs. MSC-like cells were isolated from brown adipose tissue (BAT): interscapular brown fat pads were harvested from new born wild-type mice and fractionated into mature adipocyte and stroma-vascular (BAT-MSC) fraction. We transduced BAT-MSC cells with the CMV-GFPmiR143 lentivector and differentiated the BAT-MSCs for 11 days according to a standard protocol using differentiation factors (including insuline, trijodothyronine). After differentiation, BAT-MSC derived adipocytes were analyzed for their content of triglycerides (TGs). The TG content of CMV-GFPmiR143 transduced cells was increased by 27% compared to uninfected, differentiated BAT-MSCs. In addition, typical markers for brown fat cells like PPARγ, GLUT4, UCP1 were analyzed. Taken together, our data indicate that miRNA-143 plays a role in differentiation of MSCs to adipocytes. Lentiviral expression vectors for miRNAs are powerful tool to analyze physiological and pharmacological role of miRNAs.

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310

Establishment of a non-radioactive assay to measure RISC (RNA-induced silencing complex) activity in vitro

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RNA-interference (RNAi) is an important novel gene regulatory mechanism. It is initiated by the generation of small, 22nt double-stranded RNAs through the action of ribonuclease III family proteins. The small RNAs enter the RNAi effector complex and mediate destruction of complementary mRNA targets. Alternatively small RNAs prevent protein synthesis or modulate chromatin remodelling to silence gene expression. The RNA-induced silencing complex is a multi-protein complex that mediates small RNA-induced silencing. The minimal components of RISC are members of the Argonaute family of proteins plus small RNA guides. Argonaute 2 (Ago2) is the core catalytic enzyme of RISC that mediates RNA cleavage. To identify inhibitors or activators of Ago2 it is desirable to establish cell-free in vitro assays. So far, Ago2 activity was assessed using 32P-cap-labelled in vitro synthesized mRNA targets and ATP as substrates. Cleavage products are detected after purification and separation of reaction mixtures by denaturing UREA-PAGE followed by autoradiography. This assay is time consuming, difficult to handle and only semiquantitative. Here, we report the establishment of a non-radioactive RISC assay. GST-Ago2 was purified to near homogeneity from E.Coli and incubated with an 180bp luciferase mRNA target. Cleavage reaction was started by addition of phosphorylated single stranded complementary RNA oligonucleotides. Reaction mixtures were purified using a micro RNA purification kit and separated and visualized using a small RNA Chip on an Agilent 2100 Bioanalyzer instrument. The assay was linear between 0.25 and 1h. RNA cleavage was also dependent on the ATP concentration, on temperature and on addition of phosphorylated single stranded RNA oligonucleotide. Optimized conditions resulted in complete cleavage of the target RNA. No mRNA degradation was detected using GST, or a DNA oligonucleotide. The RISC assay reported provides an important tool to assess Ago2 activity in cell-free systems. It may also be used to measure activity of endogenous Ago2 after immunoprecipitation.

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311

Atrial natriuretic peptide induces postprandial lipid oxidation in man

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Objective. Atrial natriuretic peptide (ANP) promotes human adipose tissue lipolysis through cGMP-mediated, hormone sensitive lipase activation. We hypothesized that ANP may augment postprandial free fatty acid availability, lipid oxidation, and energy expenditure, while concomitantly decreasing blood pressure. Design and Methods. Ten healthy and normal weight men received intravenous ANP infusions (25 ng/Kg/min) or placebo in a randomized, double-blind, and cross-over fashion. After 30 minutes of continuous infusion, subjects ingested a standardized fat rich test meal. We obtained venous blood sample and monitored adipose tissue and skeletal muscle metabolism using the microdialysis technique. Energy expenditure and substrate oxidation rates were assessed by indirect calorimetry. Results. Baseline mean arterial pressure was 80±2 mmHg before placebo and 81±2 mmHg before ANP administration. Thirty minutes into the ANP infusion, mean arterial pressure decreased to 76±2 mmHg ($p<0.01$ compared with placebo). With placebo, venous glycerol and free fatty acid concentrations decreased sharply in the postprandial phase. With ANP, venous glycerol remained elevated throughout the postprandial phase ($p<0.001$ and <0.05 vs. placebo). Microdialysate glycerol in adipose tissue was increased with ANP, particularly late into the postprandial phase. ANP augmented postprandial thermogenesis ($p<0.05$ vs. placebo) through increased lipid oxidation.Conclusions. We identified the ANP system as a novel pathway regulating postprandial lipid oxidation and blood pressure. ANP attenuates the postprandial decline in lipid mobilization leading to increased postprandial energy expenditure through fatty acid oxidation. 1. Franz Volhard Clinical Research Center, Medical Faculty of the Charité, Berlin, Germany, 2. Inserm-UPS Unit 586, Université Paul Sabatier, Hôpital Rangueil, Toulouse, France

312

Ten years after: implementation of a problem-based learning module as a mandatory elective unit of the pharmacology and toxicology curriculum

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In 1998, we started a pilot project of a problem-based learning (PBL) course in pharmacology and toxicology with one group of 8 students coached by a tutor. We then gradually increased the number of groups to 19 with experienced tutors recruited from former PBL courses. Starting in 1999, we implemented the PBL course as a mandatory elective unit. The PBL course is for third year medical students and takes 28 weeks with 1.5 h each. The students meet in work groups consisting of 10 participants, guided by an senior student tutor and supervised by supervisors and an expert teacher. On the basis of 12 clinical cases, the students learn pharmacological knowledge and acquire a deep understanding of the clinical context by both studying complex issues at home and by discussing problems with their peers during class. Currently, approximately 60 % of one entire grade of students (193 of 320) is enrolled in the course, the remainder is enrolled in a conventional lecture-based learning (LBL) course. The senior student tutors undergo a three-day retreat before each term, where they are trained with respect to both didactic issues and the pharmacological and clinical knowledge. Moreover, they are coached by clinical experts for each specific case. The PBL course is regularly evaluated with the following observations: PBL students (i) considered the acquired knowledge more long-lasting, (ii) experienced a higher incentive for in-depth studies of the topics covered, (iii) enjoyed the course considerably more, and (iv) hence were more motivated as compared with their previous experience with LBL courses. Concerning multiple-choice exams, the results of LBL and PBL students were not significantly different. In 2002, the PBL course received the State of Baden-Württemberg Distinguished Teaching Award (Landeslehrpreis) for Outstanding Teaching Projects.

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313

Interindividual variability of xenobiotic-metabolizing cytochrome P450 (CYP) enzymes in human lung and relevance for the risk assessment of chemicals

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Background: The lung represents an important target for the toxic effects of chemicals. Target tissue toxicity can be explained by local metabolic activation. It could be demonstrated by mRNA and Western Blot analysis, that xenobiotic metabolizing CYP enzymes are expressed in human lung. Their variation within the human population is not known, although this is an important issue for the risk assessment of chemicals. From interindividual variability, pathway-specific assessment factors can be derived. This is of special importance in the lung, where metabolism is capacity-limited due to low abundance of CYP proteins. Therefore, we determined the variability of CYPs involved in xenobiotic metabolism (CYP2A6, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP2E1) in a larger panel of individual human lung samples. Methods: Human lung tissue was obtained after informed consent. Microsomes were prepared and total protein content was determined. A Western Blotting procedure using commercially available antibodies was applied to immunoquantify the respective CYPs in 112 different individual lung samples. After detection, the blots were evaluated by densitometry. The relevant assessment factor was derived by the ratio of the 95% percentile (P95)/median. Results: With the exception of CYP2C19 and CYP2D6, the respective CYP proteins were detectable in all samples investigated. The following P95/median ratios could be determined: 1.1 (CYP2A6), 1.4 (CYP2B6), 1.8 (CYP2C9), 2.0 (CYP2C19), 13 (CYP2D6), and 1.7 (CYP2E1). It can be concluded, that interindividual variability in CYP-dependent chemical metabolism is dependent on the individual CYP form involved. For an improved risk assessment of chemicals, knowledge of the enzyme involved in their metabolism is essential. Quantification of the expression allows replacing the default value of 3.3 for the kinetic part of the intraspecies variability by a pathway-specific assessment factor. The results demonstrate that for most of the CYPs the variability in our lung is less than the default of 3.3, whereas for one CYP enzyme (CYP2D6) a higher assessment factor has to be taken in the assessment of risks to account for the larger variability.

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314

Expression of CYP2E1 and in-vitro formation of reactive metabolites in human oral cell lines

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The internalization of foreign compounds occurs through three primary routes; absorption through the skin, inhalation and ingestion. Up to date metabolism in the oral cavity is poorly characterized. Consequently, the extent to which oral metabolism may contribute to development of oral diseases remains uncertain. Several studies have examined the risk of developing oral type cancers in relation to the occurrence of polymorphic CYP genes. Still missing, however, is a comprehensive analysis of CYP expression and activity in the oral mucosa.Therefore the expression of CYP2E1 in oral cells (table 1) and its role in the metabolism of methacrylic acid (MA), a cleavage product of dental materials, was investigated.The presence of human CYP2E1 RNA was demonstrated by RT-PCR. The formation of 2,3-EMA, a epoxy metabolite of MA, was demonstrated by use of GC-MS.

Table 1: Formation of 2,3-EMA from MA in different human cell lines that express CYP2E1 (initial MA = 100 µmol/l, total reaction time 1 h).

Cell line	c(2,3-EMA) [µmol/l/h]	Error [µmol/l/h]	n	CYP2E1 expression
hum. liver microsomes	5	1	4	++++
SqCC/Y1	2.5	0.8	6	++
SqCC/Y1 (with pyrazole)	n.d.	-	3	CYP2E1 inhibited
V79hCYP2E1	1.5	0.6	6	++
V79 parenteral (negative control)	n.d.	-	4	no expression
hum. pulp fibroblasts	0.3	0.3	5	++
hum. gingival fibroblasts	0.2	0.2	4	++

n.d. = not detected

Our results reveal the generation of epoxide metabolites from dental materials right inside oral cavity cells without preliminary swallowing or digestion. The amount of orally metabolites formed can be compared to the amount formed by human liver microsomes. The risk assessment of dental materials should consider these effects.

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315

Time- and concentration-dependent CYP1A1 induction by benzo[*a*]pyrene in cells of a human bladder cancer cell line

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Next to aromatic amines, polycyclic aromatic hydrocarbons are discussed as relevant substances in bladder carcinogenesis. Cultured urothelial cells are valuable tools to study bladder-specific effects of such compounds. Previously, CYP1A1 induction by benzo[*a*]pyrene (B[*a*]P) was observed in porcine urinary bladder epithelial cells (PUBEC), and in human exfoliated urothelial cells by cigarette smoking.In the present study, we used cells of the human bladder cancer cell line 5637 as an *in vitro* model to examine their response to treatment with B[a]P. CYP1A1 mRNA was induced in a time- and concentration-dependent manner, achieving maximal mRNA levels at 1 µM B[a]P determined by real time RT-PCR. Interestingly, and comparable to results with PUBEC, only a subset of 5637 cells was inducible by B[a]P (determined by immunostaining and flow cytometry). The number of CYP1A1-positive cells increased with B[a]P concentration and with incubation time: For instance, treatment with 1 µM B[a]P resulted in 3.3% CYP1A1-induced cells after 24 h, 9.9% after 48 h, and 24.2% after 72 h, respectively. Moreover, the distribution of cells in the different phases of the cell cycle changed during incubation: In comparison to solvent-treated cells, the fraction of cells in G1/G0-phase decreased by 7% after 48 h and by 11% after 72 h B[a]P treatment, respectively. Concurrently, there was a rise in the fraction of cells in S- and G2/M-phase, pointing to an increased proliferation elicited by B[a]P. It cannot be excluded that the increased number of cells in S- and G2/M-phase of the cell cycle was a result of regenerative proliferation. It will be interesting to find out whether the CYP1A1-inducible cell subtype is more or less susceptible to bladder carcinogens compared to the non-inducible subtype.

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316

Is in-vitro induction of CYP3A1 in rat liver slices (RLS) by dexamethasone (Dex) influenced by CYP2B inducing phenoarbital (PB)?

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Precision-cut liver slices are suitable to demonstrate *in vitro* induction of several CYP forms by model inducers. For slices prepared from human liver tissue marked interindividual differences have to be expected, which might be caused by quite different CYP expression states. With RLS we could demonstrate that in vitro inducibility of CYP3A1, in contrast to CYP2B1, is not substantially influenced by preceding *in vivo* induction of the tissue donor with the same inducer. Now we demonstrate the influence of PB on the inducibility by DEX. RLS were exposed to DEX (10^{-7} - 10^{-6} M) for 24h to induce CYP3A1. PB treatment was performed either *in vivo* (3 x 60 mg/kg i.p.) with RLS preparation 24h after the last injection, or in vitro for 24h (5 or 100 µM) simultaneously with or before DEX exposure. In RLS from PB pretreated rats, CYP2B1 dependent activity was initially enhanced as expected and did not completely decrease within 24h of slice incubation. The concentration dependent CYP3A1 inducibility by DEX in vitro was not disturbed, as shown at the level of both enzyme activity and CYP3A1-mRNA expression. If RLS of untreated animals were incubated simultaneously with DEX and PB for 24h, CYP3A1 induction was not disturbed either. The same was true for a succesive *in vitro* exposure to PB for 24h, followed by DEX for further 24h, although absolute biotransformation rates and factors of induction were lower after the prolonged total incubation of 48h. Induction of CYP3A1-mRNA expression was not changed by foregoing exposure to PB.We conclude that CYP3A1 induction can be detected *in vitro* in RLS independent of preceding or simultaneous CYP2B1 induction.

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317

Expression and inducibility of cytochromes P450 in the human urothelial cell line 5637

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In recent years, it became evident that the bladder epithelium is competent in xenobiotic metabolism and, thus, is capable to generate reactive metabolites which subsequently may lead to adverse effects in this tissue. We selected the human urothelial cell line 5637 as a model system to study CYP-expression and inducibility as well as underlying mechanisms in urothelial cells. The aims of the present study are the characterization of the basal CYP-mRNA profile by RT-PCR and the examination of inducibility of several CYPs by xenobiotics. We show here by RT-PCR that untreated 5637 urothelial cells express a broad spectrum of CYPs at least at the mRNA level. Furthermore, transcripts of signal transduction molecules involved in CYP1-regulation, such as AhR, Ahr-repressor and ARNT, were detected. For untreated 5637-cells, we proved expression of the following CYP genes: CYP1A1, CYP1B1, CYP2J2, CYP2S1, CYP3A5, CYP4B1 and CYP51A1. Transcripts of CYP2B6, CYP2F1 and CYP3A4 were below detection level and could not be induced by phenobarbital or dexamethasone while CYP2E1 and CYP4Z1 were found at low levels only. Treatment of 5637 cells with benzo[a]pyrene (BaP) leads to parallel induction of CYP1A1 and CYP1B1 as shown by real time RT-PCR. Maximal transcript levels were reached after 24h treatment with 1µM BaP. The likewise AhR-regulated CYP2S1 is also induced by BaP while CYP2J2 and CYP51A1 which is overexpressed in several tumors remain unaffected. Interestingly, ethanol treatment did not increase CYP2E1-mRNA. On the protein level, induction of CYP1A1 by BaP could be demonstrated by immunoblotting and immunofluorescence microscopy.Our results indicate that 5637 cells are a potentially suitable model for urothelial cells concerning studies on CYPs and CYP-mediated effects of xenobiotics. Transcripts of relevant enzymes involved in the metabolism of bladder carcinogens are present and inducible.

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318

Effect of benzo[a]pyrene (BaP) on AhR-signaling in duodenal and colon cell lines

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The aryl hydrocarbon receptor (AhR)-pathway is involved in the regulation of developmental cellular events and mediates adaptive or toxic effects elicited by xenobiotics. In this respect, AhR regulates gene expression of cytochromes P450 in a ligand-dependent way. This process can be modulated by flavonoids present in the human diet. As a consequence, flavonoids can significantly decrease outcome of diseases associated with oxidative stress, such as cardiovascular diseases, inflammation and cancer.The final aim of this study is to examine the modulatory effect of flavonoids on components and target genes of the ligand dependent AhR signaling pathway in intestinal cells. For these experiments, we used three different cell lines, human duodenal (HuTu-80) and colon adenocarcinoma cells (CaCo-2) and rat duodenal normal epithelial (IEC-6). In a first set of experiments, cultured cells were incubated or

48 hours with BaP in the concentration range between 0.01 and 10 µM. After incubation, total RNA was isolated from the BaP-treated and from untreated confluent cells. TaqMan® real time PCR technology was applied for relative mRNA quantification by RT-PCR.In IEC-6 cells, mRNA-levels of AhRR, ARNT, Nrf2, CYP1A1 and CYP1B1 were increased by BaP whereas the level of AhR mRNA was not influenced. The dose-response experiments show that maximal transcript levels for CYP1A1 and CYP1B1 are reached at BaP concentrations as low as 0.3 µM. Remarkably, CYP1A1 mRNA was detectable in IEC-6 and HuTu-80 cells in contrast to literature data. CYP1A2 mRNA was not detectable in these cell lines. It is shown that AhR signaling components are influenced by BaP at least in IEC-6 cells and that these cells respond sensitively by CYP1A1-induction. In HuTu-80 and CaCo-2 cells, members of the AhR-pathway are constitutively expressed at the transcript level so that these two cell lines appear also suitable for our further studies.

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319

STAT-3 regulates the constitutive expression of the Ah receptor

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The Ah receptor (AhR) is a ligand-activated transcription factor which mediates the toxic effects of environmental pollutants like dibenzo-p-dioxins, dibenzo-p-furans and polycyclic aromatic hydrocarbons. Ligand-binding leads to the translocation of the AhR into the nucleus, where it dimerizes with ARNT. The resulting heterodimer binds to specific DNA motifs 5'-upstream of its target genes (e.g. CYP1A1), leading to an enhanced transcription rate. However, in contrast to the well-analyzed role of the AhR in transactivation of genes, only little is known about the regulation of the AhR itself in response to endo- or exogenous stimuli.In a previous study, we identified a core sequence of the human AhR promoter (between -1980 and -1892), which is indispensable for constitutive AhR expression. Database-driven promoter analyses revealed the existence of a putative STAT-3 recognition site. STAT-3 is the main downstream target of IL-6-type cytokines, e.g. IL-6, IL-11, and Oncostatin M (OSM). Site-directed mutagenesis of the STAT-3 motif within this AhR promoter-construct resulted in the loss of luciferase activity. Treatment of HepG2 cells with different amounts of OSM led to a dose- and time-dependent induction of AhR mRNA expression. These results were confirmed by AhR-driven reporter gene assays, displaying an enhanced luciferase activity in response to OSM exposure. Ectopic overexpression of a dominant negative STAT-3 mutant resulted in a significant decrease of constitutive as well as OSM-induced AhR mRNA expression, evidencing that STAT-3 is responsible for the observed effects. In addition, by means of gelshift analyses, we were able to demonstrate that STAT-3 binds to its recognition site within the human AhR gene. Furthermore, the gelshift experiments revealed the presence of an additional, yet unknown, protein whose existence could be confirmed by SDS-page of magnetically purified STAT-3-DNA-binding proteins. The data presented in this study disclose a direct connection between the expression of the AhR and the activated STAT-3 pathway, one of the most important signaling cascades downstream of cytokine receptors. Since the constitutive activity of both, AhR and STAT-3, are often enhanced in several types of cancer cells, the further elucidation of this interaction may be of interest.

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320

Inducibility of breast cancer resistance protein (ABCG2) by aryl hydrocarbon receptor agonists

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Breast cancer resistance protein [BCRP] is a membrane bound ABC class transporter protein which mediates the cellular export of several phase II metabolites as well as chemotherapeuticals. BCRP plays an important role at sites that form barriers e.g., colon, blood-brain- and blood-testis-barrier and placenta, where it limits the uptake of xenobiotic substances. BCRP is overexpressed in different tumors where it contributes to clinical drug resistance. Recently, BCRP was identified as an efflux-transporter of benzo(*a*)pyrene-sulfate [1]. The precursor benzo(*a*)pyrene [B(a)P] is a classical AhR agonist. The AhR mediates the metabolism of xenobiotic substances, like polycyclic aromatic hydrocarbons [PAHs]. The AhR is located in the cytosol, where it forms a multi protein complex with two molecules of HSP90, AIP and p23. After ligand binding the complex dissociates and the AhR translocates into the nucleus where it forms a heterodimer with its partner protein ARNT. This dimer induces gene expression of xenobiotic metabolizing enzymes. It has also been shown that BCRP expression is inducible by PAHs [1]. This observation assumes an involvement of the AhR in regulation of transport mechanisms. We therefore tested the inducibility of BCRP in different human celltypes like Caco 2, HCT116 and HepG2 after treatment with known AhR agonists. All three cell types showed a consitutive expression of BCRP. B(a)P and 3MC significantly increased the mRNA expression of BCRP in HepG2- and Caco 2-cells up to six- and two-fold, respectively. In contrast HCT116-cells showed no BCRP induction. Computer analysis revealed three putative xenobiotic responsive elements [XRE], the DNA binding motive of the AhR, upstream of the BCRP gene. To further elucidate the involvement of the AhR we cloned the 5' promotor region of BCRP into a luciferase vector and performed reporter-gene assays with 3MC in HepG2 cells. First results point to an AhR dependent upregulation of BCRP in response to PAHs.

[1] Ebert et al. 2005 Carcinogenesis

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321

Characterization of drug-metabolism–related gene expression in the hepatoma cell lines HepG2 and H4IIE: Effects of PXR agonists

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In drug development and environmental toxicology, the prediction of induction of drug metabolism is of outstanding interest. Relevant, rapid testing methods that can be handled easily are desired. Permanent cell lines, frequently used for this purpose, have a modified drug metabolism or some drug metabolizing enzymes are switched off. Therefore, it is important to analyze the pattern of expression of drug metabolism-related genes in these cell lines. In our study, human HepG2 and rat H4IIE hepatoma cells were treated with the prototype inducers/pregnane X receptor (PXR) agonists rifampicin and dexamethasone for 48 hours. Isolated RNA was reverse transcribed into cDNA. With TaqMan® Low Density Array cards allowing 384 simultaneous real-time PCR reactions, we analyzed gene expression of 45 phase I and II drug metabolizing enzymes as well as transporters and receptors. In HepG2 and H4IIE cells some of the 45 drug metabolism-related genes like AhR, ABCC2, CYP2A1, CYP2B6, CYP3A3/5/7, PPARα and UGT1A1/3-10 were induced by dexamethasone and/or rifampicin. Transcripts of, e.g., ABCB11, CYP2A2, CYP4A11/12 and UGT1A7 could not be detected, and others like ABCB1, CYP2A6, CYP2B3, CYP4A12, HNF4α, NR3C1 were constitutively expressed but did not show any response to the treatment. With these findings it may be possible to decide on which type of investigation HepG2 and H4IIE cells are feasible. Overall, TaqMan® Low Density Array cards are a fast and customizable method to characterize cells with respect to inducibility of drug metabolism.

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322

***In vitro* genotoxicity testing requires exogenous metabolic activation: Influence of rat strains and liver enzyme inducers**

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Since the bacterial reverse mutation test was accredited by the OECD (No.471) the use of exogenous metabolic activation by liver homogenate (S9 fraction) of induced rats was recommended for *in vitro* genotoxicity testing. So the toxicity of the compound itself and its metabolites can be assessed. For a long time Aroclor 1254 was used for hepatic enzyme induction. In the 1970s production of PCB's was baned and an alternative induction by a mixture of phenobarbitone (PB) and ß-naphthoflavone (ßNF) was included in the Guidelines. While some researchers used the new mixture, others are still using up their stocks of Aroclor. While there have been small comparison studies of both induction methods, experiences from routine labs with large data bases have not yet been published.We have investigated the influence of different rat strains and the alternative enzyme-inducing agents on the metabolic activity of S9 fractions with means of biochemical characterization and enzyme activity as well as by mutagenicity testing with the bacterial reverse mutation test.Sprague-Dawley or Wistar Han rats were treated once with Aroclor and Wistar Han rats were treated 3-times with PB/ßNF. Post mortem liver S9 fractions were prepared and analyzed for enzyme content and activities: CYP (EROD, PROD), glutathione *S*-transferase and glucuronic acid transferase (UDPGT) activity.The results showed minor rat strain dependent differences in enzyme activities. Generally, Wistar Han rats had higher enzyme activities than Sprague-Dawley rats treated with the same enzyme inducer. Using alternative induction mixture instead of Aroclor leads to a slight decrease in enzyme activities in both rat strains. Enzyme activities of PB/ßNF-treated Wistar Han rats are comparable to Aroclor-treated Sprague-Dawley rats.In the Ames test 2-aminoanthracene was clearly mutagenic in the presence of S9 mix and the revertant rates did not significantly differ when using PB/ßNF instead of Aroclor.The induction of metabolic rat liver enzymes using PB and ßNF is a useful tool for *in vitro* testing. There is no relevant difference in the outcome of Ames tests using S9 mix from rats treated with either inducer. However, the induction procedure is more time consuming than using a single i.p. application of Aroclor. But the toxicity and the costs of Aroclor are much higher compared to PB/ßNF. Overall, the use of the alternative offers a safer and more cost effective way to perform *in vitro* mutagenicity tests with external metabolic activation without distortion of the test results.

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323

Metabolic activity of skin *in vitro* systems

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The skin is a large organ and a major exposure site for many xenobiotics. The physical barrier functions of the skin are well studied, whereas less is known about the metabolic capacity of the skin and its contribution to the barrier function and the role of cutaneous first pass metabolism for the toxicity profile of dermally applied test substances.The aim of this study was to estimate the metabolic activity of skin *in vitro* systems from different species. For this, human, mini pig and rat skin sub-cellular fractions of the epidermis were analyzed for their CYP- and glutathione S-transferase -activity. Additionally, metabolism of diagnostic substrates were analyzed by HPLC. Similarities and differences of species could be demonstrated and are presented in the context of available literature data. EROD activities in all skin microsomes were approximately 0.4 pmol resorufin /min/mg protein. Whereas PROD activity was approximately 0.4 pmol resorufin /min/mg protein in human skin microsomes, but were for rats and mini pigs near the detection limit. GST activities in rat and mini pig were comparable (approximately 25 nmol CDNB /min/mg S9-fraction) and 5-fold less as compared to human activities (approximately 130 nmol CDNB /min/mg S9-fraction). The metabolism of the substrates correlated with the characterized *in vitro* system properties.In summary, human skin fractions exhibited higher metabolic activities than rat or pig skin fractions, resulting in a potentially stronger metabolic modulation of the toxicity of dermal toxicants in humans than in rats or pigs.

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324

Basal and inducible cytochrome P450 expression in β-catenin knockout mice

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β-Catenin has been shown to play an important role in gene regulation in mouse liver, particularly of genes with perivenous expression within the liver lobule such as drug-metabolizing enzymes. To analyze effects of β-catenin signaling on xenobiotic metabolism *in vivo*, expression of representative enzymes involved in drug metabolism was analyzed in livers of mice with liver-specific knockout of the Ctnnb1 gene (encoding β-catenin) and compared to their wild-type littermates. Conditional knockout of β-catenin was achieved by interbreeding of floxed Ctnnb1 mice with transgenic mice expressing Cre recombinase under control of the liver-specific albumin promoter. Mice were treated with either the aryl hydrocarbon receptor (AhR) agonist 3-methylcholanthrene or phenobarbital, an activator of constitutive androstane receptor (CAR)-mediated transcription. Differential effects were observed with regard to the basal expression of drug-metabolizing enzymes in Ctnnb1 knockout mice: expression of cytochrome P450 (Cyp) 1a2, Cyp2e1, three glutathione *S*-transferase isoforms and two sulfotransferases was significantly reduced in the knockout group, whereas other Cyp isoenzymes, namely Cyp2b10, Cyp2f2, and Cyp3a were expressed at slightly higher levels. The nuclear receptors AhR and CAR were down-regulated on the mRNA level in the knockout group, while PXR expression was not altered. Induction of the AhR- by 3-methylcholanthrene and of CAR-dependent transcription by phenobarbital revealed an important role of β-catenin in the inducibility of drug-metabolizing enzymes *in vivo*. Induction of Cyp1a1 and Cyp1a2 mRNA and protein levels by 3-MC was observed for both genotypes, but the maximum level of Cyp1a1 and Cyp1a2 expression was reduced in the knockout mice. Comparable effects were observed for phenobarbital-mediated induction of Cyp1a2, Cyp2b10, Cyp3a, and GSTm isoenzymes. The results demonstrate an involvement of β-catenin-dependent signaling basal as well as inducible expression of drug metabolizing enzymes.

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325

Effect of subchronic coexposure to arsenic and malathion on parameters indicative of oxidative stress in erythrocytes of rats

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Heavy metal arsenic is one of the most relevant environmental toxicants and contamination of water supplies with arsenic has been reported in India and several other countries. Malathion is an organophosphorus insecticide used widely in agriculture, veterinary medicine and public health programmes worldwide. The non-target species may be exposed to arsenic and malathion concomitantly through environmental processes. The present study was undertaken to examine the effects of subchronic exposure of arsenic, malathion and their combination on oxidative stress related biochemical parameters in erythrocytes of rats. Twenty four adult male rats were randomly divided into four groups each comprising of six animals. Animals of groups II, III, IV were given 4 ppm arsenic, 50 ppm malathion and 4 ppm arsenic plus 50 ppm malathion, respectively whereas animals of group I were left untreated and served as controls. Arsenic and malathion were given in water and feed for 28 days, respectively. At term, impact of the coexposure was assessed by evaluating lipid peroxidation (LPO), reduced glutathione (GSH) and activities of superoxide dismutase (SOD), catalase (CAT), and glutathione S-transferase (GST) in erythrocytes. LPO was determined by the thiobarbituric acid reaction and it was expressed as the extent of malondialdehyde (MDA) formation. Arsenic and arsenic plus malathion significantly increased LPO to the extent of 35% and 138%, respectively while the levels of GSH were significantly reduced by arsenic (21%), malathion (33%) and their combined exposure (36%) as compared with control values. The activities of SOD and CAT were significantly decreased by arsenic (55% and 33%, respectively) and arsenic plus malathion (45% and 55%, respectively) exposure wheras the GST activity was markedly increased in erythrocytes of rats co-exposed to the metal and insecticide. The results suggest that subchronic coexposure to arsenic and malathion at low levels as used in the present study induces oxidative stress in erythrocytes of rats.

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326

Characterization of the human carbonyl reductase 3 (CBR3) by site-directed mutagenesis

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Human carbonyl reductase appears in two isoforms, CBR1 and CBR3. CBR1 metabolizes a broad spectrum of endogenous and xenobiotic carbonyl compounds which are highly reactive substances that can form covalent bonds with proteins and nucleic acids. Reduction of the carbonyl moiety to the corresponding alcohol may represent the first step in a detoxification process. While CBR1 is known for more than 25 years and is well characterized, CBR3 was discovered in 1998 only and nearly no data exist regarding its enzymatic properties or physiological function. CBR1 and CBR3 share 85 % similarity on the protein level, but their substrate specificity seems to be quite dissimilar. To understand these unexpected differences we cloned and purified recombinant CBR1 and CBR3 from E. coli. Based on sequence alignments and on the crystal structures of CBR1 and CBR3 we generated 10 mutants containing one or more amino acid replacements at critical sites near the catalytic center. We used isatin as a reference substrate because isatin has been described as the only common substrate for both CBR isoforms so far. In fact, we were able to detect important regions in the CBR3 structure, which, when replaced by the corresponding CBR1 amino acid sequence, led to a considerable increase in CBR3 activity. For example, the exchange of nine amino acids from residue 236-244 raised the catalytic efficiency of CBR3 by a factor of 10. Moreover, an additional mutation at position 230 further increased the catalytic efficiency to a factor of 250. Finally, a third replacement of amino acid residues 142-143 yielded a 500-fold increase in CBR3 activity which, interestingly enough, comes close to the catalytic activity observed for CBR1 with isatin as the substrate. The same replacement strategy let mutant CBR3 to metabolize even menadione, the standard substrate for CBR1. Importantly, menadione is no substrate for CBR3 wildtype. Combined, molecular replacement studies may help to elucidate the structure-function relationships between the CBR1 and CBR3 isoforms.

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327

Identification and characterization of a new mammalian epoxide hydrolase

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Epoxide hydrolases (EHs) were firstly identified and characterized as detoxifying enzymes. Recently these enzymes proved to be also involved in the metabolism of endogenous signaling molecules and thus may act as regulators of physiological processes. Here, we report the characterization of a novel mammalian epoxide hydrolase (nEH1). The gene coding for nEH1 has been identified by us via searching genomic databases. The corresponding cDNA, coding for a 361-aa protein with a catalytic triad and a 20-aa membrane-anchor, was expressed in baculovirus-based expression system. Differential sedimentation of cellular extracts from the cells expressing the new protein confirmed association of nEH1 with the membrane fraction. Assays for enzymatic activity revealed that nEH1 does not hydrolyse the generic epoxide hydrolase substrate 7,8-styrene oxide but, instead, shows good activity with 9,10-epoxystearic acid, a fatty acid epoxide. This led us to investigate a possible role of the enzyme in the turnover of epoxyeicosatrienoic acids (EETs), compounds that have recently evolved as important modulators of physiological processes, including blood pressure, vasodilation, fibrinolysis and inflammation. Using LC-MS/MS analysis we could, indeed, show that the recombinant nEH1 efficiently hydrolyzes three of the four EET regioisomers. Analysis of the mRNA expression of the mouse nEH1 homolog revealed that this enzyme is expressed in kidney, lung, heart and brain. The very specific enzymatic activity of nEH1 and the pattern of its organ-specific expression suggest a role of the new enzyme in the EETs-mediated regulation of physiological processes. Further characterization of nEH1 will elucidate whether this enzyme could be a target in the treatment of inflammation, renal or cardiovascular dysfunctions.

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328

Identification of the amino acid residues responsible for the catalytic activity of the new human epoxide hydrolase

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Epoxide hydrolases (EHs) are enzymes that play an important role in the detoxification of genotoxic compounds. The two known human, the microsomal and the soluble EHs, share the same tertiary structure and two-step catalytic mechanism typical for the members of the α/β-fold hydrolase family. Three amino acids: a nucleophile, histidine and acidic residues,are necessary for the catalytic hydrolysis of the epoxide ring. This reaction is supported by two tyrosine residues involved in the proper orientation of the substrate toward the substrate binding cavity. In spite of the low overall sequence similarity between the members of this family the regions surrounding amino acids responsible for the enzymatic activity are highly conserved in enzymes of the α/β-fold family. We used such conserved motives for screening databases and identification of genes coding for the hitherto unknown mammalian α/β-fold epoxide hydrolases. These analyses let us to the identification of new genes coding for putative epoxide hydrolases. One of these proteins (new epoxide hydrolase 1, nEH1) expressed in Baculovirus-derived expression system displays activity towards fatty acid like epoxide containing molecules. To ascertain whether the conserved amino acid residues (D173, H337, D307, Y220, Y280 or Y281) are responsible for the enzymatic activity of nEH1 we substitute these residues by site directed mutagenesis. Analysis of the activity of the mutated forms of nEH1 revealed that substitution of D173, H337 or D307 abolishes and in the case of tyrosines decreases the enzymatic activity of nEH1. These results show that the conserved amino acid residues are essential for the activity of nEH1 and form the typical for α/β-fold epoxide hydrolases catalytical triad.

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329

Identification of a new potential human epoxide hydrolase (nEH2)

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Two human epoxide hydrolases (EH`s) are well-investigated. The microsomal EH (mEH) hydrolyzes a large variety of structurally different xenobiotic epoxides and for that reason is implicated in detoxification. The soluble EH (sEH) converts arachidonic acid derived physiological signalling molecules such as epoxyeicosatrienoic acids (EETs) to the corresponding dihydroxyeicosatrienoic acids (DHETs). Using the signature sequence RVIAPDLRGYGDSDKP that is reasonably conserved among epoxide hydrolases and many other structurally related enzymes of the α/β hydrolase fold superfamily, we could recently identify two new genes: ABHD7 and ABHD9. All features so far identified to be necessary for a functional EH, including a catalytic triad with an aspartic acid in the position of the catalytic nucleophile, as well as two catalytic tyrosines, are conserved in the primary structures of the proteins (new epoxide hydrolases: nEH2 and nEH1 respectively) predicted from these genes. The amino acid sequences of nEH1 and nEH2 share 42% identity and therefore belong to the same family being different from mEH and sEH (sequence similarity below 25%). The aim of this study is to elucidate the role of nEH2. We therefore employed a number of prokaryotic and eukaryotic heterologous expression systems in order to obtain enzymatically active nEH2. We are presently performing LC-MS/MS analysis, using different xenobiotic as well as physiologically relevant epoxides as potential substrates for nEH2. In addition, we are exploring the organ-specific expression pattern of the enzyme. First results of these ongoing studies revealed significant amounts of the transcript in mouse brain, suggesting a possible role of nEH2 in regulation and/or protection of neuronal function.

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330

Microsomal epoxide hydrolase efficiently hydrolyzes epoxyeicosatrienoic acids

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The microsomal epoxide hydrolase is traditionally thought of as a detoxifying enzyme and its metabolization efficiency of carcinogenic epoxides to their less harmful dihydrodiols is well established. But recent data in our group support earlier findings which implicate that the mEH, in addition to its central role in detoxification, also has other physiological roles; based on preliminary results we show that mEH is capable of metabolizing epoxyeicosatrienoic acids (EETs). These are a class of signaling molecules known to regulate a wide variety of physiological functions, such as blood pressure, angiogenesis, inflammation and apoptosis. Therefore, the mEH potentially participates in the regulation of these physiological processes. The two-step mechanism of the enzymatic reaction catalyzed by mEH requires a nucleophilic attack followed by hydrolysis via a water- activating charge relay (H431 and E404). An E404D mutant of mEH shows a faster metabolization rate compared to the wild type enzyme. Interestingly, this variant is exclusively expressed in Aspergillus niger and has yet not been found in higher organisms.We hypothesize that this is linked to an altered EET metabolism by the E404D variant. We have now expressed recombinant wild type as well as E404D mutant mEHs from rat, mouse and human and presently analyze their turnover rate with EETs using LC-MS/MS. We expect that our findings elucidate more details about the physiological role of mEH and answer the question of the evolutionary establishment of the less active version of mEH.

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331

Functional analysis of the potential epoxide hydrolase MEST

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Epoxide hydrolases are a family of structurally related enzymes that catalyze the hydration of chemically reactive epoxides to their corresponding dihydrodiol products which is an important step in the detoxification of electrophilic products of CYP catalyzed epoxidations. In mammalian species, there exist at least five epoxide hydrolase forms which can be differentiated due to their substrate spectra. Furthermore, on the basis of amino acid sequence similarity analysis new enzymes can be placed into the family of epoxide hydrolases, among these MEST. Initially the protein MEST (mesoderm specific transcript also known as PEG1 for paternal expressed gene 1) has been analyzed due to its differences in paternally and maternally expression unveiling it as one of the first known so called “paternally imprinted gene” products. In these studies MEST was associated with altered embryonic growth and maternal behavior. Experimental proof of any enzymatic function of MEST is still missing. Based on the sequence similarities we are trying to prove its function as an epoxide hydrolase. Here we present strategies to express full length as well as truncated MEST in bacterial and insect cell expression systems. Additionally we show results of different protein purification procedures and screening assays searching for the enzyme physiological substrate(s).

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332

Characterization of the human sEH phosphatase active site by site directed mutagenesis and LC-MS/MS

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Human soluble epoxide hydrolase (sEH) is a multifunctional enzyme and well investigated in its classical role as xenobiotic metabolizing enzyme. Moreover, sEH emerges as important regulator of diverse physiological processes such as blood pressure regulation or inflammation. sEH is implicated in such functions due to the brakedown of arachidonic acid derived signalling molecules like epoxyeicosatrienoic acids (EETs) by its C-terminal epoxide hydrolase domain. Recently, others and we discovered that the sEH N-terminal domain, which is structurally related to the haloacid dehalogenases (HADs), displays a novel phosphatase activity. sEH accepts the generic substrate 4-NPP as well as some lipid and isoprenoid phosphates, but the physiological role remains uncertain to date. The phosphatase domain contains three highly conserved sequence motifs, including the potential catalytic nucleophile Asp9, and several residues implicated in substrate turnover and/or Mg^{2+} binding. To enlighten the proposed catalytic mechanism of dephosphorylation we constructed sEH phosphatase active site mutants by site directed mutagenesis. A total of 18 mutants were recombinantly expressed as soluble proteins, purified and subsequently analysed for their kinetic properties. An exchange of Asp9, Lys160, Asp184 or Asn189 results in a complete loss of phosphatase activity, emphasising the absolute requirement of these amino acid residues for catalysis, whereas a substitution of Asp11, Thr123, Asn124 and Asp185 leads to sEH mutant proteins with residual phosphatase activity. The proposed catalytic mechanism will be discussed. To finally prove the role of Asp9 as catalytic nucleophile we presently analyse the presumed phosphoester intermediate by means of LC-MS/MS. The dual phosphatase and epoxide hydrolase activity further highlights the role of human sEH in regulatory processes.

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333

Reconstruction of *N*-acetyltransferase 2 haplotypes using PHASE

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The genotyping of *N*-acetyltransferase 2 (NAT2) by PCR/RFLP methods yields in a considerable percentage ambiguous results. To resolve this methodical problem a statistical approach was applied. PHASE v2.1.1, a statistical program for haplotype reconstruction was used to estimate haplotype pairs from NAT2 genotyping data, obtained by the analysis of 7 SNPs relevant for Caucasians. In 1011 out of 2921 (35%) subjects the haplotype pairs were clearcut by the PCR/RFLP data only. For the majority of the data the applied method resulted in a multiplicity (two to four) of possible haplotype pairs. Haplotype reconstruction using PHASE v2.1.1 cleared this ambiguity in all cases but one, where an alternative haplotype pair was considered with a probability of 3%. The estimation of the NAT2 haplotype is important because the assignment of the NAT2 alleles *12A, *12B, *12C or *13 to the rapid or slow NAT2 status has been discussed controversially. A clear assignment is indispensable in surveys of human bladder cancer caused by aromatic amine exposures. In conclusion, PHASE v2.1.1 software allowed an unambiguous haplotype reconstruction in 2920 of 2921 cases (>99.9%).

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334

Metabolism studies of the PHENION® Full Thickness Skin Model compared to other *in vitro* models

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Skin metabolism has a great influence on the risk potential of substances applied topically in terms of transforming them into harmless metabolites or in contrast to convert them into sensitizers or genotoxic compounds.Several phase I and phase II enzymes are involved in the metabolism of a wide range of exogenous compounds. Like in other organs cytochrome P450 are the most important phase I enzymes. Also a range of flavin-containing monooxygenases (FMO1 – FMO5) seem to play an important role in skin metabolism, however much less is known about them e.g. their regulation or substrate specificity especially in the dermal compartment.Since human native skin is not easy to obtain and exhibit a high donor variability standardized produced 3D-Human Skin Models might not only be a good tool for risk assessment like skin irritation or corrosion but also might provide a good tool for investigating dermal xenobiotic metabolism.The Phenion® Full Thickness Skin Model consists of a dermal and epidermal layer. The interaction of both compartments is essential for cell differentiation, regeneration as well as xenobiotic metabolism.To investigate the suitability of *in vitro* models with regard to human native skin we compared the commercially available Phenion® Full Thickness Skin Model, an in house produced epidermis model and two monolayer cultures represented by fibroblasts and keratinocytes. To exclude possible donor variabilities all four *in vitro* models were constructed with cells from the same donor. In order to evaluate the suitability of the different models for analyzing skin metabolism *in vitro* we analyzed gene expression of phase I and II enzymes with quantitative realtime PCR. In addition to basal levels also the inducibility of several enzymes by typical inducers like β-naphthoflavone and all-trans retinoic acid was analyzed. Further approaches will comprise the analysis of additional phase I and II enzyme on gene expression-, protein expression level and enzyme activity as measured by analyzing biotransformation reactions with known model compounds.

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335

Acrylamide: formation of glycidamide and genotoxic effects in genetically modified V79 cell lines expressing CYP2E1 from different species

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There is some concern about possible health risks from dietary exposure to acrylamide (AA). AA has been shown to be neurotoxic, genotoxic and carcinogenic. In laboratory animals AA is metabolized to glycidamide (GA) catalysed primarily by CYP2E1. AA and its metabolites are rapidly eliminated in urine, mainly as mercapturic acid conjugates of AA and GA. GA is suggested to be much more reactive than AA with DNA. In a recent study we addressed the question if and to which extent GA is formed from AA in human and monkey liver microsomes as well as in genetically modified V79 cells expressing human CYP2E1. We now extended our studies using in addition genetically modified V79 cells expressing CYP2E1 from the rat or the mouse. In addition we investigated the genotoxic potency of AA and GA in these cells by using the COMET Assay. Incubations have been performed according to conventional procedures. Special emphasis was laid on the analytical detection of GA. ^{13}C-Glycidamide and D_3-acrylamide were added as external standards. GA and AA were extracted and refined from protein suspensions by using organic solvents and by elution through activated carbon-aluminium oxide-columns. The detection of GA was performed using gas chromatograph/mass spectrometer (Finnigan Mat SSQ 710) equipment. The results show that AA is metabolized to GA in genetically modified V79 cells expressing CYP2E1 from different species (human, rat, mouse) at various but low amounts. Formation of GA could be inhibited by e.g. MAB-2E1 antibody (monoclonal, raised in mouse, human CYP2E1 selective) to about 80% in cells expressing human CYP2E1. Significant DNA damaging potency could not be demonstrated when cells were incubated with AA despite the fact that GA could be determined. However when GA was added in various concentrations genotoxic effects were clearly detectable.

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336

Isolation and characterization of a steroid degrading bacterial strain

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Steroid contamination of sea water is an ever growing problem and impacts population dynamics of all kinds of sea animals. We have long experience with the soil bacterium *Comamonas testosteroni* which is able to catabolize a variety of steroids and polycyclic aromatic hydrocarbons, and which might be used in bioremediation of contaminated soil [1,2]. For our studies we use 3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) as a reporter enzyme, since it is the key enzyme in steroid degradation [3,4]. Moreover, the expression of the corresponding gene, hsdA, is induced by environmental steroids. In previous investigations we have identified and described several genes being involved in hsdA regulation [1,2].In this work we isolated several bacterial strains from the Baltic Sea at Kiel, Germany, which degrade steroids and which are able to use

steroids as carbon source. One of them, strain H5, was characterized as being gram negative and showing resistance against ampicilline and carbenicilline. It could be best grown in SIN medium supplemented with 1.6–4.1% NaCl. More than 80% of cholesterol was digested when the strain was grown in SIN medium with 0.05 mM cholesterol. Western blots revealed that this salt water strain H5 expresses a 3α-HSD/CR orthologous enzyme. Interestingly, 3α-HSD/CR expression in strain H5 increased after induction with 0.3 mM testosterone, 0.3 mM estrogen or 0.05 mM cholesterol. Therefore, strain H5 might be used for the bioremediation of steroid contamination in sea water. Isolation of the 3α-HSD/CR orthologous gene, as well as studies on its regulation and the generation of homologous hsdA knock-out strains of H5 are currently in progress. In addition, the exact characterization and systematic classification of this marine steroid degrading bacterial strain is envisaged.

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337

Isolation and activity identification of the LysR gene

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Comamonas testosteroni 3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) is a key enzyme in the degradation of steroid compounds and polycyclic aromatic hydrocarbons in soil, and may therefore play a significant role in the bioremediation of harmful compounds in the environment [1]. We previously reported the cloning and isolation of two repressors and an activator which regulate the gene expression of 3α-HSD/CR in *C. testosteroni* [2, 3]. After steroid induction 3α-HSD/CR gene expression increased. In the present study, we have identified the LysR gene as another and new activator gene for 3α-HSD/CR gene regulation. LysR was isolated from the chromosomal DNA of *C. testosteroni*. The LysR gene consists of 912 bp and locates 3.360 kb downstream from the 3α-HSD/CR gene. To produce purified LysR protein, the LysR gene was cloned into plasmid pET-15b and the overexpressed protein purified by its His-tag sequence on metal chelate chromatography. SDS PAGE revealed a molecular mass of the LysR protein of 33.6 kDa (303 aa). For functional studies and cotransformation experiments, the Tac promoter was cloned upstream of the LysR gene into pK18 to yield plasmid pKtac-lys1. Interestingly, 3α-HSD/CR expression increased when pKtac-lys1 was cotransformed with plasmid pAX1 (harbouring the 3α--HSD/CR gene) into *E. coli* HB101. We then produced knock-out mutants of the LysR gene in *C. testosteroni*. As expected, these knock-out mutants expressed low levels of 3α-HSD/CR, even after testosterone induction. From these results we conclude that LysR is an important player in the regulation of the steroid degradation pathway in *C. testosteroni*.

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338

A LC–MS/MS metabonomics investigation of mercapturic acid patterns in urine samples of smokers and non-smokers

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Metabonomics approaches are an emerging field, all having in common a screening method for the detection of metabolite changes and biomarkers in biofluids. Mercapturic acids (MA) are a known class of effect markers, resulting from detoxification processes of reactive compounds. Thus, a good correlation between MA-levels and the electrophilic burden of an organism, caused by e. g. tobacco smoke is anticipated. A LC-ESI-MS/MS method based on theoretical multiple reaction monitoring was used to screen the urine of 26 heavy smokers (11–x cigarettes/day), 56 moderate smokers (1–10 cigarettes/day) and 40 non-smokers for MA-levels. In addition, 18 quality control samples were analyzed to ascertain stability of the measurements. Sample cleanup and on-line enrichment of analytes was performed with a column-switching unit. To generate an aligned data matrix, the chromatograms were processed using MarkerView software 1.2.0.1. To correct for differences in total urine volume and detector sensitivity, every signal was normalized to specific gravity and scaled to the mean area of the four internal standards. Data was then subjected to multivariate data analysis performed with SIMCA-P+ software 11.5. Orthogonal partial least squares discriminant analysis (OPLS–DA) of pareto scaled data could easily separate heavy smokers from non-smokers. The model showed good characteristics of $R^2(X) = 0.33$, $R^2(Y) = 0.83$ and $Q^2 = 0.61$ for the three significant principle components. The corresponding loadings plots and S-plots provided 57 potential markers responsible for the group discrimination. Dose dependency of makers was evaluated using shared and unique structure (SUS) plots. Five markers were subsequently identified by comparison of their fragmentation patterns to authentic standards. Levels of acrylamide-MA, 2-cyanoethyl-MA, 3-hydroxypropyl-MA, 2-carboxy-1-methylethyl-MA and 3-hydroxy-1-methylethyl-MA were found to have higher intensities in the smoker groups or were completely absent in the non-smoker group, respectively. As can be concluded from these results, MA-screening shows great promise for the rapid and non-invasive detection of metabolite changes and biomarkers *in vivo*.

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339

***In vitro* stability of methyl methacrylic acid facing esterases**

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As already known from previous studies methacrylic acid (MA) is an intermediate in the metabolism of unpolymerisized dental restorative materials containing monomers like Bisphenol-A-glycidylmethacrylate (Bis-GMA) and comonomers like triethyleneglycoledimethacrylate (TEGDMA). These (co)monomers can be released into the oral cavity and thus they are able to enter the human body. In general these (co)monomers are methacrylic acid esters.Because of the chemical structure of these esters two different chemical pathways were suggested: saponification of TEGDMA or Bis-GMA leading to free MA as an ionic intermediate or enzymatically epoxidation leading to lipophilic intermediates, respectively. Whereas ionic intermediates are eliminated predominantly renal, lipophilic intermediates can be saved in fatty tissue and may cause long lasting effects.Therefore the stability of (co)monomers facing esterases was investigated in this study.Experiments have been performed in an isolated system with pig liver esterase in phosphate buffer using methylmethacrylate as a substitute for other methacrylates. It was found that saponification of (co)monomers depends on concentration of esterases thus the system acts like a Michaelis-Menten system. The fastest time for complete saponification was less than 5 sec and the slowest time was in the range of about 30 sec. These times were observed for both (co)monomers.Earlier studies revealed that completing the epoxidation of MA and its related esters took about 5 min or even longer therefore it must be concluded from the present results that the predominantly reaction pathway occurs via saponification with an ionic intermediate. Nevertheless it can not be excluded that epoxidation of lipophilic intermediates and storage of these intermediates in fatty tissue plays probably a minor role in metabolism of dental materials.

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340

Clearance of dental composite component BisGMA in guinea pigs

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Bisphenol-A-glycidyldimethacrylate (BisGMA) is used in many resin-based dental materials. It was shown *in vitro* that BisGMA was released into the adjacent biophase from such materials during the first days after placement. In this study the uptake, distribution, and excretion of [^{14}C]BisGMA applied via gastric and intravenous administration at dose levels well above those encountered in dental care were examined *in vivo* in guinea pigs to test the hypothesis that BisGMA reaches cytotoxic levels in mammalian tissues. [^{14}C]BisGMA was taken up rapidly from the stomach and intestine after gastric administration and was widely distributed in the body following administration by each route. Most [^{14}C] was excreted within one day as $^{14}CO_2$. The peak equivalent BisGMA levels in guinea pig tissues examined were at least one-thousand-fold less than known toxic levels. The peak urine level in guinea pigs that received well in excess of the body weight-adjusted dose expected in humans was also below known toxic levels. The study therefore did not support the hypothesis.

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341

Development of a simplified sample preparation procedure for in vivo analysis of TEGDMA and some of its related metabolites

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As shown in former studies dental materials based on methacrylic acid (MA) like triethyleneglycoledimethacrylate (TEGDMA)can be released in the oral cavity and thus into the organism after application. Subsequently these methacrylates can be metabolized *in vivo* as well as *in vitro* which was demonstrated in ^{14}C-experiments. In some of these studies several (toxic) metabolites like 2,3-expoxymethacrylic acid (2,3-EMA) were proved to be formed .The presented study was performed in order to develop two sample preparation procedures based on solid phase extraction (SPE) and solid phase micro extraction (SPME), respectively for some important analytes involved in the metabolism of TEGDMA. The sample matrix was Krebs-Henseleit-buffer, commonly used for *in vivo* experiments, e.g. liver perfusion. Gas chromatography-mass spectrometry was used for analysis.SPE was found to be the optimal preparation method for sample analytes TEGDMA and triethyleneglycole (TEG).

SPE cartridge	Bakerbond® Carbon 500 mg/6 ml; J.T. Baker
Conditioning	5 ml H_2O followed by 5 ml methanol
Sample volume	3 ml
Adsorption time on cartridge	5 min
Washing	4 x 2 ml H_2O
Drying conditions	vacuum, 5 min
Elution	acetone, 3 ml
Elution time on cartridge	5 min

To analyze samples containing methylmethacrylic acid and 2,3-EMA headspace-SPME was proven to be the most suitable method.

SPME fiber	65 µm PDMS/DVB coating
Matrix; matrix pH-value	Krebs-Henseleit-buffer; 7.4
Volume of sample	3 ml
Headspace above sample	7 ml
Equilibration of sample in water quench	20 min
Temperature during adsorption	46-48 °C
Adsorption time	15 min
Temperature of GC-injector	270 °C
Desorption time in GC-injector	2.3 min
Immersion depth in headspace/injector	3.8 cm

For the investigated analytes the SPE recovery rate was found to be in the range 80-110 % (TEGDMA 20 %).With these optimized sample preparation parameters it is possible to work up samples obtained from *in vivo* experiments using Krebs-Henseleit-buffer, in order to reveal the *in vivo* metabolism of MA based dental materials.

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342

Ethylene inhibits its own metabolism in liver and lung microsomes from male Fischer 344 rats and B6C3F1 mice

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Ethylene (ET), a gaseous olefin, is an important industrial chemical. It is produced endogenously in plants and mammals. In rodents, ET is biotransformed via CYP to ethylene oxide (EO). Metabolism of this epoxide could be catalyzed by epoxide hydrolase (EH). The aim of the present work was to investigate quantitatively the metabolism of ET and EO in microsomes of livers and lungs from mice and rats. Microsomal incubations with ET or EO were carried out at 37°C in closed headspace vials. An NADPH generating system was added to ET containing incubations. In addition, some incubations were carried out with heat-inactivated microsomes. ET and EO were monitored in the headspace using GC/FID. In some experiments, metabolically produced EO was detected by GC/MS. Microsomal CYP2E1 and EH activities were verified using chlorzoxazone and propylene oxide, respectively.Only when high microsomal protein concentrations (10 mg/ml) were used, an initial decline of atmospheric ET could be detected in incubations with rat liver microsomes. In all ET incubations with active microsomes, metabolically formed EO appeared immediately in the gas phase reaching plateau values. Initial rates of EO formation from ET followed Michaelis-Menten kinetics. Maximum rates of EO formation were 0.82 and 1.6 (nmol/min/mg) in liver microsomes of rats and mice, respectively. In lung microsomes, maximum rates (nmol/min/mg) of EO formation were substantially lower, 0.05 (rat) and 0.04 (mouse). Neither a decline of ET nor any EO was observed when incubating ET with heat-inactivated microsomes. In all EO incubations practically no loss of the EO concentrations occurred.The results hint to a suicide inactivation of the ET metabolizing CYP species in livers and lungs of rats and mice leading to a complete loss of EO formation. Furthermore, if at all, EO is only a poor substrate for microsomal EH of rodents. Financially supported by the American Chemistry Council.

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343

Changes in intraepithelial lymphocytes of gut associated lymphoid tissue in AhR-deficient mice

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The majority of lymphocytes in the body is found in the gut associated lymphoid tissue (GALT) as intraepithelial lymphocytes (IEL). The gut is continuously exposed to harmless nutrients and potentially harmful pathogens, and GALT maintains a balance of immunity and tolerance. The arylhydrocarbon receptor (AhR) is a sensor of small molecular weight chemicals, which convert the AhR to its active form as a transcription factor. The immune system is sensitive to systemic over-activation of the AhR. However, GALT has not been studied so far, although AhR-binding substances (e.g. flavonoids and indoles) are abundant in food (e.g. apples or onions), and oral uptake is the major route of exposure for the AhR-ligand dioxin. We determined the frequencies of lymphoid cell subpopulations in GALT in C57BL/6 wild-type and AhR-deficient mice (wt, AhR-ko). Cellularity did not differ in both cases in intraepithelial lymphocytes (IEL), Peyer's Patches (PP) or mesenteric lymph nodes (MLN). Between AhR-ko and wt mice, lymphocyte subset frequencies were comparable for PP and MLN. However, significant differences in IEL subset frequencies were detected. In IEL from AhR-ko mice CD8αα receptor T-cells were reduced from approximately 60% to 20%. As only this subset contains gdTCR-T-cells, this population is decreased as well (from appr. 30% in wt to about 10% in AhR-ko mice). Concomitantly, the regular CD8αβ-αβTCR+-T-cells increased in frequency and absolute number. We fed mice with 10µg TCDD/kg body weight, and analysed IEL cells 2 days later. Co-stimulatory markers CD28 and CD25 were increased on T-cells from PP and MLN. In conclusion, loss of the AhR affects IEL subset frequency, including a loss of potentially immuno-modulating CD8+ gdTCR+ cells while over-activation affects functionally relevant parameters on T-cells from MLN and PP. We are currently investigating further changes in IEL of AhR ko and of TCDD-exposed mice.

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344

Effects of concurrent exposure to arsenic through drinking water and endosulfan via diet in broiler chickens: a subchronic immunotoxicological study

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The aim of the present study was to evaluate the effects of concurrent exposure to the global groundwater metal contaminant arsenic and the organochlorine insecticide endosulfan on the immune system of broiler chickens. Day-old chicks were exposed to arsenic (3.7 ppm) via drinking water at the maximum groundwater contamination level reported in West Bengal, India, and to 30 ppm of endosulfan-mixed feed either independently or concurrently for 60 days. All birds were vaccinated against Ranikhet disease virus (F-strain; RD-F) on days 1 and 30. None of the treatments altered the absolute body weight or body weight gain, except arsenic significantly reduced weight gain on day 60. Absolute, but not the relative, weights of spleen, thymus, bursa of Fabricius, kidneys, heart and brain were significantly reduced in all treatment groups. Absolute weights of lungs were decreased by arsenic and endosulfan given alone, but their relative weights remained unaffected. The absolute weight of liver was not affected but its relative weight was increased in birds exposed to arsenic or endosulfan. In vitro lymphocyte proliferation assay after stimulation with the antigen RD-F or the mitogen concanavalin A, and the *in vivo* delayed type hypersensitivity reaction against 2, 4-dinitro-1-chlorobenzene or the mitogen, phytohaemagglutinin-P indicated that cell-mediated immunity was significantly suppressed, and results of ELISA showed suppression of humoral immunity with all these treatments. The reduction in nitrite production by mononuclear cells after stimulation with RD-F or lipopolysaccharide indicated that the treatments also affected non-specific immunity. Interestingly, the effects of the concurrent exposure on the immunological parameters were mostly not statistically different from those produced by either one of the agents. It can be concluded that exposure to arsenic, at the groundwater contamination level, or to endosulfan alone caused immunosupression in broiler chickens almost comparable to that produced by their concurrent exposure.

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345

Effects of the dental monomer TEGDMA on the cytokine secretion in murine RAW264.7 macrophages

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Monomers like triethylene glycol dimethacrylate (TEGDMA) are released from polymerized dental composite materials. These compounds influence the homeostasis of exposed cells including those of the immune system. In a recent study we found that TEGDMA down-regulated the lipopolysaccharide (LPS) induced expression of the surface antigen CD40 (T-cell interaction), whereas CD54 (cell adhesion) was up-regulated by LPS and TEGDMA. Besides these surface antigens, pro- and anti-inflammatory cytokines like TNF-α, IL-6, and IL-10 play a pivotal role in the immune response against pathogens and other foreign substances. Therefore, murine macrophages (RAW264.7) were exposed to TEGDMA (0.125-2.0mM) for 6, 24 and 48h in the presence and absence of LPS. Cytotoxicity of TEGDMA and LPS/TEGDMA was detected photometrically using the crystal violet assay. The release of TNF-α, IL-6, and IL-10 from cell cultures was determined by enzyme-linked immunosorbent assay (ELISA). Statistical analyses were performed using the Mann-Whitney-U-test ($p<0.05$). LPS alone strongly induced secretion of all cytokines after the various exposure periods. Amounts of TNF-α, IL-6, and IL-10 were increased 200-fold, 600-fold, and 15-fold compared to untreated controls after a 6h exposure. The amounts of TNF-α and IL-6 were further increased about 2-fold after 24h and 48h, and IL-10 levels increased about 100-fold. TEGDMA alone had no influence on cytokine secretion. However, LPS-stimulated cytokine secretion was inhibited by increasing TEGDMA concentrations at all time points. The addition of 0.125mM TEGDMA to LPS-stimulated cell cultures reduced TNF-α, IL-6, and IL-10 to 92%, 87%, and 75% compared to LPS alone, while 2.0mM TEGDMA even decreased these values to 0.7, 2.4%, and 1.2% after 6h. Inhibition of cytokine release by TEGDMA was even higher after 24 and 48h. Furthermore, complementary crystal violet staining showed that the reduction of cytokine secretion by TEGDMA was only partly caused by reduced cell numbers and mainly by the reduced ability of each cell to produce cytokines. We conclude that TEGDMA has the ability to suppress LPS-stimulated responses of the cells of the innate immune system.

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346

Effects of perfluorinated fatty acids (PFCA) on lymphocytes and dendritic cells *in vitro*

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Perfluorinated fatty acids (PFCAs) are widely used as anti-wetting agents, plasticizers, corrosion inhibitors, and fire extinguishers. We studied the immunomodulatory potential of three PFCAs, ammonium perfluorooctanoate (APFO), perfluorooctanesulfonic acid (PFOS) and perfluorodecanoic acid (PFDA) using two different *in vitro* models. First, lymphocytes isolated from spleens of 4 months old BALB/c mice were incubated in the presence of PFOS, APFO and PFDA at 0.1, 1, 10, or 100 µM for 24, 48, and 72 hrs, and changes in percentages of subpopulations of lymphocytes (CD4+, CD8+, CD45+) were monitored by flow cytometry. Incubation of cells with PFOS at 1 µM for 48 h led to a significant decrease of CD4+ cells (5.7%) and CD8+ cells (14.4%) while percentages of CD45+ cells increased (24%). Similar effects were observed with higher concentrations (10 and 100 µM) and also after 72 h of incubation. At concentrations of 0.1 µM, PFOS still significantly increased CD45+ cells (30%) at 72 hr, as compared to controls. PFDA caused a significant decrease (5.5%) in CD4+ cells at 24 h of incubation. Incubation with 1 µM APFO for 48 h showed a significant decrease (6.3%) in CD45+ cells. Additionally, immature human monocyte-derived dendritic cells (DCs) obtained by culturing CD14+ cells in the presence of GM-CSF and IL-4 for 6 days were incubated with APFO (1, 10, or 100 µM) for 2 days and simultaneously activated by the addition of pro-inflammatory cytokines. AFPO did not affect the expression of phenotypic markers such as CD14, CD25, CD80, CD83, CD86 and HLA-DR, the IL-12 secretion of the cells, or their capacity to stimulate allogeneic T cell-proliferation. These results show the immunomodulatory effects of PFCAs on mouse lymphocytes, while human monocyte-derived DCs were not affected under our experimental conditions. Further characterization is ongoing.

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347

The estrogenic potency of the heavy metal cadmium depends on the route of administration

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Humans are exposed to cadmium (Cd) from many sources, food being the most relevant source of Cd exposure in non-smokers. The toxic heavy metal shows estrogenic activity in cultured cells, and can elicit typical estrogenic responses, e.g. uterine weight increases in rats and in mice upon i.p. injection. Estrogenicity of Cd has not been documented with other, more relevant routes of exposure, although it is known that its distribution in the body is strongly affected by the application route. Thus, we

investigated and compared estrogenic activity of Cd in Wistar rats, after per os and i.p. administration, in typical estrogen target organs and in the intestine, a non-classical target tissue.Changes in estrogen-regulated gene expression and uterine weight are suitable markers for estrogenicity in ovariectomized rats. Using real time PCR progesterone receptor, estrogen receptor and proliferating cell nuclear antigen mRNA level were analyzed in the uterus, and expression of vitamin D receptor (VDR) in the intestine. The analysis revealed pronounced differences in hormonal potency of Cd for the two routes of administration: A single i.p. dose of Cd (0.5 µg/kg bw and 2000 µg/kg bw) resulted in clear increases of uterine weight, similar to those elicited by i.p administration of estradiol (E2). E2 is also effective upon oral dosing, but, per os administration of Cd (4 d up to 4 w) did not result in an increase of uterine weight. Liver wet weights decreased after a Cd administration of 143 mg/l in drinking water. VDR mRNA expression was stimulated by both Cd and E2 at doses of 2000 µg/kg bw upon i.p. application, whilst exposure to 0.5 µg Cd/kg bw per os led to a decrease in VDR mRNA expression in the small intestine.In summary: In agreement to published data our *in vivo* study confirmed estrogenic effects of Cd on the uterus upon i.p. injection. But, oral short and long-term administration of Cd had no estrogenic effect on the uterus indicating low systemic bioavailability by this route. Yet, modulation of VDR expression demonstrates that oral intake of Cd induces estrogenic signalling in the small intestine.
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348

Modulation of estrogen-dependent gene expression in breast and colon cancer cell lines by the heavy metal cadmium

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The toxic heavy metal cadmium (Cd) is an important environmental pollutant, thought to act also as an endocrine disruptor. Previously, it was shown that Cd mimics the effects of estradiol (E2) in MCF-7 cells, evidenced by an activation of the estrogen receptor α (ERα) along with increased cell proliferation, a decrease in ERα mRNA expression, and induction of some estrogen-regulated genes. Since the intestine is the most important organ for interactions between dietary contaminants and the body, the aim of our project is to investigate the ER-mediated Cd effects in the intestine in comparison to classical estrogen-sensitive tissues.To examine ER-mediated Cd signalling in vitro, the expression of estrogen-sensitive marker genes (ERα, ERβ), progesterone receptor (PR), vitamin D receptor (VDR)) were analysed in the colon carcinoma cell lines HT-29 and HCT-116 as well as in MCF-7 breast cancer cells by semi-quantitative and real time-PCR. Also, expression of metallothionein was studied.In MCF-7 cells, treatment with E2 (0.01 µM) led to an increase of PR mRNA and a down-regulation of ERa expression. Cd exposure (0.0001 up to 10 µM) resulted also in down-regulation of ERa, but did not stimulate PR mRNA expression. This is a distinctive antiestrogen expression pattern. In colon HT-29 and in HCT-116 cells the vitamin D receptor (VDR) mRNA level were decreased upon treatment with Cd (0.0001 µM up to 1 µM) and E2 (0.01 µM). Interestingly, cells, expression of ERα tended to be down-regulated in HT-29 cells after treatment with E2 whereas Cd led to an up-regulation of ERα expression. Metallothionein 2a (MT2a) expression was clearly increased in the colon cells after treatment with 1 µM Cd, indicative of an adaptation to higher Cd concentrations. The hormonal effects in the colon cell lines occurred at Cd concentrations where MT-expression is unaffected.These data show that Cd alters the expression of classic molecular markers for estrogenicity and the VDR in breast and in colon cells. Interestingly, the detected gene expression pattern appears to be indicative for an antagonistic interaction of the heavy metal with the ER. This has to be confirmed in future investigations.
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349

Effects of triphenyltin on brain aromatase activity in male and female rats after in utero and postnatal exposure

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Introduction: The organotin compound triphenyltin is used extensively as a pesticide in agriculture as well as in marine antifouling paints. It has been shown that TPT can cause changes in male and female reproductive organs of rodents and it is theorized that inhibition of aromatase might be one possible mode of action. Materials and methods: Gravid Wistar rats were treated with triphenyltinchloride (TPTCl) per gavage from gestational day (GD) 6 until the end of lactation at dose levels of 0 or 2 mg TPTCl/kg b.w./day. Vehicle control received 5 ml/kg peanut oil. After weaning offspring were divided into two groups per dose level. In group A treatment was continued, in group B animals received no further treatment. A separate control group was assigned to both group A and B. Male offspring were sacrificed on PND 58 and female offspring were sacrificed in estrus determined through vaginal smear. Subsequently aromatase activity in the preoptic area of the brain was determined in both sexes of all dose groups. Results and conclusion: Females in group A exhibited a statistically significant increase in brain aromatase activity compared to control, while no change in aromatase activity was observed in females of group B (Group A: Control 15.5±5.4, 2 mg 43.0±24.8; Group B: Control 26.9±15.6, 2 mg 24.5±13.3; fmol/mg protein/15 min, mean±SD). Male offspring in group A exhibited a statistically significant decrease in brain aromatase activity while in males of group B no alterations occurred (Group A: Control 25.5±5.8, 2 mg 18.1±5.4; Group B: Control 26.2±4.9, 2 mg 27.1±6.3; fmol/mg protein/15 min, mean±SD). We conclude that exposure to 2 mg TPTCl/kg b.w. from GD 6 until early adulthood resulted in significant changes in brain aromatase activity in male and female offspring. While in males a significant decrease was observed, females exhibited a significant increase in aromatase activity. In contrast, after in utero and lactational treatment no changes were observed in either sex at early adulthood. These results are consistent with effects of TPT treatment on sexual development in male and female rats we reported earlier.
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350

Impact of para-phenylenediamine on cyclooxygenases expression and prostaglandin formation in human immortalized keratinocytes (HaCaT)

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Para-phenylenediamine, a monocyclic arylamine, is a frequently used chemical and common contact allergen but informations on its effects are limited. Cylooxygenases, the key enzymes in prostaglandin synthesis, exhibit manifold functions in the skin, including inflammation and immune responses, proliferation and differentiation of keratinocytes. We therefore studied if para-phenylenediamine impacts on the cyclooxygenase pathway in human immortalized keratinocytes (HaCaT). We analyzed COX-1, COX-2 and cPLA2 steady state mRNA levels for 100µM to 400µM PPD after 2-24h and found significant COX-2 induction for 400 µM PPD after 24h, while cPLA2 and COX-1 levels were increased dose-dependently between 8h and 24h. Increased expression was accompanied by enhanced prostaglandin E_2 and $F_{2\alpha}$ formation. Specific involvement of COX enzymes was confirmed by prostaglandin estimation in the presence of exogenous arachidonic acid and inhibition experiments using COX inhibitor NS-398. In addition, para-phenylenediamine-induced prostaglandin formation was completely inhibited in cells pre-stimulated with the anti-oxidant *N*-acetyl cysteine. We found that HaCaT have intrinsic NAT1 acetylation capacities, although they seemed saturated by the applied para-phenylenediamine concentrations. Further investigations of generated amounts of mono-acetyl-PPD, di-acetyl-PPD and the auto-oxidation product Bandrowski's base during para-phenylenediamine treatment revealed that the latter compounds were not able to impact COX pathway and prostaglandin E_2 formation. In sum, our results demonstrate that para-phenylenediamine or early formed instable derivatives impact on COX expression and activity in target cells, likely via oxidative processes. Moreover, acetylation capacities which possibly anticipate oxidative processes considerably influence PPD-mediated COX induction.
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351

UVB-induced COX-2 expression in HaCaT keratinocytes: Modulation at the posttranscriptional level

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Cyclooxygenase-2 (COX-2) is a pivotal player in inflammatory processes, the expression of which in skin cells is known to be stimulated by ultraviolet (UV) radiation. It is demonstrated here that exposure of HaCaT human keratinocytes to UV-B radiation (280-320 nm) causes an enhanced expression of COX-2, as shown both by RT-PCR and Western blotting, whereas UV-A (320-400 nm) was ineffective. Exposure of cells to UVB resulted in a dramatic increase in stability of COX-2 mRNA. Furthermore, p38MAPK was strongly activated under the irradiation conditions chosen. Stabilisation of COX-2 mRNA as well as the elevation of COX-2 steady-state mRNA and protein levels following UV-B irradiation were prevented by inhibitors of p38MAPK, suggesting that UV-B-induced COX-2 expression is brought about by p38MAPK-dependent COX-2 mRNA-stabilisation. A well-known mediator of p38-dependent mRNA stabilisation, hnRNP A0, was demonstrated not to be involved in COX-2 induction by siRNA depletion experiments. In contrast, the stress-responsive ELAV-family mRNA stabilising protein HuR was found to be essential in bringing about COX-2 expression upon exposure to UV-B. The mode of action of HuR under the influence of UV-B is presently being explored.
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352

Circadian gene expression in human keratinocytes

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Circadian regulation of gene expression is a common phenomenon that has been observed in many organisms ranging from plants to animals. Circadian rhythmicity is thought to adapt the organism to ambient environmental conditions by regulating physiological and behavioral responses. Not only the brain regulates circadian rhythm of the organism, but also peripheral cells and organs possess rhythmicity that respond to environmental stimuli like ultraviolet light (UV). The basic molecular players of the circadian clock are the PAS-related (homologous to Per/ARNT/Sim) proteins Period, Clock and Bmal-1 and the non PAS proteins Cryptochrome and Timeless.Chronobiological effects seem also to be important for regulation of skin homeostasis under healthy and diseased conditions and may have an impact on the pharmacotherapy. However the mechanisms of regulation of the circadian rhythmicity in the skin are not well understood.In the past we reported that ultraviolet light (UVB, 100 J/m^2) induces a shift in circadian gene expression in HaCaT cells. This was not observed when the cells were deficient for the Arylhydrocarbon Receptor (AhR). In order to characterize this observation in more detail we started to optimize our cellular system in order to achieve circadian oscillation over a period of more than 24 hours. After starving HaCaT keratinocytes for 24 hours in serum free medium, followed by a two hour serum shock (10 or 50% serum), we could show that HaCaT keratinocytes display a stable circadian expression of the clock genes Period-1 and Bmal-1 over 72 hours. Moreover, in our system starvation of the cells seems to be the key factor for circadian synchronization. We are currently working on translating this procedure into primary human keratinocytes.The role of the AhR in circadian gene expression of HaCaT keratinocytes and primary human keratinocytes will then be subject of further investigations.
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353

Effects of the upper respiratory tract of male healthy non-smokers exposed to 0.21 ppm ozone

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Introduction: In-vitro studies on nasal tissue described increased concentrations of cytokines, especially IL-1beta and IL-8, due to ozone exposure compared to filtered air. Also a negative effect of ozone on the ability to smell is known. Our research's aim was to analyse the effect of short term ozone exposure near high environmental ozone concentrations on the ability to smell and certain nasal inflammatory markers. Methods: The ability to smell and the concentrations of IL-1beta and IL-8 in nasal secretion in healthy, normosmic adult men were determined in a randomised parallel group design. The "ozone group" (n=15, median age 23 years) was exposed to 0.21 ppm ozone while the control group (n=13, median age 24.5 years) was exposed to filtered air in an exposure chamber. Exposure time was 2 hours. The ability to smell was determined by Sniffin' Sticks *n*-butanol odor threshold before and 70 minutes after exposure. Nasal secretion was collected by foam rubber pads at least one week before and 85 minutes after exposure. Concentration of IL-1beta and IL-8 was determined by commercial ELISA kits. The study was approved by the local ethics committee. Results: The medians of the differences of the n-butanol odor threshold values after and before exposure accounted -1.25 for "ozone group" and 0 for control group ($p<0.001$, Mann-Whitney-Test). There were no significant differences of the IL-1beta and IL-8 concentrations after and before exposure between both groups (median IL-1beta values ozone vs. control: -20 pg/ml vs. -13 pg/ml, p=0.306, Mann-Whitney-Test; mean IL-8 values: -342 pg/ml vs. -245 pg/ml, p=0.44, t-Test). Subjective respiratory symptoms were shown by using a standardised questionnaire. Discussion: The increased odor threshold after ozone exposure is a subclinical effect. This could be possibly caused by an inflammatory edema that affected the diffusion of odor molecules at the olfactory mucosa. For a detectable increase of cytokines the duration of exposure and period after exposure until sampling the nasal secretion were not as long as in other studies that used even higher ozone concentrations. Since cytokine levels were showing a high interindividual variation in this study, a larger number of subjects might be required to reveal small effects. Measuring the olfactory threshold seems to be a sensitive method to detect irritating effects of gases and vapors.

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354

Inhalation toxicity of nano-scale zinc oxide in comparison with pigmentary zinc oxide using short-term inhalation test protocol

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With the rapid development of the nanotechnology, concerns about the possible health effect of these new materials rise. Among the possible effects on human health, inhalation of aerosols from nanomaterials, is of the highest concern (based on existing experience with fine and ultra-fine dusts). To ensure the product safety of nanomaterials, BASF developed a testing strategy to examine the potential inhalation toxicity of manufactured nanomaterials. The standard protocol for inhalation toxicity provides a 5 day inhalation exposure (6 hours/day) in male Wistar rats and examinations at two time points: immediately after the last exposure and three weeks thereafter, histopathological evaluation of the respiratory tract and the examination of blood parameters are scheduled. Bronchoalveolar lavage is scheduled three days after the last exposure and after a recovery period of three weeks. The parameter set examined in blood and lavage fluid was developed earlier (Landsiedel et al. 2007) and has been predictive for effects after long-term exposure with nano-TiO2. Further studies with various nanoscale materials are ongoing to proof the standard short-term inhalation test for nanomaterials. In this context we studied the inhalation toxicity of nanoscale ZnO as a partly soluble material according to this protocol. To evaluate the impact of the particle size on the inhalation toxicity, pigmentary ZnO was tested. Rats were exposed to 0.5, 2.5 and 12.5 mg/m^3 nano-ZnO and 12.5 mg/m^3 pigmentary ZnO for five days. Additional to the parameters for toxic effects, we looked at the kinetics of inhaled ZnO by examination of the organ burdens in seven tissues immediately after the last exposure and three weeks thereafter. An extensive set of parameters (cytokine profiles, indicators of oxidative stress and indicators of complement activation) was analyzed in lung lavage fluid and in blood. Moreover, the cell proliferation rate was determined in the lung compartments.Summarizing the result, we observed cytotoxic effects on upper and lower respiratory tract and interstitial inflammation in the lungs. All effects were reversible within 3 weeks. The effect of the nanomaterial was comparable to that of the pigmentary material.

Landsiedel R, Ma-Hock L, Burkhardt S, Strauss V, Gamer A, Wiench K, van Ravenzwaay B: Toxicity of Nanoparticles: Developing a standard short-term inhalation test. 48th Spring Meeting of the German Society for Pharmacology and Toxicology, Mainz, 2007 Abstract: Naunyn-Schmiedeberg's Arch Pharmacol Volume 375 (Supplement 1) 2007, 369

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355

***In vitro* and *in vivo* screening of the cytotoxic, pro-inflammatory and genotoxic potential of quartz-containing ceramic dusts**

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Inhalation of respirable crystalline silica (RCS) may lead to lung diseases, e.g. chronic inflammation, silicosis and cancer. Therefore, occupational exposure levels for quartz are set across the European Union. But, depending on quartz polymorph and industrial sector, lung-reactivity of RCS-containing dusts may differ substantially. Compre-hensive physicochemical and biological data sets are thus strongly needed to allow discrimination. In the European Collective Research Project SILICERAM an *in vitro* screening battery with primary rat alveolar macrophages was established and used to evaluate the cytotoxic, genotoxic, and inflammatory potential of 5 RCS-containing ceramic dusts. Aluminum lactate, a well-known quencher of biological quartz effects enabled estimation of quartz-specificity. Dust samples (3.1 to 8.1% quartz) collected at divers industrial sectors/production steps, i.e. brick (BR), tiles (TI), refractory ware (RW), and tableware granulate (TG) and cast (TC), were tested and compared to Al_2O_3 (negative control) and quartz DQ12 (positive control). After 2h of incubation, DQ12 and TC strongly increased lactate dehydrogenase levels, while all other dusts mediated only moderate, mostly not quartz-specific impairment of membrane integrity. In the Comet assay BR and TI exhibited the highest DNA-damaging potential, followed by DQ12>TG>TC>RW. Clastogenic potential was more or less quartz-related, but without clear correlation with the quartz content. After 4h of incubation, all dusts induced PGE2-liberation (DQ12>BR>TC>TG>TI>RW). In a subsequent 28-day *in vivo* study, DQ12, TG, and a contrived sample (CS: 30% DQ12, 20% feldspar, 50% china clay) were intratracheally instilled in rats, using equal amounts of quartz (0.29 mg/animal). TiO_2 (rutile) served as negative, saline as vehicle control. Interestingly, endpoints in bronchoalveolar lavagate and histopathology demonstrated substantial differences between TG, CS and DQ12. In conclusion, *in vitro/in vivo* data pointed to striking distinctions in the toxic potential of various RCS-containing ceramic dusts, probably due to a varied composition and differences in the quartz-covering effects of dust components.

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356

Hydrogen peroxide influences barrier function and mucociliary function: a human bronchial co-culture model

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Ozone (O_3) is a major component of smog and on inhalation is a toxicant to the lung. O_3 rapidly reacts with the airway epithelial cell membrane phospholipids to generate lipid ozonation products. Ozone is believed to cause damage to biological tissues either by direct reaction and/or through the formation of free radicals and reactive oxygen intermediates like hydrogen peroxide.After an explant-outgrowth culture of epithelial cells from small bronchi pure populations of primary isolated normal human bronchial epithelial cells (NHBE) were cultured with lung fibroblasts as bilayer on a 24-well HTS-Transwell filter plate. The cells were grown on a collagen type I and maintained at an air-liquid interface (ALI) by feeding basolaterally with medium. Barrier properties and morphological phenotype were studied over a period of 31 days. The NHBE formed confluent layers, expressing functional tight junctions as measured by transepithelial electrical resistance (TER). H_2O_2 is unstable and may be rapidly decomposed in the cell culture medium or released by cells. To determine if the treatment with H_2O_2 influences the co-culture, cells were treated apically and basolaterally with H_2O_2 in concentrations between 0.1 to 0.5µM and were then further cultivated for 24h. TER was measured every hour after addition. Depending on the different concentrations TER decreased in the first 5h up to 20% of the control. Cells recovered subsequently and TER increased back to 100%. An increased release of LDH was observed in comparison to the untreated controls. Immunfluorescence labelling showed that H_2O_2 disrupts the epithelial cell-cell junctions. Ciliary structure was destroyed and mucus production was induced. H_2O_2 also showed a stimulatory effect on the proinflammatory cytokines IL-6 and IL-8. In summary, H_2O_2 induced significant changes in barrier functions and morphological structures. These data indicate that our *in vitro* model reflects important characteristics of H_2O_2 mediated cytotoxicity effects and could be a useful system to study pathomechanisms of lung toxicity.

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357

Effect of cytokines on multidrug resistance related proteins (MRPs) in human lung cells in culture

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In acute inflammation, a group of inflammatory mediators termed the pro-inflammatory cytokines are released from macrophages in a coordinated manner. Among them, IL-6, IL-Iß, and TNF-alpha, are the principal mediators of the acute phase response (APR). There have been numerous studies which demonstrate that acute inflammation and cytokines have an influence on the expression of MRP family members in human colon carcinoma cell lines and rat hepatocytes. We wanted to establish whether similar regulatory pathways exist in humans. We therefore examined the effect of IL-6, IL- Iß and TNF-alpha on MRP expression at both protein level using western blot test and functional level by means of single cell fluorometry techniques in human lung cells in culture. Treatment of A549 cells with IL-6, IL Iß from 24 to 72 hours increased the expression a of MRP1 (1.3 and 1.5 fold, respectively) and MRP2 (1.3 and 2.1 fold, respectively) while no alteration of MRP3 expression was detected. On the other hand TNF-alpha has had no effect on MRP1 expression. In cultivated normal human bronchial epithelial cells (NHBEC), MRP1 expression was increased (1.8 fold) upon IL-6 treatment from 24 to 72 hours but not with IL-1ß or TNF-alpha. In NHBEC cells treated with IL-6, IL- Iß and TNF-alpha no change in both MRP2 and MRP3 expression was seen. As compared to controls, IL-6, IL- Iß and TNF-alpha caused an up-regulation of MRP-mediated CDF efflux in NHBEC cells from 24 to 72 hours (1.6, 1.5, 2 fold respectively) indicating higher MRP activity. Similary A549 cells showed an induced MRP activity after 24 to 72h of IL6 and IL- Iß treatment. As opposed to that, TNF-alpha imposed a down-regulation of MRP-mediated CDF efflux in A549 cells after 72 hours. We conclude that these cytokines may play a role in the regulation of the tested transporters.

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358

Glucocorticoid treatment of alveolar epithelial type II-like cell lines increases oxidative stress induced by tertiary butyl hydroperoxide

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Treatment with glucocorticoids is a common therapeutic option in inflamma-tory processes and such processes often are accompanied with cellular oxidative damage. Glucocorticoid treatment of alveolar epithelial type II-like (AE II) cells is followed by an increase in zinc-mediated toxicity caused at least partly by a decrease in cellular glutathione content. The question arises whether the glucocorticoid treatment enhances the susceptibility towards oxidative stress of other causes than zinc in this cell type, too. Cells of two AE II cell lines (L2, A549) were pretreated with 7.5 µmol/l dexamethasone for 72 h, then incubated with hydrogen peroxide (H_2O_2) or tertiary butyl hydroperoxide (tBHP). As parameters to assess toxicity the methionine incorporation, reduced glutathione content and changes in oxidised glutathione ratio were measured. Additionally, influence of de novo glutathione synthesis was estimated by increasing its synthesis by *N*-acetyl cysteine (NAC) administration. Dexamethasone decreased the GSH content, protein content and glutathione reductase activity of the cell layers (without peroxide). In both cell lines toxicity of tBHP was increased as assessed by all parameters tested. H_2O_2 toxicity was non-significantly increased in L2 cells. In this cell line toxicity of tBHP was clearly dependent on glutathione synthesis, while H_2O_2 toxicity did not seem to be influenced by increasing the glutathione synthesis due to NAC treatment as compared to the treatment with the D-enantiomere of NAC lacking glutathione synthesis probability. The results demonstrate an increase in glutathione dependent oxidative stress in AE II cells after glucocorticoid treatment. Because of the important functions of this cell types (surfactant synthesis, stem cells of type I alveolar epithelials, edema resorption) such an effect would be detrimental *in vivo* as well, especially in respect to pulmonary restitution.

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359

Human lung cell cultures as model system for the effects of various heavy metals

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Even though inhalation is not the main route of exposure to heavy metals, human lung is constantly exposed to low levels of various heavy metals from diverse sources. This situation might among other effects lead to increased expression of multidrug resistance associated proteins (MRP), which might protect lung cells by exporting glutathione complexes of heavy metals. We used primary cultures of normal human bronchial epithelial cells (NHBEC), peripheral lung cells (PLC) and the human lung tumour cell line H322 to study the effects of As(III), Cd(II), Co(II), Cu(II), Hg(II) and Pb(II) on cell viability. Viability was measured by MTT-Assay. Cultures from higher generations of NHBEC from different donors were more sensitive to Cd(II) (24h) (65% viability at 10 µM vs. 90% viability at 10 µM) than cultures at start. NHBEC treated with Co(II) (24h) did not show altered tolerance to Co(II) depending on generation or passage of culture. Incubation (24h) of NHBEC with concentrations of Pb(II) close to maximum solubility (35 µM) did not decrease cell viability. We treated NHBEC and H322 cells with MK571, an inhibitor of MRP1-4 and As(III), Cu(II) or Hg(II) respectively for 24h. MK571 increased sensitivity of NHBEC and H322 to As(III) and Hg(II), but not to Cu(II). We exposed NHBEC from a single donor to As(III) (2.5 and 5 µM) for four weeks and analysed expression of MRP1-5 using real-time PCR. After the first week MRP1, 3 and 4 were decreased (0.5 to 0.6 fold). The second week of treatment down-regulated MRP1, 3, 4, and 5 (0.4 to 0.5 fold). The third week did not affect MRP1, 4 and 5, but decreased MRP3 (0.6 fold). The fourth week did not alter expression of MRP1, 3, 4 and 5. The analysed MRP isoforms were detectable throughout the whole experiment and cells did not show any signs of toxic effects. We conclude from these results that long term exposure to As(III) alters expression of MRP transporters in primary cultures of NHBEC.

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360

Ultrafine particles evoke a release of catecholamines in the isolated heart

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In our recent research on the effects of ultrafine particles (UFP) on isolated Langendorff hearts of guinea pigs we could detect an increase in heart rate and the occurrence of arrhythmia. We hypothesized that these effects are caused by a release of catecholamines from the heart nerve endings. (N-S Arch. Pharmacol. 371, R434 (2005), N-S Arch. Pharmacol., 372, R 384 (2006)). To prove this we performed experiments with the betablocker metoprolol and later on with reserpine pretreated animals. Hearts of guinea pigs were prepared for mounting them in a Langendorff equipment. To get rid of blood residues and stress hormones which are discharged from the heart during preparation, the isolated heart was eluted with Krebs Henseleit buffer (KHB) for one hour. Afterwards the KHB was recirculated an additional hour until reaching a stable heart rate. UFP were added and circulated for the next hour followed by one hour of treatment with 3 µg/ml metoprolol.In other experiments animals were pretreated with reserpine (5 mg/kg bodyweight) which depletes almost all catecholamine reservoirs. TiO_2 evoked an increase in heart rate by 10% and Printex 90 by 8%. After addition of metoprolol the heart frequency decreased in both cases and reached nearly the rate before addition of UFP. This is a hint that the increase in heart rate was evoked by catecholamines. To ensure our hypothesis experiments with hearts of reserpine-pretreated animals (for 24 h) were done. In these cases no significant increase in heart frequency after addition of UFP could be observed. This is further evidence that UFP affect the release of catecholamines.The UFP evoked increase in heart rate can be explained by a release of catecholamines, e.g. noradrenaline out of nerve endings in the hearts. This result can also explain mechanisms of the epidemiological findings on cardiovascular diseases. Therefore the isolated heart is an appropriate model to test the toxicity of nanomaterials.

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361

Possible signalling pathways for the cancer promotion through fumonisin B_1

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Fumonisins are a group of structurally related mycotoxins produced as secondary metabolites by species of the genus Fusarium, fumonisin B_1 (FB_1) being the most abundant one. This fungal metabolite causes several diseases in animals and chronic feeding to rats leads to both cancer initiation and promotion in the liver. One of the important toxic effects of FB_1 is the disruption of sphingolipid metabolism due to the structural similarity to sphingosine. Elevation of intracellular sphingosine-1-phosphate (S1P) comes along with inhibition of ceramide synthase. It was the aim of this study to further clarify the mechanisms of FB_1 carcinogenicity by identifying genes or pathways leading to the aforementioned effects. Primary rat hepatocytes were incubated with different concentrations of FB_1 for 24 h in order to perform microarray experiments and to investigate alterations in gene expression patterns. Total RNA was extracted and converted into cDNA. After labelling, the targets were hybridized to rat whole genome oligo microarrays. Data analysis of the microarrays shows a total of 96 significantly up-regulated as well as down-regulated genes. The majority of the affected genes are involved either in cell-cell adhesion or in S1P signalling. Since S1P was suggested to act as a suppressor of apoptosis in rat hepatocytes, both topics are in close relationship with plausible mechanisms of tumor promotion in rodent liver. Although the results have to be validated with reverse transcription real-time polymerase chain reaction (RT-PCR) and Western Blotting, the microarray experiments give a hint to signalling pathways possibly involved in the toxicity and carcinogenicity of FB_1 in primary rat hepatocytes.

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362

Withdrawn

363

Oxidative stress and DNA damage in human colon epithelial cells by engineered nanoparticles

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Nanoparticles are defined as particles with a diameter smaller than 100 nm in at least one dimension. They are typically characterised by an increased biological activity compared to larger particles of the same chemical composition. Inhalation and ingestion are considered as the major exposure routes for nanoparticles. Nanosized particles in ambient air have been shown to cause inflammation in the respiratory tract and have been associated with chronic inflammatory diseases and risk of cancer development. The new features of engineered nanoparticles (ENP) offer many advantages to different fields of research and industry, and can for instance be applied to optimize food and food packaging. Presently, very little is known about the potential toxic effects of ENP within the gastrointestinal tract and their possible effects on inflammatory and malignant diseases, such as ulcerative colitis, Crohn´s disease and cancer. In the present study we have investigated the oxidative and genotoxic effects of a panel of ENP on the human colon epithelial cell lines Caco-2 and HCT116. Cytotoxicity was analysed by the LDH assay, whereas total cellular glutathione (GSH) was determined as a marker of oxidative stress. The formamidopyrimidine glycosylase (fpg)-modified comet assay was used to investigate oxidative DNA-damage. To determine the potential effects of ENP in the inflamed colon, we also investigated their ability to activate human neutrophils, by using lucigenin-enhanced chemiluminescence. Our current results revealed DNA strand breakage and oxidative DNA damage induction by TiO_2 and ZnO in both colon epithelial cell lines. Among all particles tested, SiO_2 showed the highest cytotoxicity whereas a marked neutrophil activation was found with MgO. Further research is needed to unravel the responsible mechanisms and to determine their relevance in relation to actual human exposure levels to specific ENP.

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364

Evaluation of putative biomarkers of nephrotoxicity after exposure to ochratoxin A *in vivo* and *in vitro*

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The kidney is one of the main targets of xenobiotic induced toxicity, but early detection of renal damage is often difficult. Recently, several sensitive biomarkers of acute kidney injury have been identified by transcription profiling *in vivo*, including kidney injury molecule-1 (Kim-1), lipocalin-2, tissue inhibitor of metalloproteinases-1, clusterin, and osteopontin. However, it is not known if these cellular marker molecules may also be useful to predict chronic nephrotoxicity or to detect nephrotoxic effects *in vitro*. In this study, a panel of new, promising kidney biomarkers was assessed via quantitative realtime PCR, immunohistochemistry in kidneys of rats treated with the nephrotoxin ochratoxin A (OTA) for up to 13 weeks. In addition, changes in the expression of putative biomarkers were determined in proximal tubule cell cultures (NRK-52E) in response to sub-lethal concentrations of OTA. Repeated administration of up to 210 µg/kg bw OTA to male F344/N rats for 14, 28, or 90 days resulted in a dose- and time-dependent increase in the expression of genes suggested as candidate biomarkers of renal injury. Changes in the expression of novel biomarkers were found to correlate with the progressive histopathological alterations observed in kidneys of OTA treated animals and appeared to precede effects on traditional clinical parameters indicative of impaired kidney function. Induction of Kim-1 mRNA expression was the earliest and most prominent response observed, supporting the use of this marker as sensitive indicator of chronic kidney injury. In contrast, no significant increase in the expression of putative marker genes was evident in NRK-52E cells after exposure to OTA for up to 48 hours, suggesting that they may not be suitable endpoints for sensitive detection of nephrotoxic effects *in vitro*.

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365

A combined GC-MS, LC-MS and NMR metabonomics approach for early detection of ochratoxin A nephrotoxicity

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Within the last years, metabonomics has shown great potential for the early noninvasive detection of toxicity. In this study, ochratoxin A, a potent nephrotoxin and renal carcinogen was used to investigate time- and dose-dependent changes in urine composition in relation to alterations in clinical chemistry and histopathology. Male F344/N rats (n = 5 per group) were administered 0, 21, 70, 210 µg/kg bw in corn oil for 14, 28, 90 days for 5 days/week. 24 h urine samples were collected prior to sacrifice on day 14, 28 and 90. Urinary metabolites were screened using three complementary analytical approaches, i.e. GC–MS, LC–MS and ^{1}H–NMR. GC–MS required methoximation and silylation with *N*-methyl(trimethylsilyl) trifluoroacetamide. Positive and negative electrospray ionization LC-MS experiments were run on a C18 column with hydrophilic endcapping and were diluted to unit osmolarity to minimize ion suppression effects. ^{1}H–NMR samples were buffered with 1 M phosphate buffer to prevent pH dependent signal shifts and were referenced to D_4–trimethylsilylpropionic acid. For all three approaches, multivariate data analysis tools such as orthogonal partial least squares discriminant analysis (OPLS–DA) resulted in good separation of control and respective dose groups. Changes in urinary patterns were evident as early as 14 days after start of treatment in high dose animals and correlated well with the histopathological findings consisting of single cell degeneration and regeneration within the proximal tubule epithelium. In contrast, changes in traditional clinical chemistry parameters were evident only at later time points. Potential markers responsible for group discrimination were extracted from S–plots and shared and unique structure (SUS) plots and were subsequently identified with the aid of various databases. Glucose, pseudouridine, *myo*–inositol, pyroglutamate and *N,N*–dimethylglycine were found to be increased in a dose-dependent manner, while the concentrations of citrate, 2-oxoglutarate and hippurate decreased with dose. These results demonstrate that metabonomics may serve as a sensitive, noninvasive method for the early detection of drug-induced nephrotoxicity.

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366

Lipocalin 2, clusterin and Kim 1 as potential early, non invasive biomarkers of nephrotoxicity

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The kidney is one of the main targets of xenobiotic induced toxicity, but early detection of renal damage is often difficult. This study was designed to investigate the use of urinary lipocalin 2, clusterin and Kim 1 as early, non invasive biomarkers of nephrotoxicity. Male Wistar rats were treated with 0, 60 or 120 mg/kg/bw gentamicin (s.c.) for 7 days. Urine was collected daily for clinical chemistry and analysis of novel biomarkers by western blot and ELISA. A clear dose and time dependent increase in urinary excretion of lipocalin 2, clusterin and Kim 1 was observed, which correlated with increased expression of lipocalin 2, clusterin and Kim 1 mRNA in kidneys of treated animals as determined by qRT-PCR. Kim 1 was not detected in urine of control animals but was increased after 5 days of treatment with the high dose. A significant increase in urinary clusterin was detected as early as 2 days after treatment with 120 mg/kg/bw Gentamicin and from day 3 on after low dose treatment by both ELISA and western blot. In this study, lipocalin 2 was found to be the most sensitive indicator of nephrotoxicity, with a 1.7-fold increase as early as day 1 in high dose animals and a 3.1-fold increase after 3 days of low dose treatment. In contrast, marked changes in clinical chemistry parameters (glucose, protein, g-GT, creatinine) were only evident at later time points. Taken together, results from this study suggest that lipocalin 2 and clusterin may serve as early urinary non invasive biomarkers of nephrotoxicity.

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367

The presence of an estrogen receptor α (ER α)-like protein in the amphipod, *G. fossarum* is regulated by 17α-ethinylestradiol

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In vertebrate reproductive endocrinology estrogens play a pivotal role via binding to estrogen receptors (ER). The origin and relevance of estrogens in invertebrates remains unclear. It is believed that ER orthologs are lost within the ecdysozoan clade since, within protostomes, ER receptor genes and proteins have only been found in molluscs. But, evidence exists from environmental regulations and testing programs that endocrine-disrupting industrial chemicals with estrogenic-activity (xenoestrogens) affect the development and differentiation of some insects and crustaceans. Here we present the identification, gender-specific expression, and induction of an estrogen receptor alpha ortholog in an ecdysozoan species, the crustacean *Gammarus fossarum*. We targeted two protein sequences within the ER amino acid sequence (which are highly similar to the vertebrate ERs) exclusively in adult females while they were absent in males and juveniles. Exposure to 17α-ethinylestradiol (EE2) induced ER expression in adolescent females, but failed in males and adult females. Our data clearly indicate that ER-like protein deriving from a common ancestor established before the branching which gave rise to the ecdysozoans, lophotrochozoans, and deuterostomes were not lost in at least a basal group of ecdysozoans offering the possibility that sex steroid receptors could have evolved also in arthropods and nematodes. In contrast to the ER ortholog from the mollusc, *Aplysia californica*, this ER alpha-like protein seems to have estrogen-dependent functional activity.

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368

ReProTect: a novel approach in hazard assessment of reproductive toxicity

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The new European legislation "REACH" is asking for a dramatic increase in assessing reproductive toxicity of existing chemicals. Due to the large amount of animals involved, the integration of alternative tests into testing strategies is foreseen. Within the 6th Framework Programme of the EU an Integrated Project (ReProTect, www.reprotect.eu), involving 32 partners from industry, academia and governmental institutions, has been set up aiming to develop and optimize *in vitro* tests that are able to detect toxic effects and mechanisms associated with reproductive toxicity. However, due to the complexity of the mammalian reproductive cycle, it is not possible to model the whole cycle in one *in vitro* system in order to detect adverse effects to mammalian reproduction. Individual tests should be used as building blocks to compose testing batteries and strategies. Within the last three years the Integrated Project ReProTect explored the predictive power of a range of pioneering *in vitro* tests, addressing various adverse effects such as testicular toxicity, female germ cell toxicity, endocrine disruption and teratogenicity. The consortium explored the predictive power of the developed/optimsed *in vitro* tests by testing more than 100 peer-reviewed reproductive toxicants with different toxicological mechanisms. Seventeen tests are assessing adverse effects on mammalian fertility by modeling various toxicological mechanisms related to leydig/sertoli cells, folliculogenesis, germ cell maturation, the motility of sperm cells, fertilisation and on the preimplantation embryo, placentation and uterus functions. Furthermore, the relevance of inter-species variations has led to a strong focus on the use of human and murine embryonic stem cell for identifying embryotoxicity by evaluating most predictive toxicological endpoints and optimizing differentiation protocols. Advanced tests for the identification of (anti) estrogenic and (anti) androgenic compounds have been optimised and currently assessed for entering in the formal validation process under the umbrella of the Validation Management Group "non animal" of the OECD.

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369

Furocoumarins in phytomedicines: Is there a phototoxic risk?

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Furocoumarins occur in plants used as food (e.g. grapefruit, parsley, parsnip) or in phytomedicines (*Ammi majus, Angelica archangelica*). In combination with UV light they can lead to phototoxic and genotoxic effects. The latter are caused, e.g., by UV-induced covalent binding of furocoumarins to the pyrimidine bases of the DNA. In the absence of light, a genotoxic effect was not detectable in our preliminary experiments using the comet assay to detect possible DNA damage induced by 5-methoxypsoralene in the human A-431 epidermoid carcinoma cell line and the human HepG2 hepatoma cell line. According to the literature [1,2], in humans, phototoxic effects were observed at 10 mg 8-MOP plus 10 mg 5-MOP (oral dose; 0.25 mg/kg b.w 8-MOP equivalents/kg b.w. in adults) or 14 mg 8-MOP (oral dose; 0.23 mg/kg b.w. in adults). The average daily furocoumarin intake per adult via food was estimated to be 1.45 mg in Germany [3] (24 µg/kg b.w.). Thus the daily intake of furocoumarins via food lies about 10-fold above the phototoxic 'threshold dose' in humans. With an assumed level of total furocoumarins in *Angelica* tincture of 1 mg/ml, and a daily dose of 1.5 g according to the German commission E monograph, a total daily intake of about 1.5 mg furocoumarins via phytomedicine can be estimated. For an adult, this corresponds to an additional daily intake of 25 µg/kg b.w. being in the same range as the average intake via food. With respect to the phototoxic 'threshold dose', the intake via a phytomedicine as described would be in the range of 10 %. In conclusion, intake of a phytomedicine containing *Angelica* root extract as described does not contribute in a relevant way to phototoxicity and is within the range of the average intake via food.

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370

Ultrastructural alterations induced by linezolid in W3 Sertoli cells *in vitro*

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Linezolid is the first oxazolidinone available for the treatment of infections by Gram-positive pathogens; it is not recommended for pediatric indications. In male rats linezolid causes infertility which is irreversible if animals are treated during postnatal development. The mechanism of the reproductive toxicity of linezoid is not known. In the present study we evaluated the effects of linezolid on Sertoli cells. Rat derived W3 Sertoli cells were incubated with linezolid at therapeutically relevant concentrations, i. e. within the range of concentrations achieved in plasma during therapy (0.1; 1.0; 10.0 mg/l). Cells were cultured in DMEM supplemented with 5% fetal calf serum. After 1, 2 and 5 days in culture, cells were harvested, fixed in Karnovski`s solution and studied by transmission electron microscopy. At all concentrations tested, we observed degenerative changes such as swollen and dilated mitochondria and enlarged rough endoplasmic reticulum. In untreated controls multiple intact tight junctions, which are typical features of these cells, were observed. The number of cells with morphological signs of damaged tight junctions was determined by scoring 200 cells from 30 different microscopic fields. The number of tight junctions was significantly reduced in linezolid-exposed cells and this effect was time- and concentration-dependent. For example, after 5 days in culture, 2% of the control cells exhibited damaged tight junctions, incubation with linezolid resulted in a pronounced increase of such alterations to 35% (0.1 mg/l), 54% (1.0 mg/l) and 84% (10.0 mg/l) of the cells. Our data indicate that linezolid causes ultrastructural changes in tight junctions of Sertoli cells in a time- and concentration-

dependent manner. The resulting impairment of the blood-testis barrier and spermatogenesis may be responsible for the fertility impairment observed after treatment with linezolid in male rats.
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371

Different effects of nucleoside reverse transcriptase inhibitors on mitochondrial gene expression
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Mitochondrial dysfunction is a major toxicity of nucleoside reverse transcriptase inhibitors (NRTIs) which are used for treatment of HIV infection. Impairment of mitochondrial (mt) DNA synthesis has been thought to be mainly responsible for mitochondrial toxicity of these agents but also direct effects on mitochondrial function have been reported. In addition, it has been hypothesized that NRTIs may also affect mtRNA production.The aim of this study was to investigate effects of different NRTIs on expression of mtDNA-encoded genes in HepG2 cells in parallel to mtDNA content and cellular lactate production.HepG2 cells were treated over 14 days with the NRTIs zalcitabine (ddC), didanosine (ddI), stavudine (d4T), and zidovudine (AZT) at concentrations between 1 and 100 µM. Relative expression of mtDNA-encoded proteins and mtDNA content were analysed by real-time PCR. Mitochondrial function was determined by measurement of cellular lactate production. Expression of mtDNA-encoded proteins in HepG2 cells was very different among the various NRTIs tested. DdC induced an increase in gene expression at 1 µM for most genes, whereas at 100 µM a decrease was observed. For d4T a concentration-dependent decrease in gene expression was found. DdI also induced reduction of gene expression beginning at 1 µM with no further decrease at higher concentrations. AZT treatment reduced gene expression at 1 and 10 µM. At 100 µM weaker or no reductions were found. Alterations in gene expression did not always correlate with changes in mtDNA content and cellular lactate production. Whereas ddC and ddI depleted mtDNA concentration-dependently, AZT induced an increase. No significant change was found for d4T. Lactate production increased concentration-dependently for all NRTIs.This study shows that treatment with NRTIs induced alterations in expression of mtDNA-encoded proteins. Further investigation is needed to ascertain whether these are direct or indirect effects of NRTIs.
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372

Influence of mitochondrial toxic drugs on mitochondrial gene expression *in ovo*
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Mitochondrial toxicity, through depletion of mitochondrial (mt) DNA, is caused by many drugs like nucleoside reverse transcriptase inhibitors (NRTIs), DNA intercalating agents and antibacterial type II topoisomerase inhibitors. As a consequence of a decrease of mtDNA, mitochondrial function and mtRNA production can be altered. The aim of this study was to investigate expression of mtDNA-encoded genes in our recently established *in ovo* model after treatment with drugs causing mtDNA depletion. A correlation of change in gene expression and the results of mtDNA content and mitochondrial function was sought to be established. The NRTIs zalcitabine (ddC), didanosine (ddI) and zidovudine (AZT), the DNA intercalating agent ethidium bromide (EtBr) and the antibacterial type II topoisomerase inhibitor nalidixic acid (NA) were administered into incubated hens' eggs at different doses at days 5, 7 and 9 of incubation. Mitochondrial toxicity was investigated at day 11 of incubation. Relative expression of mtDNA-encoded proteins and mtDNA content of the embryos' livers were determined by real-time PCR. For determination of mitochondrial function blood lactate level was measured. Treatment with AZT and EtBr resulted in significant decrease of expression for most mitochondrial genes in liver cells at the highest dose, whereas NA showed an increase of gene expression at the highest doses. After ddC treatment a yet not significant decrease of gene expression was found for most genes. For ddI no clear change in gene expression could be determined. Analysis of mtDNA showed a significant dose-dependent depletion after treatment with ddI, ddC, and EtBr. No change was found for NA. Blood lactate levels increased dose-dependently for all drugs. This study shows that the *in ovo* model is useful for studying effects of NRTIs and other agents on expression of mtDNA-encoded genes as well as on mtDNA and mitochondrial function.
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373

***In vivo* toxicity and gene targeting efficacy of polyethylenimine (PEI)/siRNA nanoplexes for therapeutic inhibition of tumor-relevant genes**
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RNA interference (RNAi) is a powerful method for the inhibition of the expression of pathologically relevant genes. Since it is mediated through small interfering RNAs (siRNAs), their delivery, most desirably through the systemic, non-viral administration of non-toxic formulations, poses a major challenge in therapeutic *in vivo* applications of RNAi. The non-covalent complex formation of siRNAs with polyethylenimines (PEI) may represent a promising method for therapeutic siRNA delivery. Here, we explore the toxicity / biocompatibility, safety and therapeutic efficacy of PEI/siRNA-mediated gene targeting in mice. We performed biodistribution studies to optimize administration strategies of intact siRNAs *in vivo*, and radioactive siRNA quantitation in various organs reveals distinct biodistribution profiles being dependent on the route of injection. For 25 kDa PEI, we demonstrate the induction of erythrocyte aggregation and hemorrhage in the lung upon i.v. injection, which is dependent on the N/P ratio in the complex, and acute toxicity at the highest N/P ratio. Furthermore, we show that in liver and spleen, but not in lung and kidney, siRNA levels are dependent on macrophage activity. In vitro, the recently introduced PEI F25-LMW, a low molecular weight PEI prepared through the size exclusion chromatography-based fractionation of 25 kDa PEI, demonstrates lower toxicity and higher efficacy of siRNA delivery. Additionally, it shows favourable features regarding the preparation, handling and long-term storage of the complexes, being important for reproducible treatment regimens. In vivo toxicity studies reveal the absence of side effects of the PEI F25-LMW/siRNA complexes or of the free carrier at the concentrations used for therapeutic applications in mice. Finally, anti-tumorigenic efficacies are demonstrated by the direct comparison of (i) PEI/siRNA-mediated targeting of the growth factor VEGF, (ii) treatment with the therapeutic antibody Avastin and (iii) a combination of both.
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374

Interferons as a modulator of the radio- and chemosensitivity of different tumor cell lines
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Type I interferons (IFNs), including IFN-α and IFN-β, belong to the family of cytokines and are widely used either alone or in combination with ionizing radiation or chemotherapy. Clinical studies on patients suffering of pancreatic cancer showed that IFNs can improve post-operative therapy when combined with ionizing radiation (IR). Also in the treatment of melanomas IFNs are used, and from studies on glioma cells it is known that IFNs can sensitize resistant cancer cell lines to the alkylating agent temozolomide (TMZ). To extend these studies, we determined whether pancreatic cancer cells and melanoma cells respond to IFN treatment and whether IFN has an effect on the efficiency of IR and TMZ treatment. For our studies on the sensitization effect of IFNs on IR we used a panel of pancreatic cancer cell liens derived from primary ductal pancreatic adenocarcinomas, or metastases of pancreatic adenocarcinomas. Cells were treated with 300 I.U./ml of either IFN-α or IFN-β. IFN alone caused a decrease in cellular survival, ranging between 20% and 70%, in eight out of the ten cell lines used, as measured by colony survival. Generally, IFN-β was more effective than IFN-α as, on average, 15% more cell kill was observed with IFN-β. A sensitization effect of IFN on IR was only observed in three out of the eight cell lines, and ranged between a 5% and 20% increase in cell kill compared to IR alone. Again, IFN-β was more effective. Mathematical analysis of the survival curves of four pancreatic cancer cell lines by the linear-quadratic equation revealed a significant change in the alpha values. For investigating the chemosensitization properties of IFN-ß we used two different melanoma cell lines and four pancreatic cancer cell lines. Cells were pretreated for 24 h with 300 I.U./ml IFN-ß and the subG1 fraction was meassured by FACS analysis 144h or 96h after TMZ treatment in the presence or absence of O^6-BG. While we could see a sensitization effect in melanoma cells of IFN-ß on TMZ in the absence of MGMT, we were not able to detect any sensitization effect in pancreatic cancer cells. Collectively, the data show that IFN sensitize pancreatic cancer cell lines to IR, but not TMZ, whereas in melanoma cells IFN enhances the cytotoxic effect of TMZ in an MGMT independent way.
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375

***In vitro* examinations on the compatibility of magnesium alloys as a basis for the development of biodegradable implants**
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Magnesium alloys as promising new material for the development of biodegradable implants for use in both hard tissue (e.g. bone surgery) and soft tissue (e.g. stents) were examined concerning their biocompatibility. The release of ions from different alloys into cell culture medium was determined using inductively coupled plasma (ICP) analysis. In cell culture experiments effects of relevant ion concentrations of magnesium, calcium, aluminium and lithium supplemented as chlorides to the culture medium were determined on the viability of fibroblasts. For assessment of the compatibility in the intact tissue further examinations using the isolated perfused bovine udder [1] were performed. The alloys were implanted subcutaneously (connective tissue) and into the teat cistern (mucosal tissue). Tissue viability and irritation were determined using a methyltetrazolium (MTT) assay and by measuring the release of pro-inflammatory mediators (e.g. prostaglandins) into the surrounding tissue. The cell culture experiments showed a good tolerance of magnesium and lithium (at least about 10 mmol/l). Calcium and aluminium showed cytotoxic effects at concentrations about 10 mmol/l and 0.1 mmol/l, respectively. The studies on the isolated perfused bovine udder showed differences between the examined magnesium alloys. From the materials used pure magnesium and the alloy containing 0.8 % calcium were most promising. The present *in vitro* studies demonstrate the suitability of magnesium alloys as a potential new implant material in biotechnology, with regard to their biocompatibility. These results may be used as basis for the selection of promising candidates for *in vivo* trials. Finally, in the development of new medicinal products magnesium alloys are promising candidates as scaffolds for active compounds in drug eluting implants.
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376

Comparison of hepatic coumarin concentrations following dermal vs. oral exposure – a PBPK modelling approach
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Coumarin (1,2-Benzopyron, CAS 91-64-5) is present in plants such as woodruff, sweet clover, and Cassia cinnamon. With regard to its health effects, the toxicity in liver is most

relevant. In 2004, the EFSA derived a TDI level of 0.1 mg/kg bw based on data obtained in dogs. By assessing clinical human data this value has been confirmed in 2006.In addition to its occurrence in food, coumarin is used as a fragrance in cosmetic products which may contribute to the overall exposure of humans. In performing route to route extrapolation the tissue doses have to be considered. In this respect, the dose metric related to the hepatotoxic effect is of crucial importance (Cmax vs. AUC in the target organ). We therefore applied a PBPK modelling approach to calculate concentrations of coumarin in blood and organ tissues after oral or dermal exposure to this compound. The hepatic tissue concentrations were compared for doses in the TDI range when applied orally or onto the skin. The model utilized and the physiological parameters have been described by Abraham et al. (Arch Toxicol 2005, 79:63-73). The chemical specific parameters were taken from Rietjens et al. 2007 (Species differences in coumarin metabolism. Unpublished report); absorption rates were taken from the literature.The human model predicted concentration-time-courses in blood which are in agreement with experimental data of Ritschel et al. (Int J Clin Pharmacol Biopharm 1979, 17:99-103). After oral exposure to 0.1 mg/kg bw, the Cmax in liver was reached within minutes, declining with a short half-life. Upon dermal exposure to the same dose, the Cmax was found 10 times lower and was achieved with delay. As expected, the AUC was identical for both scenarios. With a rat PBPK model (taken from Rietjens et al. 2007), we calculated Cmax and AUC values for exposures used in gavage- and diet studies with coumarin. The relation between the different dose metrics and the effect revealed that in this case Cmax is better related to the hepatotoxic effect than AUC. Our results indicate that the TDI may be very conservative regarding dermal exposure to coumarin.
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377

Effects of dietary flavonoids in Hct116 cells: Investigation on cellular uptake, antioxidant capacity and toxicity

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A diet rich in fruits and vegetables has been linked to a lower risk for various diseases like colon cancer and cardiovascular disease. Since these beneficial effects are suggested to be mediated by dietary polyphenols (e.g. flavonoids, phenolic acids) the use of these compounds as food supplements has become increasingly popular. However, the exact mechanisms of flavonoid action still remain unclear.We investigate absorption and metabolism of flavonoids in cell culture and *in vivo*. Bioavailability and metabolism of flavonoids are essential to identify those flavonoids that possess high biological activities. In this study we used Hct116 human colon carcinoma cells to investigate the uptake as well as cellular effects of structurally related flavonoids (e.g. quercetin, luteolin, kaempferol and fisetin). These flavonoids were taken up rapidly but differed in their intracellular amount. For example quercetin showed a high intracellular accumulation while other flavonoids reached relatively low concentrations. The rate of metabolism in Hct116 cells was generally low, especially when compared with H4IIE rat hepatoma cells. Further on we determined the antioxidant capacity in a cell free assay (DPPH) as well as the cytotoxic potential (MTT) of the flavonoids and compared these data with intracellular ROS levels (DCF) and the uptake data. The antioxidative effects correlated well with structural characteristics and gave a possible explanation for differences in toxicity. Cellular uptake was also shown to correlate with biological effects of these secondary plant compounds in Hct116 cells.

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378

Role of dietary flavonoids in the modulation of the antioxidant responsive element (ARE) and the induction of phase II drug metabolizing enzymes

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Flavonoids are polyphenolic compounds that occur ubiquitously in foods of plant origin. This class of compounds has become increasingly popular in terms of health protection because flavonoids possess a remarkable spectrum of biochemical and pharmacological activities. In epidemiological studies it was found that flavonoids are associated with a reduced incidence of coronary heart disease and may protect against distinct forms of cancer; for this reason, flavonoids are used in food supplements at relatively high doses. The biological actions of flavonoids have long been thought to be due to their antioxidant potential but although some flavonoids act as powerful antioxidants, it was also shown that in high concentrations they can generate ROS. ROS can activate the ARE which is found in the promoter region of a battery of genes encoding detoxification enzymes of phase II xenobiotic metabolism, e.g. GST and HO-1. The activation of this response element protects cells against oxidative stress. We compared effects of structurally related flavonoids (e.g. quercetin, kaempferol, luteolin and apigenin) on the activation of the ARE using Hct116 human colon carcinoma cells (induction of GST, HO-1). Further on, the antioxidant potential of the investigated flavonoids was detected in a cell free assay system (DPPH assay) and the cytotoxic potential of these flavonoids was compared. In summary, this study provides data showing cellular effects of flavonoids on activation of the ARE in Hct116 cells. These results may be important to estimate the effect of the ARE activation in human colon carcinoma cells compared to antioxidative effects.

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379

Influence of flavonoids on the catalase expression in human hepatoma cells

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The antioxidant enzyme catalase belongs to the cellular antioxidant defence system and is responsible for detoxifying H_2O_2 by catalyzing its degradation to H_2O and O_2. Results from our previous work indicate, that the isoflavone daidzein increases the transcription of the catalase gene via interaction with the proximal part of the promoter. The present study was performed to investigate the effect of the flavonoids quercetin, kaempferol and morin on the expression of the antioxidant enzyme catalase in the HepG2 human hepatoma cell line. HepG2 cells were treated with various concentrations of flavonoids (0-100µM) for 48h and cell viability was assessed by MTT assay. Quercetin and kaempferol induced a dose-dependent cytotoxicity with EC50 values at 50µM. Morin showed up to 100µM no detectable cytotoxicity. To quantifying the antioxidant activity of the flavonoids cells were preincubated with flavonoids and were then loaded with H_2DCF-DA for 30 min. The decrease in cellular fluorescence was compared to the control cells as a measure for the antioxidant capacity of the compounds. Quercetin had the highest antioxidant activity, followed by kaempferol and morin. HepG2 cells were transiently transfected with the human catalase promoter fused upstream of the reporter gene for the secreted embryonic alkaline phosphatase (SEAP). By measuring the activity of SEAP in the medium it is possible to quantify modulation of catalase expression after treatment with flavonoids. Our preliminary results indicate that treatment of HepG2 cells with flavonoids for 48h impair catalase promoter activity in a dose-dependent manner. To support these findings catalase expression at transcript and protein level will be analyzed by RT-PCR and western blotting. We conclude that flavonoids are able to modulate the expression human catalase. Further work is needed to make clear if the mechanism of action involve interaction with the human catalase promoter.

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380

Molecular effects of herbal flavonoids on the model organism *Caenorhabditis elegans*

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Flavonoids are secondary plant metabolites and their ubiquitous presence in foods of herbal origin makes them an integral part of human diet. Although it is well known that they possess a broad spectrum of biochemical and pharmacological properties the precise mechanisms of their action have not been finally elucidated yet.The nematodic organism *Caenorhabditis elegans* is an important model in different areas of biomedical research and many key discoveries with relevance for mammals including man were first made in the worm. Because of the remarkable strong conservation in molecular and cellular pathways between *C. elegans* and mammals we considered this organism as a suited model to study the effects caused by herbal flavonoids in the context of the complex physiology within complete animals. We demonstrated that in *C. elegans* several flavonoids can increase stress resistance. Quercetin was even found to prolong the lifespan. These protective effects were accompanied in living worms by reduced intracellular levels of reactive oxygen species and a delayed accumulation of the age pigment lipofuscin. In order to ensure the bio-availability and thus the possibility of a systemic action of the flavonoids we verified their uptake by the worms. To shed light on the mechanisms by which the flavonoids confer their effects we determined the influence of these substances on the expression of genes involved in stress response, like the mitochondrial superoxid dismutase (SOD-3) and a small heat shock protein (HSP-16.2), and in phase II metabolism, like a glutathione *S*-transferase (GST-4) and a glutamylcysteine synthetase (GCS-1). We also observed flavonoid-induced changes in the subcellular distribution of the corresponding *C. elegans* transcription factors DAF-16 and SKN-1 that are homologous to mammalian FoxO and Nrf2 proteins. Additionally, we have started to analyse the impacts of flavonoids on upstream signalling molecules.

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381

Influence of flavonoids on the stress response of *Caenorhabditis elegans*

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The nematode *C. elegans* shows a remarkable strong conservation in many basic molecular and cellular processes to mammals and thus it is widely used in various areas of biological and medical research. Recently, this organism is increasingly employed to study beneficial and toxic effects of substances as well as to identify new targets for pharmacological intervention. In our study we take advantage of *C. elegans* as a simple animal model that offers the possibility to directly monitor the effects of flavonoids in living worms. Flavonoids ubiquitously occur in foods of plant origin and are assumed to contribute to the beneficial impact on health of a diet rich of vegetables and fruits. Although many of these polyphenolic substances exhibit an antioxidative capacity and possess a broad spectrum of biochemical and pharmacological properties the precise mechanism of their action is still under investigation.The FoxO transcription factor DAF-16 that has a pivotal function in the control of stress resistance and lifespan in *C. elegans* was translocated into the nucleus in response to treatment with catechin and luteolin. In accordance with this observation these two flavonoids induced the upregulation of the mitochondrial superoxide dismutase SOD-3, a known target of DAF-16, and they reduced the intracellular accumulation of reactive oxygen species in worms stressed with heat. Additionally, the heat-induced upregulation in expression of the small heat shock protein HSP-16.2 was diminished by treatment of the worms with luteolin and myricetin but not with catechin. Luteolin and myricetin also delayed the accumulation of lipofuscin, a biomarker for ageing, indicating a possible influence of these flavonoids on the lifespan of *C. elegans*.Our results suggest that the flavonoids have the capacity to affect processes associated with stress response even if they revealed differences in their profile of action.

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382

Examination of the influence of quercetin on signaling in *Caenorhabditis elegans*

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Flavonoids are secondary plant metabolites that occur in foods of herbal origin and it is assumed that they contribute beneficial effects on health. In many *in vitro* studies a variety of biochemical and pharmacological properties of flavonoids were discovered but data about their effects in whole animals are restricted. In order to overcome this limitation we used the established model organism *C. elegans* to elucidate the effects of flavonoids in living animals. In previous studies we demonstrated that quercetin, a representative flavonoid that is found at high concentrations in many herbal edibles, has the capacity to protect the worms against stress conditions and to prolong their lifespan. Treatment of the worms with quercetin also resulted in an alteration of the localisation of the *C. elegans* transcription factors DAF-16 and SKN-1 that have essential functions in the control of stress response and ageing like their homologs FoxO and Nrf2 in mammals. Based on this observation we have started an attempt to investigate the impact of quercetin on components of signaling pathways that may be responsible for the regulation of these transcription factors. Like in mammals, different kinase cascades are involved in this process. The FoxO protein DAF-16 is a target of a pathway transducing the signal from the insulin/insulin growth factor-like receptor DAF-2 down to Akt-kinases and another MAP kinase cascade ending at the c-jun N-terminal kinase JNK-1. The stress responsive transcription factor SKN-1 depends on the activity of the *C. elegans* p38 homolog PMK-1. To examine whether the activity of these pathways is influenced by quercetin we perform Western Blot analysis with heterologous antibodies directed against the phosphorylated form of the mammalian homologs. The specifity of the antibodies for the *C. elegans* proteins is ensured by the use of mutants deficient for the respective gene product and by RNAi-mediated gene silencing.

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383

Enniatins A1, B, B1 and Beauvericin induce apoptotic cell death in H4IIE hepatoma cells accompanied by inhibition of ERK phosphorylation

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Enniatins A1, B, B1, isolated from *Fusarium tricinctum* and Beauvercin isolated from *Fusarium begoniae* are cyclic peptides. These mycotoxins have an important impact on human health, e.g. as contaminants of cereals, but are also discussed as possible anti-cancer agents. The aim of this study was to investigate the toxic effects of these mycotoxins on different cancer cell lines. The Enniatins and Beauvericin were highly toxic in rat hepatoma H4IIE cells (EC_{50}-values approximately 1 – 2.5 µM) and also showed toxic effects in HepG2 human hepatoma, Hct 116 human colon carcinoma and C6 rat glioma cells. Enniatins and Beauvericin increased caspase 3/7 activity and nuclear fragmentation, marker for apoptotic cell death. Enniatin A1, Enniatin B1, Beauvericin and, to a lesser extent, also Enniatin B decreased the activation of ERK kinase (p44/p42), a mitogen activated protein kinase which is associated with cell proliferation. Furthermore, Beauvericin, Enniatins A1 and B1, but not Enniatin B were able to moderately inhibit TNF-α-induced NF-κB activation. Screening of 24 additional protein kinases involved in signal transduction pathways (cell proliferation, survival, angiogenesis and metastasis) showed no inhibitory activity of enniatins.We conclude that Beauvericin, Enniatins A1 and B1 and, to a lesser extent, Enniatin B may possess anticarcinogenic properties by induction of apoptosis and disruption of ERK signalling pathway. Further analysis of these substances is necessary to analyze their usefulness for cancer therapy.

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384

The *Fusarium* toxin enniatin B: studies on its genotoxic potential and cytotoxicity

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Fusarium species are prevalent toxin-producing fungi of the northern temperate region. Well known *Fusarium* mycotoxins, e.g. deoxynivalenol, fumonisins, trichothecenes and zearalenone, can cause a variety of toxic effects. In contrast, the secondary fungal metabolite enniatin B, produced by several species of *Fusarium*, has not been well characterized in this respect, although it is now recognized as a frequent contaminant of grains [1]. Enniatins possess interesting biological activities: They show antibiotic activity, can act as inhibitors of drug efflux pumps and are known for their ionophoric, phytotoxic and anthelmintic effects. They exert also potent cytotoxic activity against cancer cell lines [2], and thus may be developed further for therapy. But, at present published data on the genotoxic potential of these compounds is lacking. Therefore, we have investigated enniatin B in a battery of short term tests for its genotoxic potential: For mutagenicity testing in bacteria, the compound was deployed in the *Salmonella typhimurium* assay (Ames assay) with the strains TA 98, TA 100, TA 102 and TA 104, in the presence and absence of an external metabolizing enzyme system (rat liver S9). No mutagenicity was found in a concentration range of 0.1 to 100 µM in the Ames assay. Likewise, mutagenicity tests in mammalian cells, i.e. the HPRT assay with V79-cells, did not show a significant concentration-dependent increase in mutant frequency for enniatin B (0.3 up to 30 µM). Tests on other types of genotoxicity, i.e. clastogenicity and chromosomal damage, are currently conducted: Preliminary results from alkaline single cell gel electrophoresis (Comet assay) and the micronucleus assay in V79 cells argue against a significant genotoxic potential of enniatin B. However, enniatin B exerts clear cytotoxic effects in V79 cells: The IC_{50} (50% inhibitory concentration) of 4.0 µM, determined by neutral red uptake assay for 48 h exposure, is about 4-fold higher than that of the more potent cytotoxin deoxynivalenol (IC_{50} of 0.9 µM) but clearly lower than that of a number of other mycotoxins.

[1] Uhlig, S. et al. (2007), Int J Food Microbiol 119: 17-24, [2] Firáková, S. et al. (2007), Pharmazie 62: 563-568

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385

Proapoptotic compounds isolated from *Erythrina addisoniae*

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The genus *Erythrina* (Fabaceae) contains more than 100 different species, which are distributed in all tropical areas of the world. Diverse *Erythrina* species are used against cancer, e.g. stomach cancer in traditional medicine. In spite of these therapeutic effects of *Erythrina* species, little is known about pharmacologically active substances and the corresponding anticancer mechanisms. Therefore the ground root bark of the Ghanaian medicinal plant *Erythrina addisoniae* was extracted in a Soxhlet apparatus and different compounds were purified yielding different prenylated flavonoids (known substances like Abyssinon IV and Abyssinon V as well as the new flavonoids 3'-(2-hydroxy-3-methyl-but-3-enyl)-licoflavanone and its 4'-methylether), prenylated isoflavonoids (e.g. parvisoflavone A) and pterocarpanes (e.g. neorautenol, phaseolin). We analyzed the cytotoxic potential of these compounds in rat and human hepatoma cells (HepG2, H4IIE): Some of the prenylated flavonoids and also the pterocarpanes exerted a high toxicity in both cell lines (IC_{50} value in low micromolar range). We further investigated whether the cell death was mediated via apoptosis analyzing caspase-3/7 activation (Apo-ONE assay) and nuclear fragmentation: Some of the compounds (e.g. pterocarpane neorautenol) showed a high proapoptotic potency. We further investigated cellular mechanisms of this proapoptotic effect: For example, neorautenol (1 µM) strongly reduced phosphorylation of the ERK MAPK, first effects are found after 30 minutes (10 µM). We conclude that the ground bark of *Erythrina addisoniae* contains several pharmacologically active compounds. Distinct prenylated flavonoids as well as pterocarpanes exhibited a prominent toxicity in both H4IIE and HepG2 cells inducing apoptotic cell death. In case of neorautenol, apoptosis may be mediated via disruption of the ERK signalling pathway, this effect may be responsible for the anticarcinogenic actions of the plant extract. Further analysis of these substances may lead to new pharmacons to be used in cancer therapy.

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386

Apoptosis induction and cell cycle arrest of human HepG2 cells by 4-methylthiobutyl isothiocyanate: telomerase suppression as a new component in the signaling pathway?

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A number of ITCs have been identified as strong inhibitors of cell proliferation and apoptosis inducing agents which presents the chance for therapeutic impact of ITC-treatment on malignant transformed cells. Although much is known about ITC-induced apoptosis, many questions remain to be answered. The present study aimed to investigate the chemopreventive properties of 4-methlythiobutylisothiocyanate (MTBITC), which are still largely unknown despite its substantial quantitative presence in food plants. Thereby, this study focused on the hypothesis that p53-dependent induction of apoptosis leads to suppression of telomerase. Human telomerase is stably expressed in the majority of cancer cells but absent in normal tissues which outlines the importance of this enzyme and its compounds in the process of carcinogenesis and provides a promising target for a selective therapeutic approach of malignancies. As a result, this study showed for the first time the suppression of the telomerase catalytic subunit hTERT and the holenzyme activity in malignant transformed cells mediated by an ITC, accompanied by cell cycle arrest and apoptosis induction. Thereby, DNA strand breakage, induced by MTBITC-treatment probably presented the initial step in the growth suppression machinery. This DNA damage was followed by an increase in the protein level of the tumor suppressor p53 and subsequent by the initialization of $p21^{WAF1}$ protein expression. As a conclusion, the present findings support the idea of an existing extra-telomeric, cell cycle regulation function of telomerase in HepG2 cells. The results of this study also showed that irrespective of the intense degradation kinetics of MTBITC, the strong cytostatic effect of the ITC was not markedly affected by it and suggests that although ITCs are only present at maximum concentrations in a living system for a rather short time, this might be sufficient to exert their therapeutic effects.

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387

Curcumin induces mitotic breakdown in various human cancer cells

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Curcumin is an important constituent of turmeric, which is the yellow pigment from the rhizomes of the Asian plant *Curcuma longa*. Turmeric has long been used in Ayurvedic medicine as well as a spice, e.g. in curry. Curcumin exhibits anti-oxidative, anti-proliferative and anti-carcinogenic properties. Recent studies in our laboratory have shown that curcumin is an unstable compound, which is metabolized to hexahydro-curcumin and to an unstable curcumin-glucuronide in mammalian cells. In the present study, the genotoxic potential of curcumin and effects on the cell cycle were investigated in various human cells, i.e. HepG2 cells derived from liver carcinoma, HT-29 colon carcinoma cells and Ishikawa endometrial adenoma cells. All three cell lines exhibited a concentration- and time-dependent arrest in G2/M transition, leading to a decrease in cell number with increasing curcumin concentration. Longer post-incubation times did not abolish the arrest. Curcumin itself appeared to be responsible for this effect, because neither a mixture of the decomposition products of curcumin nor the main metabolite hexahydro-curcumin decreased the number of viable cells or showed an arrest of the cell cycle. Furthermore, studies of the cells were conducted using immunofluorescence staining of chromosomes with DAPI, microtubuli with anti-α-tubulin antibodies and kinetochores with CREST-antibodies. After incubation with curcumin, cells exhibited damage of microtubuli, fragmentation of the nucleus and formation of

micronuclei containing whole chromosomes. This mitotic breakdown was more pronounced in Ishikawa and HepG2 than in HT-29 cells. In summary, this study has shown that curcumin arrests cancer cells in the G2/M phase of cell cycle and induces mitotic breakdown. These effects may be related to the anti-carcinogenicity of curcumin.
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388

Menadione (2-methyl-1,4-naphthoquinone, vitamin K_3) diminishes gap junctional intercellular communication: role of adherens junctions

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Gap junctions are clusters of intercellular channels connecting the cytoplasmic compartments of adjacent cells and allowing for the direct exchange between cells of ions, signalling molecules and nutrients of low molecular mass. Gap junctional channels consist of two connexin (Cx) hexamer hemichannels provided by each of the connected cells. Gap junctional intercellular communication (GJIC) has been hypothesized to play a critical role in the regulation of carcinogenesis and cellular proliferation/differentiation. We have previously demonstrated that the redox cycler and alkylating agent menadione potently attenuates GJIC in rat liver epithelial cells by causing EGF-receptor-dependent activation of the mitogen-activated protein kinases ERK1 and ERK2 that in turn phosphorylate Cx43, the major Cx in this cell line. We here demonstrate (i) that menadione also affects adherens junction integrity and that (ii) depletion of adherens junction proteins (E-cadherin and beta-catenin) strongly attenuates GJIC. Exposure of rat liver epithelial cells to menadione for 30 min caused phosphorylation of Cx43 and a 50-75% decrease in GJIC. Moreover, the total amount of E-cadherin and beta-catenin was lowered after menadione treatment, as shown by immunocytochemistry. In line with reports on tyrosine phosphorylation of E-cadherin and beta-catenin induced by oxidative stress, general tyrosine phosphorylation of cellular proteins was strongly enhanced in cells exposed to menadione, with particularly prominent phosphorylation in the cell membrane. To further investigate the role of beta-catenin in the regulation of GJIC, beta-catenin was depleted from rat liver epithelial cells using a siRNA approach. The mere depletion of beta-catenin caused a loss in GJIC of up to 60% relative to control cells. The loss of beta-catenin further resulted in a reduction of Cx43 and E-cadherin levels in regions of intercellular contact and a partial internalization of Cx43, as demonstrated by immunocytochemistry. In summary, it is hypothesised that menadione affects the integrity of adherens junctions, with consequences on gap junctional intercellular communication. This disruption of intercellular signaling may contribute to menadione toxicity.
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389

Aberrant expression of key regulators of mitosis and chromosome segregation in rat kidney following exposure to ochratoxin A

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Ochratoxin A (OTA) is a potent renal carcinogen, but little is known regarding the mechanism of OTA carcinogenicity. Early histopathological alterations induced by OTA, which include single cell death, increased cell turnover and prominent nuclear enlargement, suggest that aberrant mitosis may be the principal cause of cell death and subsequent trigger for cell proliferation to compensate for cell loss. To gain further insight into the molecular mechanism of OTA toxicity, we used custom made qRT-PCR arrays to investigate expression of genes involved in cell cycle control and mitosis in kidneys of male F344 rats treated with 0, 21, 70 and 210 µg/kg bw OTA. Treatment with OTA at doses of 70 and 210 µg/kg bw for 90 days resulted in overexpression of several genes implicated in progression of mitosis and chromosome segregation, including Polo-like kinase 1, Aurora B kinase, cyclin-dependent kinase 1 (Cdk1), cyclin B, cyclin E, cyclin A2, $p21^{WAF1/CIP1}$, 14-3-3sigma, topoisomerase II and Bub1b. Immunohistochemical analysis used to confirm results obtained by qRT-PCR also demonstrated increased expression of Cdk1, $p21^{WAF1/CIP1}$, topoisomerase II, and increased phosphorylation of histone H3, a target of Aurora B kinase, in S3 proximal tubule cells, the target site of OTA toxicity. No effects were evident at the low dose or in the liver, which is not a target for OTA carcinogenicity. In the high dose group, alterations in gene expression occurred within 2 weeks of treatment and, therefore, before the onset of cell proliferation, suggesting that overexpression of mitotic regulators is an early event and not a consequence of enhanced cell turnover. Results from this study support our hypothesis that disruption of mitosis, resulting in blocked or asymmetric cell division, accompanied by an increased risk of aneuploidy acquisition, may play a critical role in OTA carcinogenicity and point to several key regulators of mitosis and chromosome segregation as potential mediators of these effects.
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390

Mafosfamide induces apoptosis by inhibition of transcription and DNA replication via the ATM/ATR-Chk1/Chk2-p53 pathway

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Interstrand cross-links (ICLs) induced by the bifunctional alkylating agent cyclophosphamide are considered to be responsible for the cytotoxicity of the anticancer drug. The mechanism of cell death, however, remained unclear. We applied the cyclophosphamide derivative mafosfamide, which requires no metabolic activation, in TK6 ($p53^{wt}$) and WTK1 ($p53^{mt}$) cells and show that mafosfamide induces apoptosis in lymphoma cells and that p53 exerts a clear sensitizing effect. We elucidated two upstream events that induce cell death: transcriptional inhibition and DNA replication blockage. The transcriptional inhibitor α-amanitin induced apoptosis on its own, and no addtitive effect was observed for combination treatment with α-amanitin and mafosfamide, indicating that transcriptional inhibition contributes to mafosfamide induced apoptosis. Furthermore, mafosfamide inhibited DNA replication and caused an accumulation of cells in S-phase. Comparing resting and proliferating P493-6 lymphoma cells, clearly more apoptosis was observed in proliferating cells, indicating a role for DNA replication blockage in the apoptotic response. At low dose of mafosfamide (2 µg/ml) DNA replication was inhibited more severely than RNA transcription, while at high dose of the drug (10 µg/ml) replication and transcription were reduced to similar level. At low dose of mafosfamide, capases were preferentially activated in S-phase, while in cells treated with a high dose caspases were similarly activated in all cell cycle phases. These results suggest that for low dose treatment with mafosfamide DNA replication blockage is the dominant trigger of apoptosis causing caspase activation in S-phase, while with increasing doses transcriptional inhibition also comes into play leading to caspase activation in an S-phase-independent way. Replication blockage resulted in activation of ATM/ATR and Chk1/Chk2. In turn p53 was stabilized by phosphorylation at Ser37, Ser15 and Ser20. Inhibition of ATM/ATR or Chk1/Chk2 impaired accumulation of p53 and, correspondingly, decreased the apoptotic response in $p53^{wt}$, but not $p53^{mt}$ cells. Collectively, the data show that mafosfamide-induced ICLs cause a block of transcription and DNA replication. The apoptotic signalling originating at the stalled DNA replication fork is propagated by ATM/ATR to p53 either directly or via Chk/Chk2.
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391

Death and repair induced by the anticancer drugs temozolomide and fotemustine in malignant melanoma cells

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Malignant melanomas exhibit a high level of resistance to chemotherapeutic drugs. Two alkylating drugs are used in first line chemotherapy: temozolomide (TMZ) and fotemustine (FM). Here, we examined the mechanism of cell death provoked by these agents in a panel of different melanoma cell lines. Using three independent methods of detecting apoptosis (i.e. sub-G1 and annexin V/PI double-staining, caspase-3 and -7 activation, and PARP cleavage) we show that melanoma cells clearly undergo apoptosis following treatment with the methylating drug TMZ and the chloroethylating agent FM. We further show for two melanoma lines, which differ in their sensitivity towards these drugs, that they undergo apoptosis via the mitochondrial and not the death-receptor pathway. For FM, we measured in both cell lines a similar level of DNA interstrand crosslinks (ICL) 20 h after exposure, using a method based on the alkaline comet assay. The level of DNA double-strand breaks (DSBs) was determined by phosphorylated H2AX (γH2AX) by immuno-blotting. It is high in the sensitive and low in the resistant cell line. This could be confirmed by immuno-histochemistry, counting γH2AX foci which represent DSBs. Here, we found a dramatically increase of foci 48 h after treatment with a lower level in the resistant melanoma cell line. The data provide evidence that alkylating agents induce death of malignant melanoma cells by apoptosis via the mitochondrial pathway and that resistance of malignant melanoma cells to FM and related drugs could be due to repair of secondary DNA lesions such as DSBs.
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392

Lack of functional NBS-1 leads to an increased induction of necrosis in human fibroblasts and lymphoblastoid cells after treatment with alkylating agents

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NBS-1 encodes for the protein Nibrin, which causes in case of deficiency the autosomal recessive disease Nijmegen-breakage-syndrome. It is a member of the MRN complex and has essential functions in DNA double-strand break (DSB) recognition and DSB processing, especially by homologues recombination. Further, it takes part in cell cycle checkpoint control by interaction with the ATM kinase. In this study, we show that NBS-1 defective human fibroblasts and lymphoblastid cells are more sensitive to alkylating agents (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and methyl methanesulfonate (MMS)) than the corresponding wild-type. Interestingly, annexin V/propidium iodide double staining revealed that alkylating agent-induced death of NBS-1 cells occurred mainly via induction of necrosis. Since the expression levels of the mismatch repair (MMR) proteins MutLα (MLH1, PMS2) and MutSα (MSH2, MSH6) did not differ significantly, the differences observed as to cell sensitivity appears to be independent from the MMR status. Similar to human NBS-1 deficient cells, human ATM fibroblasts are hypersensitive to MNNG and show a higher level of necrosis. The data suggests that NBS-1 and ATM interact, triggering the same apoptotic pathway and, therefore, are involved in the protection of cells against alkylating agent-induced cell death by necrosis. The exact mechanism of necrosis execution remains to be determined.
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393

Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on the expression of apoptosis-related genes in primary rat hepatocytes

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TCDD is a multiple-site carcinogen in various experimental models. In the liver of rats pretreated with a genotoxic carcinogen, TCDD acts as a potent tumor promoter. This effect is believed to be caused by the clonal expansion of pre-neoplastic hepatocytes due to an inhibition of apoptosis. In cDNA-microarray experiments in mouse liver, TCDD was found to modulate the expression of several apoptosis-related genes, notably an induction of the anti-apoptotic gene bcl-xL was observed. An enhanced expression of the Bcl-xL protein could lead to an inhibition of apoptosis and, therefore, result in the survival of pre-neoplastic hepatocytes, ultimately giving rise to liver tumors. So we tested if TCDD induces Bcl-xL in a time- and concentration-dependent manner in primary cultures of rat hepatocytes. Following incubation with TCDD a clear dose- and time-dependent induction of CYP1A1 mRNA and protein was observed. The induction of CYP1A1 by TCDD served as a control for aryl hydrocarbon receptor activation. None of the TCDD concentrations used showed any effect on mRNA or protein levels of Bcl-xL at any time-point. Next we tested the effect of TCDD on the expression of apoptosis-related genes performing qRT-PCR-arrays. Primary rat hepatocytes were incubated with 1 nM TCDD for 24 or 48 hours, RNA was isolated, transcribed to cDNA and used for PCR-arrays. Here we found several genes being regulated. The two IAP-(inhibitor of apoptosis) genes birc1b and birc3 showed an approximately two-fold induction following

incubation for both 24 and 48 hours, tumor-necrosis factor (TNF)-α was induced almost three-fold at both time-points, and the gene pycard was induced two-fold after 24 hours and almost 8-fold at 48 hours. The pycard gene encodes an adaptor protein that is a member of the six-helix bundle death domain-fold superfamily which mediates the assembly of large signalling complexes in the inflammatory and apoptotic signalling pathways via the activation of caspases. The results indicate that TCDD affects the expression of a variety of anti-apoptotic pathways which needs further investigation.
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394

Quantification of DNA Adducts of 1-Methlypyrene by Liquid Chromatography-Tandem Mass Spectrometry: Multiple Reaction Monitoring vs. ^{32}P-Postlabeling
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The alkylated polycyclic aromatic hydrocarbon, 1-methylpyrene (1-MP), is a hepatocarcinogen in rodents and has been detected in cigarette smoke condensates and car exhausts. It is activated metabolically by benzylic hydroxylation to 1-hydroxymethylpyrene (1-HMP) followed by sulfonation to yield an electrophilic sulfuric acid ester, 1-sulfoxymethylpyrene (1-SMP), that is prone to form DNA adducts. A liquid chromatography-tandem mass spectrometry (LC-MS/MS) method using multiple reaction monitoring (MRM) of fragment ions has been developed for specific detection and quantification of N^2-(1-methylpyrenyl)-deoxyguanosine (MPdG) and N^6-(1-methylpyrenyl)-deoxyadenosine (MPdA) adducts formed in DNA in the presence of 1-SMP. DNA samples were spiked with stable isotope internal standards, [$^{15}N_5$,$^{13}C_{10}$]MPdG and [$^{15}N_5$]MPdA, followed by enzymatic digestion to 2´-deoxynucleosides and solid-phase extraction to remove unmodified 2´-deoxynucleosides prior to analysis by MRM. The detection limit of the method was 5 fmol MPdG or 0.5 fmol MPdA (40 MPdG or 4 MPdA per 10^8 2´-deoxynucleosides) using 100 µg of herring sperm DNA as the matrix. The method was validated with herring sperm DNA reacted with 1-SMP *in vitro*. Liver DNA was analyzed from rats that were dosed intraperitoneally with 9.3 mg/kg 1-SMP and killed after various time periods. Levels of MPdG and MPdA in rat liver were found to increase reaching maxima at 6 h and then decrease over time. A good correlation was observed between the results obtained for DNA samples using LC-MS/MS MRM as compared to those from ^{32}P-postlabeling. The MRM method allowed the more precise quantification of specific 1-MP adducts, in addition to a time reduction of the analysis when compared with the ^{32}P-postlabeling method.
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395

Aldosterone causes oxidative DNA damage
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The mineralocorticoid aldosterone controls the electrolyte and fluid balance and subsequent blood pressure homeostasis. In normal physiological situations, aldosterone is regulated inversely with the salt status. An inappropriate increase of the aldosterone level may be induced by a stimulated renin-angiotensin system, predominantly in patients with heart failure or in a subgroup of hypertensive patients. Epidemiological studies exploring the connection between hypertension and cancer incidence find a higher cancer mortality in hypertensive patients and an increased risk to develop kidney cancer. We could already show the *in vitro* genotoxicity of angiotensin II in porcine renal proximal tubular cells (Schupp et al. Am J Physiol 2007). Since both angiotensin II and aldosterone are increased in many hypertensive patients, we also studied the potential genotoxic effects of aldosterone in cultured renal cells. While there was no significant change in cell vitality and proliferation, aldosterone concentrations starting from 10 nM caused a significant increase of single and double strand breaks monitored with the comet assay. Aldosterone concentrations starting from 100 nM also caused micronuclei. Flow cytometric measurements showing oxidative stress caused by aldosterone hint to the mechanism by which aldosterone causes DNA damage. The aldosterone-induced DNA damage could be reduced by the addition of the steroidal mineralocorticoid receptor antagonist eplerenone. Further the damage could be prevented by apocynin, an inhibitor of NADPH oxidase, suggesting that the DNA damage is caused by reactive oxygen species. Time course experiments show an onset of DNA lesions already after 5 minutes. Addition of DNA transcription and protein translation inhibitors will clarify, if the genotoxic effect of aldosterone is a classical genomic or a rapid non-genomic action.
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396

Angiotensin II is not only genotoxic *in vitro* but also causes DNA strand breaks in the isolated perfused kidney
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Increased activity of the renin angiotensin system with enhanced levels of angiotensin II (AngII) leads to oxidative stress with endothelial dysfunction, hypertension and atherosclerosis. Newer epidemiological studies revealed a higher cancer mortality and an increased kidney cancer incidence in hypertensive patients. Among other factors, an elevated AngII level might contribute to carcinogenesis, in particular of the kidney. We could show that AngII in concentrations, which can be expected in primary urine of hypertensive subjects, induces DNA damage in several cell lines analysed by the comet assay and the micronucleus frequency test (Schupp et al. Am J Physiol 2007). This observation could now be confirmed at the organ level in isolated perfused mouse kidneys. As little as 1 nM AngII caused a significant increase of DNA strand breaks. This damage was independent of the lower perfusate flow caused by AngII, since a similar decrease of the flow with the thromboxane mimeticum U-46619 did not lead to DNA breaks. Antagonists of the two angiotensin II receptor subtypes AT_1 and AT_2 were tested to identify the receptor responsible for the induction of DNA damage, showing a role only for the AT_1 receptor and its downstream signalling. The DNA strand breaks in the isolated kidneys were also prevented by the use of an AT_1-receptor antagonist. AngII-binding to AT_1 activates the NADPH oxidase which results in the production of reactive oxygen species (ROS). The antioxidant *N*-acetyl cysteine could prevent AngII-induced DNA damage, indicating the involvement of ROS. The type of DNA damage induced by ROS involves single strand breaks, double strand breaks, abasic sites and 8-OHdG adducts, and all of these were detected *in vitro*. The participation of the superoxide-generating enzyme NADPH oxidase was shown by a decrease of damage after the application of specific inhibitors. First experiments exploring the signal transduction pathway from AT_1 to the activation of the NADPH oxidase suggest an involvement of phospholipase C and protein kinase C.
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397

The mycotoxin alternariol acts as a topoisomerase poison, preferentially affecting the IIα isoform
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Alternariol (AOH), a mycotoxin formed by Alternaria alternata, has been reported to possess genotoxic properties. However, the underlying mechanism of action is unclear. In the present study, we tested the hypothesis that interactions with DNA-topoisomerases play a role in the DNA damaging properties of AOH. First we compared DNA-damaging properties of AOH with other Alternaria mycotoxins such as alternariol monomethyl ether (AME), altenuene and isoaltenuene. AOH and AME significantly increased the rate of DNA strand breaks in human carcinoma cells (HT29, A431) at micromolar concentrations, whereas altenuene and isoaltenuene did not affect DNA integrity up to 100 µM. Next, we selected AOH as the most DNA-damaging Alternaria metabolite for further studies of interactions with DNA topoisomerases. In cell-free assays, AOH potently inhibited DNA-relaxation and stimulated DNA cleavage activities of topoisomerase I, IIα and IIβ. Stabilisation of covalent topoisomerase II-DNA intermediates by AOH was also detectable in cell culture, and here, the IIα-isoform was preferentially targeted. AOH is thus characterized as a poison of topoisomerase I and II with a certain selectivity for the IIα-isoform. Since topoisomerase poisoning and DNA-strand breakage occurred within the same concentration range, poisoning of topoisomerase I and II might at least contribute to the genotoxic properties of AOH.
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398

Nucleotide excision repair and cisplatin sensitivity in human tumor cell lines
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Nucleotide excision repair (NER) acts on a variety of DNA lesions, including damage induced by many chemotherapeutic drugs such as cisplatin. Therefore the repair capacity of cancer cells might affect the efficacy of chemotherapeutic treatment. We investigated the importance of NER on cisplatin sensitivity in bladder cancer cell lines and testis tumor cell lines. We asked whether down-regulation of the NER protein ERCC1 would affect cellular sensitivity and repair capacity of cisplatin-resistant MGH-U1 bladder cancer cells. MGH-U1 was treated with siRNA against ERCC1. We found that ERCC1 levels could be reduced to less than 20 % of untreated control cells. Down-regulation of ERCC1 led to an increase in apoptosis induction and decrease in survival after cisplatin treatment. ERCC1 forms a tight complex with its partner XPF. Using a bi-cistronic mammalian expression vector we over-expressed both ERCC1 and XPF in the testis tumor cell lines 833K and SuSa. However, in these cell lines over-expression of the ERCC1-XPF complex had only a minor effect on apoptosis induction and cellular survival after treatment with cisplatin. Currently we are investigating whether down-regulation of ERCC1 in bladder cancer cells and over-expression of ERCC1-XPF in testis tumor cells affects the capacity to repair cisplatin-induced DNA damage.
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399

Trex1 is c-Fos dependent regulated and interacts with DNA polymerase eta: a novel cell protection mechanism
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Cells deficient in c-Fos are hypersensitive to genotoxic stress such as ultraviolet (UV) light, alkylating agens and benzo[*a*]pyrene diolepoxide (B(a)P). Here we analysed the c-Fos dependent regulation of DNA repair genes in mouse fibroblasts upon exposure to UV light. Microarray analysis revealed a strong UV provoked induction of the three prime exonucleases I (Trex1) and II Trex2, which was verified by real time PCR and protein expression studies. Experiments using the transcriptional inhibitor α-amanitin showed that the induction of Trex1 is caused by promoter activation, whereas the induction of Trex2 is caused by stabilization of the mRNA. The UV induced induction of Trex1, but not Trex2, is abrogated in c-Fos deficient cells, as shown by RT-PCR and protein analysis. The Trex1 promoter was cloned and shown to be inducible by UV light. It contains an AP-1 binding site, which is recognized by c-Fos (AP-1) as shown by chromatin immunoprecipitation (CHIP). Beside UV, Trex1 and Trex2 are also induced by different chemical genotoxic stimuli, such as B(a)P and H_2O_2. These agents also induce c-Fos in the same cells at high level. To study the biological role of Trex1, we performed co-immunoprecipitation experiments to demonstrate physical interaction with other proteins. We were able to show that Trex1 interacts with the translesion polymerase eta (PolH). The data strongly suggest a role for Trex1 in the tolerance of UV and B(a)P-induced DNA damage.
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400

Human monocytes are hypersensitive to alkylating genotoxins due to a DNA repair defect

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Monocytes and dendritic cells (DCs) are key players in the immune response. We studied the sensitivity of monocytes and monocyte-derived DCs against various genotoxic agents and found monocytes to be more sensitive to overall cell kill and apoptosis upon exposure to methylating agents (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, temozolomide, methyl methanesulfonate) than DCs. The data indicated a defect in the repair of DNA methylation damage in monocytes. Because no influence of the repair protein O^6-methylguanine-DNA-methyltransferase on sensitivity of monocytes was observed, we investigated the base excision repair (BER) pathway. Monocytes showed an accumulation of DNA single-strand breaks upon treatment, indicating DNA repair patches cannot be sealed or ligated. Expression studies revealed that monocytes lack XRCC1 and ligase IIIa, whereas DCs express these repair proteins at a high level. Lack of XRCC1 and ligIIIa may explain the observed accumulation of DNA breaks after alkylation. Further, we investigated the expression of XRCC1 and ligase IIIa in $CD34^+$ blood stem cells and different cell types of the myeloid and lymphoid lineage. $CD34^+$ stem cells express XRCC1 at lower level and lack detectable ligase IIIa. Human macrophages express both proteins at lower level, whereas T helper cells (Th), cytotoxic T cells (CTL) and B cells express both proteins at similar level than DCs. The data revealed that human monocytes exhibit a base excision DNA repair defect that makes them valuable to methylating genotoxins, including various anticancer drugs (Ref.: Briegert and Kaina, Cancer Res., 67, 26-31, 2007).

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401

Homologous recombination protects against O^6-methylguanine-triggered apoptosis, DNA double-strand break formation and chromosomal aberrations, but not against sister chromatid exchange formation

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Genotoxicity caused by many carcinogens and anti-cancer drugs with alkylating properties is due to the formation of O^6-methylguanine (O^6MeG). O^6MeG-triggered genotoxicity is dependent on reversal repair by O^6-methylguanine-DNA methyltransferase (MGMT) and mismatch repair (MMR) and is executed by a strong induction of apoptosis. The model for O6MeG-induced genotoxicity and apoptosis implicates the formation of DNA double-strand breaks (DSBs). If this is true, repair of DSBs would play a key role in the defense against alkylating agent-induced O^6MeG lesions. This study was aimed at elucidating the contribution of the main pathways of DSB repair, non-homologous end joining (NHEJ) and homologous recombination (HR), on cellular protection against O^6MeG. NHEJ defective cells (DNA-PKcs mutated, and Ku80 mutated) only showed a marginal increase in apoptosis following O^6MeG induction while HR defective cells (Xrcc2 mutated, and Brca2 mutated) showed an 8-11 fold increase in apoptosis compared to wild-type controls. Stable transfection with the repair protein MGMT completely abrogated the apoptotic response in all cell lines used. NHEJ defective cells were able to repair the DSBs arising from the processing of O^6MeG lesions while HR defective cells were unable to do so. We also showed that DSBs after treatment with alkylating agents arose from O^6MeG lesions, as MGMT transfected cells did not display DSB formation. The consequence of lack of DSB repair in HR defective cells after MNNG or temozolomide treatment was a huge increase in chromosomal aberrations compared to wild-type controls, while there was no difference in clastogenicity between NHEJ defective cells and wild-types. Using synchronized cells we show that in the second cell cycle following O^6MeG induction recombination events occurred which can be visualized as sister chromatid exchanges (SCEs). Formation of SCEs is dependent on HR and is required for protection against O^6MeG-induced genotoxicity. Collectively, the data clearly demonstrate that HR is required for O^6MeG tolerance by a mechanism that involves recombinational bypass of O^6MeG/T mispaires in the second cell cycle following genotoxic treatment.

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402

Cockayne syndrome B (CSB) deficiency enhances transcriptional inactivation caused by small number of 7,8-dihydro-8-oxoguanine bases in the transfected plasmid DNA

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Transcription inhibition by oxidised DNA bases is of dual importance. First, it is potentially toxic for the cell. Second, it may signal the switch between base and nucleotide excision DNA repair (BER and NER) pathways if RNA polymerase complexes stall at the base damage site. Since these effects are technically hard to address, they are poorly studied in mammalian cells. We have applied a host cell reactivation assay combined with flow-cytometry to measure the effects of oxidised guanine on transcription.To randomly generate oxidised guanines, reporter plasmids were damaged by visible light in presence of photosensitisers methylene blue or Ro18-8022. To incorporate single 7,8-dihydro-8-oxoguanine (8-oxoG) in defined position, the non-transcribed strands were purified from M13 phage particles. The complementary strands were generated by extension of a modified oligonucleotide containing a synthetic 8-oxoG, and ligated plasmids were purified from agarose gels. Damaged plasmids encoding for green fluorescent protein were co-transfected with a reference plasmid encoding for dsRed protein.Presence of 8-oxoG in the reporter plasmids treated with photosensitiser detectibly impaired the transgene expression in human and murine cells. Most likely, this effect was not caused by RNA polymerase stalling at the 8-oxoG sites, since it was observed even at very low lesion densities (less than one base modification per transcribed DNA strand). Even though, functional CSB protein, an important component of transcription-coupled NER machinery, alleviated – but not completely restored – transcription of damaged DNA. In time-course studies, transcription of 8-oxoG containing plasmids did not differ from the control plasmid till 8 hours after transfection, but descended in the time interval between 12 and 24 hours. We thus suggested that processing of oxidised guanine by DNA repair enzymes can result in transcription inhibition. In support of this notion, we found that 8-oxoguanine DNA glycosylase 1 (OGG1) contributes to the transgene inactivation, at least in CSB-deficient cells. We thus conclude that 8-oxoG repair intermediates can block transcription.

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403

A role for iron-sulfur cluster proteins in mutagenicity

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Oxidative damage to DNA is thought to contribute to the pathogenesis of various diseases including several types of cancer as well as aging. Reactive oxygen species are formed as by-products during normal cellular metabolism, but can also be formed by genotoxic chemicals and ionizing radiation. Cells have several defence systems against DNA damage caused by oxidative stress. One of the protection mechanisms is DNA repair, which removes oxidative DNA damage and restores correct genetic information. Some of these DNA repair enzymes depend on the activity or expression of iron-sulfur (Fe/S) cluster containing proteins. The aim of this work was to find out whether overexpression of the mitochondrial protein frataxin, which is involved in the maturation of Fe/S cluster containing proteins, leads to an enhanced DNA repair and a reduced mutagenicity. The antimutagenic effect of frataxin was analyzed with the Ames test, a standard assay used to test the ability of a compound to induce mutations in bacteria. Salmonella typhimurium strains expressing the Friedreich Ataxia protein in the wildtype and I154F form were generated. *S. typhimurium* strain TA104 expressing the Friedreich Ataxia protein not only showed reduced numbers of spontaneous revertants but also an enhanced protection against mutagenic effects of benzo[*a*]pyrene 4,5-oxide, 1-nitropyrene and glycidamide. The antimutagenic effect of frataxin in eukaryotic cells was tested by using the forward HPRT gene mutation assay. V79 Chinese hamster cells were transfected with the mammalian expression vectors pCI-neo and pCI-neo-hFX (the latter containing a human frataxin cDNA). V79 cells overexpressing human frataxin exhibited reduced mutation frequencies when exposed to potassium bromate, a complete carcinogen and model substance for the induction of oxidative stress. Taken together these data suggest that the activity or expression of Fe/S-cluster containing proteins might play an important role in the repair of damaged DNA.

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404

Histone deacetylase inhibitor trichostatin A modulates transcriptional inactivation by oxidative purine lesions in DNA

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We and others have previously found that oxidative purine modifications induce gene inactivation in transfected cells. These findings are hard to reconcile with *in vitro* data that shows very week capacity of these lesions to block transcription. Since vectors can be inactivated by epigenetic mechanisms that involve histone deacetylation and subsequent DNA methylation, we tested the relevance of this mechanism for transcription of oxidatively damaged DNA.Reporter plasmids were damaged by visible light in presence of methylene blue as a photosensitiser to generate one to three oxidised purine bases – mostly 7,8-dihydro-8-oxoguanine (8-oxoG) – per plasmid molecule. The lesions were quantified by treatment with formamidopyrimidine-DNA glycosylase (Fpg). Damaged plasmids encoding for green fluorescent protein (GFP) were co-transfected with a reference plasmid encoding for dsRed, and GFP expression was compared after 24 hours between the cells that were treated with HDAC inhibitors, namely trichostatin A (TSA), valproic acid and splitomicin. Host cells were mouse embryonic fibroblast (MEF) cell lines derived from wild type, $ogg1^{-/-}$, $csb^{mut/mut}$ and $csb^{mut/mut}/ogg1^{-/-}$ mice. In all tested cell lines, transcriptional block by oxidative DNA lesions was significantly enhanced by TSA that is an inhibitor of HDAC classes 1 and 3. At the same time, valproic acid had a very weak effect, and a HDAC class 2 inhibitor splitomicin had no detectable effect on expression of damaged reporter gene. It is known that TSA induces hyperacetylation of histones and thus increases nucleosome mobility and modulates overall chromatin structure. This can determine better accessibility of damaged DNA to the repair factors and other sensor proteins. Capability of TSA to regulate transcriptional inactivation by oxidative lesions in DNA indicates that accessibility of the lesion can be critical for the transcriptional status of the damaged genes.

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405

Expression of PARP-1 protein under the influence of cigarette smoke condensate in cultivated human lung cells

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Cigarette smoke exposure has been reported to induce DNA damage in several cell types. Poly (ADP-ribose) polymerase-1 (PARP-1), is a nuclear enzyme that has been included in the cellular response to DNA injury, and is supposed to play a role in determining whether DNA damage lead to repair or to apoptosis. Therefore, this study was directed to examine the PARP-1 expression in normal human bronchial epithelial cells (NHBEC) grown directly from normal human lung tissue after exposure to Cigarette Smoke Condensate (CSC) for 24h and 3 weeks to compare the effect of acute and chronic exposure and the correlation between the activity and expression of this protein under the influence of smoking. PARP-1 expression in (NHBEC) and tumor cell line A549 could be detected by Western Blotting. To study the protein activity a functional

fluorescence assay for measurement of PARP-1 activity has been used, after induction of ADP-ribose polymers formation by H_2O_2 (100 µM). Treatment of NHBEC with (CSC) 0.5 mg/L for 24h increased PARP-1 expression by factor 1.6 compared to the control (basal cellular PARP-1 expression), and by factor 1.8 for cells treated for 3 weeks with the same dose. (CSC) alone has induced PARP-1 activity by factor 1.2 compared to the control (basal cellular PARP activity) and has increased H_2O_2 (100 µM) -induced PARP-1 activity from 1.3 fold to 1.8 fold. Also the activity was increased after 3 weeks treatment with (CSC) by factor 1.2 more than the basal activity induced by H_2O_2. The used concentration of CSC was within the subtoxic range as indicated by MTT-assay. We can conclude that PARP-1 expression and activity in (NHBEC) were increased by (CSC) in acute and chronic exposure.

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406

Aprataxin is a component of DNA single-strand break repair and interacts with poly (ADP-ribose) polymerase 1

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Aprataxin has recently been identified as the protein mutated in the hereditary disease ataxia with oculomotor apraxia type 1 (AOA1). This is a mainly neurological disorder characterized through cerebral atrophy, gait disorder, early onset and the characteristic oculomotor apraxia. Cells from AOA1 patients are sensitive to different DNA-damaging agents like hydrogen peroxide, methyl methanesulfonate and camptothecin. In vitro, aprataxin has been shown to remove abortive 5'-AMP-intermediates generated during ligation of strand-breaks. The purpose of this study was to test wether the proposed mechanism for aprataxin has an effect on the cellular DNA single-strand break repair after hydrogen peroxide treatment and to obtain insights in a possible functional interaction with poly (ADP-ribose) polymerase 1 (PARP-1) which has been shown to interact directly with aprataxin. Our results indicated that the repair of single-strand breaks in lymphoblastoid cell lines derived from AOA1 patients was only slightly delayed as measured with the alkaline elution assay. In non-proliferating (confluent) mouse embryonic fibroblasts from AOA1 knockout mice the repair retardation was found to be more pronounced. This could indicate that aprataxin is more relevant for short-patch repair than for long-patch repair, since the latter pathway is less active in the G0 phase of the cell cycle. To check the interaction with PARP-1, we treated cells with a PARP-inhibitor (DPQ) before exposure to hydrogen peroxide. DPQ inhibited single-strand break repair in all cell lines, but the effect was significantly higher in AOA1 cells than in the controls. The inhibition of PARP activity (poly-(ADP-ribosyl)ation) by DPQ was the same in both cell lines, as indicated by a flow cytometric based immunofluorescence assay.

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407

Poly(ADP-ribose) polymerase 1 dependent chromatin remodeling is associated with the efficient repair of oxidative DNA base modifications

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The posttranslational modification of nuclear proteins by poly(ADP-ribose) polymerase 1 (PARP1) is an immediate response of mammalian cells to the infliction of various types of DNA damage. We have shown previously, that the efficient repair of oxidative DNA modifications involves a mechanism dependent on PARP1, CSB and XPA proteins. However, the exact role of PARP1 in the DNA damage response is only poorly understood.Here we report that PARP1 in the pathway seems to be required for an efficient block of transcription, since the repair retardation observed in the absence of PARP1 activity, either in *parp1*$^{-/-}$ cells or by inhibition of the catalytic activity by the PARP inhibitor DPQ, can be compensated by either a-amanitin, a RNA pol II inhibitor, or a transcriptional "road block" by a high dose of UVB. In addition, we have quantified the extent of histone acetylation (H3; H4) using specific antibodies in western blot and FACS analysis and observed that after DNA damage induction with the photosenstizer Ro 19-8022, PARP1 (together with CSB) alters chromatin structure by increasing the DNA damage dependent acetylation of histones. We conclude that PARP1 acts as a sensor of oxidative DNA base damage in combination with the transcription complex and thereby facilitates the accessibility and global repair of DNA modifications.

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408

Impact of zinc on cellular poly(ADP-ribose) formation

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Poly(ADP-ribosyl)ation is a reversible posttranslational modification of nuclear proteins and represents an early cellular response to DNA damage generated by endogenous and exogenous damaging agents in mammalian cells. The synthesis of poly(ADP-ribose) chains covalently attached to target proteins is catalysed by poly(ADP-ribose)polymerases (PARPs), using NAD^+ as substrate. Poly(ADP-ribosyl)ation is involved in several cellular processes including DNA repair and maintenance of genomic stability. Poly(ADP-ribosyl)ation is mostly catalyzed by PARP-1, an abundant nuclear enzyme that binds via its zinc finger motifs to DNA with single or double strand breaks. Zinc binding has shown to be essential for activation of purified PARP-1 *in vitro*. A decrease in cellular poly(ADP-ribosyl)ation has been implicated in the aging process. Its decreased function might be related to age-related zinc deficiency. Our aim was to determine cellular poly(ADP-ribosyl)ation capacity as function of age and zinc status in humans. Therefore healthy old subjects from various European countries were recruited in the framework of the EU FP6 project "ZINCAGE" and supplemented orally with 10 mg zinc for 7 weeks. Blood was taken before and after zinc supplementation and the plasma zinc level was determined by inductively-coupled-plasma mass-spectrometry (ICP-MS). Cellular poly(ADP-ribosyl)ation capacity was assessed as function of age and nutritional zinc status by using a recently established flow cytometry based assay. Our results reveal a positive correlation between cellular poly(ADP-ribosyl)ation capacity and zinc status in human peripheral blood mononuclear cells (PBMC) ($p<0.05$). We could also confirm a decrease of PARP-1 activity with donor age, highlighting the role of PARP in the aging process. The results demonstrate that zinc supplementation in elderly people can increase the cellular poly(ADP-ribosyl)ation capacity of their PBMC. We speculate that this may help maintain integrity and stability of the genome more efficiently and so contribute to an extension of "healthspan".

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409

Age-associated pathology and impaired survival in a novel mouse model with ectopic expression of human poly(ADP-ribose) polymerase-1

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Poly(ADP-ribose) polymerase-1 (PARP-1) exhibits a key role in the regulation of nuclear functions. By using NAD^+ as a substrate, PARP-1 modifies a plethora of nuclear proteins with the biopolymer poly(ADP-ribose), thereby regulating a variety of cellular processes such as DNA repair, genomic stability, chromatin remodeling, gene transcription, and cell death. Its diverse functions on the cellular level are reflected by its contribution to multiple physiological and pathophysiological conditions on an organismal level. We generated a novel mouse model with ectopic expression of the human-PARP-1, which exhibits a 2-5fold higher poly(ADP-ribosyl)ation capacity than its rodent orthologue. In agreement with the higher poly(ADP-ribosyl)ation capacity of the human enzyme, genetically modified mouse embryonic stem cells, which do express human as well as murine-PARP-1 exhibit decreased basal NAD levels yet show normal DNA repair capacity. Our current phenotypic analyses reveal that starting from week 4, hPARP-1-expressing mice, which are on a mixed genetic background (B6x129P2), display approximately 10% increased body weight than wild-type littermate controls, exhibit hyperglycemia, and display impaired survival rates. By the age of 12 months, less than 5% of the wild-type littermate controls died, whereas more than 25% of the genetically modified animals deceased. Such animals often showed age-related kyphosis, which is considered a sign of osteoporosis. The unexpected phenotype of our hPARP-1 mice suggests a novel role of PARP-1 in age-associated alterations of energy metabolism, which could form a new basis for pharmacological intervention.

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410

Poly(ADP-ribose) interacts non-covalently with Werner syndrome protein and inhibits its helicase activity

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Poly(ADP-ribose) polymerase 1 (PARP-1) is a molecular DNA damage sensor catalyzing the synthesis of the complex biopolymer poly(ADP-ribose) [PAR] under consumption of NAD^+. PAR is engaged in fundamental cellular processes such as DNA repair, transcriptional regulation and mitosis, and it interacts non-covalently with specific binding proteins involved in DNA repair. The Werner syndrome protein (WRN) is a RecQ helicase involved in several DNA repair pathways. Its deficiency leads to premature aging. WRN interacts with PARP-1 and is thought to functionally co-operate with PARP-1 in DNA repair pathways.The objective of our project was to characterize WRN overexpressed in Sf9 insect cells and to study a potential interplay with PAR.WRN helicase was overexpressed in Sf9 cells using the baculovirus system and purified. Coomassie and silver staining of affinity-purified recombinant WRN revealed 85 % homogeneity. Isolated WRN was then analyzed with respect to its helicase and exonuclease activities using novel non-radioactive assays. The protein catalyzed the unwinding of a biotinylated forked 37mer duplex in a concentration-dependent manner, with a concentration of 9 nM WRN resulting in 53 % unwinding. In addition, the exonuclease activity of WRN was assessed using a biotin-labeled 49mer forked duplex and displayed exonucleolytic degradation of the substrate. 45 nM WRN was sufficient to digest 50 % of the used forked duplex in 3'-5' direction. PAR was biosynthesized *in vitro* and purified according to a DNA extraction protocol. Next, the non-covalent binding of PAR to WRN was monitored by PAR overlay blots and revealed a tight interaction which was resistant to high-salt washes (1M NaCl) underscoring the specificity. Moreover, PAR binding was demonstrated to negatively regulate WRN's helicase activity and to inhibit the unwinding of the forked duplex substrate. Taken together, we established a novel link between the RecQ helicase WRN and PARP-1 via the PARP-1 product, PAR, which contributes to our understanding of how PARP-1 and WRN co-operate in regulating DNA repair.

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411

Werner protein protects against topoisomerase I, but not topoisomerase II inhibitors, by preventing DNA double-strand break formation

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Werner syndrome helicase/3'-exonuclease (WRN) is majorly involved in DNA repair and replication. To analyze whether WRN plays a role in the repair of topoisomerase-induced DNA damage, we utilized U2-OS cells expressing WRN-specific si-RNA (*wrn-kd*) and the corresponding non-transfected cells (*wrn-wt*). We show that cells not expressing WRN are hypersensitive to treatment with the topoisomerase I inhibitor topotecan, as revealed by WST assay, colony formation and induction of apoptosis. Interestingly, there is no cross-sensitivity to topoisomerase II inhibitors, since *wrn-kd* cells show the same sensitivity to etoposide than *wrn-wt* cells. Upon exposure to topotecan, *wrn-kd* cells displayed enhanced DNA replication inhibition and S phase arrest, whereas upon etoposide exposure, both cell lines were equally arrested in the

G2 phase of the cell cycle. The most pronounced difference between WRN and wild-type cells was observed by measuring DNA single and double-strand break formation and strand break repair upon topotecan exposure. Topotecan induced DNA single-strand breaks peak 6 h after treatment and were repaired in both *wrn-wt* and *wrn-kd* cells at similar kinetics. However, only in *wrn-kd* cells single strand breaks were converted into DNA double-strand breaks (DSBs) at high frequency, as shown by neutral comet assay and by phosphorylation of H2AX. Our data provide evidence that WRN helicase is involved in the repair of DNA damage induced by topoisomerase I, but not topoisomerase II. This is most likely due to preventing the conversion of DNA single-strand breaks into DSBs during the resolution of stalled replication forks. Therefore, we suggest that in tumor cells the WRN status could impact on anticancer therapy with topoisomerase I, but not topoisomerase II inhibitors.
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412

Cyto- and genotoxicity studies with disperse orange 3 and its metabolites
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Among colorants used in foods, cosmetics, toys and textiles azo dyes represent a major class of substances. According to previous results certain azo dyes may be a potential source of exposure of consumers to aromatic amines. These amines are possibly formed during metabolism of the azo dyes on and in the skin and may cause various toxic effects, particularly genotoxicity, carcinogenicity and allergy. The azo dye disperse orange 3 (DO 3) is a well known contact allergen. DO 3 may migrate from coloured polyamide, polyester and acetate textiles. It can be split into the amines *p*-phenylene diamine (PPD) and 4-nitroaniline (4-NA). This reaction can take place in the liver (P450-mediated), in the gut (by bacteria) as well as on (by bacteria) and in (e.g. by keratinocytes) the skin. The resulting amines were described as genotoxic *in vitro*. In this study the potential cytotoxic and genotoxic effects of DO 3, its metabolites and migrates from DO 3-coloured textiles were investigated. Various fabrics (acetate, polyester, PE-microfiber) were coloured with diperse orange 3 and characterised based on textile chemistry. The various colouring methods resulted in fabrics with different colour fastness. The released amounts of DO 3 depend on the extraction method, on the textile substrate and the color content. Treatment of freshly isolated human keratinocytes with DO 3, PPD or 4-NA (up to 200 µM) resulted only in minor cytotoxic effects (reduction of viability of 33%, 7% and 7%, respectively). However, combined treatment with DO 3 and PPD induced a synergistic cytotoxic effect, while mixtures of DO 3 with 4-NA had no additional effect. The genotoxic potential of DO 3 and 4-NA was investigated in *S. typhimurium* TA 98, TA 100, TA 1537 and TA 1538 strains. Clear dose related mutagenic effects in the plate incorporation test were observed with DO 3 in the absence and presence of a metabolic system (hamster-S9) and with 4-NA in the presence of S9 only. In the *in vitro*-micronucleus test with concentrations of DO 3 between 1.56 and 50 µg/ml no increased frequency of micronuclei in V79 cells was observed. The results of these studies show that DO 3 migrates from consumer products, *e.g.* textiles, to human skin. It was demonstrated that DO 3 and its metabolites have mutagenic and cytotoxic effects. Furthermore the allergenic potential of DO 3 and PPD is well known. Because of the toxic, particularly mutagenic and allergic potential the azo dye DO 3 and its metabolites, the use in consumer products is of concern.
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413

Cytotoxic and genotoxic effects of three representative reprographic toner dusts and their dimethyl sulfoxide (DMSO) extracts on cultured human epithelial A549 lung cells *in vitro*
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In today's information society, the handling of toners for photocopiers or printers has become an everyday task. In principle, these toners do not pose any health hazards. However, some people have reported to react sensitively when exposed to toner dust, displaying allergic reactions of the skin, eyes and respiratory tract. Furthermore, the reported results concerning the mutagenicity and genotoxicity of reprographic toners are inconsistent. In this study the cytotoxic and genotoxic potency of three representative black reprographic toner dusts (A, B, C) were investigated by exposing human cells (A548) *in vitro*. For comparison, the known genotoxic compounds benzo[*a*]pyrene (BaP) and quartz DQ12 were studied. Cultured human epithelial A549 lung cells were exposed to toner particles in suspensions or to their dimethyl sulfoxide (DMSO) extracts at final concentrations corresponding to 80 µg toner dust/cm^2 up to 1000 µg toner dust/cm^2 for 24 h at 37°C. Cytotoxicity was assessed by the lactate dehydrogenase (LDH) assay, whereas genotoxicity was assessed using the micronucleus assay and the alkaline single-cell gel electrophoresis (comet assay). The toner particles and the toner extracts (except toner A) of all three toners tested showed significant cytotoxicity. Furthermore, all three toners as particles and as extracts (except toner A) were able to cause significant DNA damage in the comet assay and to induce micronuclei formation in the micronucleus assay, although to a different extent. Taken together, our results have shown that the three reprographic toner dusts tested were cytotoxic and genotoxic in A549 human lung cells *in vitro* using LDH assay, micronucleus assay and SCGE assay. To evaluate a possible human health risk further studies are necessary.
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414

Ochratoxin A induced genotoxicity in human urothelial 5637 and Chinese hamster V79 cells is not related to cytotoxicity
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Ochratoxin A (OTA), a frequent contaminant in various foods and feeds, is known to cause porcine nephropathy and is suspected to be involved in Balkan endemic nephropathy in humans. The mycotoxin is a potent carcinogen in rats, with kidney and urinary bladder as major target organs. Its mode of action is not fully understood, although recent evidence supports a non-DNA-reactive mechanism of OTA genotoxicity *in vivo* and in cultured cells. In this study we addressed the question whether OTA-mediated genotoxicity occurs in the absence or presence of notable cytotoxicity. To this end, human urinary bladder carcinoma (5637) and Chinese hamster lung fibroblast (V79) cells were exposed to graded concentrations of OTA: Cytotoxicity was determined with cell titer blue (mitochondrial activity) and neutral red (lysosomal activity) assays while genotoxicity was assessed by single cell gel electrophoresis (Comet assay, with and without FPG enzyme) and by means of the micronucleus assay. OTA exerted cytotoxicity in both 5637 and V79 cells in a concentration- and time-dependent manner. Cytotoxicity was seen at low micromolar OTA concentrations, with 5637 cells being about 5 times more sensitive than V79 cells. OTA-induced free radical generation in 5637 cells, assessed with dichlorofluorescein as fluorogenic probe, was detected only at or above cytotoxic OTA concentrations (> IC20). In contrast, induction of micronuclei in V79 cells occurred clearly below cytotoxic concentrations of OTA. Also, significant DNA damage was detectable in the FPG-modified Comet assay below cytotoxic levels of OTA in both cell types. Our results indicate that OTA can cause genotoxicity in the absence of notable cytotoxic effects in cells. This conclusion appears to conflict with that of other studies where rather high OTA concentrations were applied to cultured cells, far above urine or blood levels of this mycotoxin reached under realistic exposure scenarios. Studies to further characterize the OTA-mediated genotoxity should focus on such low levels.
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415

Genotoxicity and mutagenicity of the furan metabolite *cis*-2-butene-1,4-dial in L5178Y mouse lymphoma cells
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Furan is found in various food items and is cytotoxic and carcinogenic in the liver of rats and mice. Metabolism includes the formation of a dialdehyde, *cis*-2-butene-1,4-dial (BDA). In view of the multifunctional electrophilic reactivity of BDA, adduct formation with protein and DNA may explain some of the toxic effects. Short-term tests for genotoxicity of furan in mammalian cells are inconclusive, little is known for BDA. We investigated BDA, generated by hydrolysis of 2,5-diacetoxy-2,5-dihydrofuran, for genotoxicity in L5178Y mouse lymphoma cells with the comet assay, the micronucleus test, and the mouse lymphoma thymidine kinase gene mutation assay. BDA was tested at 0, 6.25, 12.5, 25, 50, and 100 µM. Cytotoxicity was remarkable; cell viability at 50 µM was reduced to 50 %. Up to 25 µM, cell viability was >90%, and measures of both comet assay and thymidine kinase mutations were increased about two-fold over control. The dose response showed linearity with a significant slope. Compared to methyl methanesulfonate used as positive control, BDA was slightly more potent. An increase in apoptotic cells measured as annexin V-positive cells by flow cytometry was observed at all BDA concentrations with a linear dose response. Furan added to the cells to give initial concentrations of up to 60 mM was neither cytotoxic nor genotoxic. The lack of effect could be explained by deficiencies of the used cell line in metabolic activation to BDA. A potential DNA-crosslinking activity of BDA was investigated by checking whether gamma radiation-induced DNA migration in the comet assay could be reduced by pretreatment with BDA. As opposed to the effect of the positive control glutaraldehyde, BDA treatment did not reduce the comets. On the contrary, an increase was observed at ≥100 µM BDA, which was attributable to early apoptotic cells. We conclude that although BDA was a relatively potent direct genotoxic agent in terms of the concentration necessary to double the background measures, effects such as cytotoxicity and induction of apoptosis will have to be taken into account in the discussion of modes of toxic and carcinogenic action of furan metabolites.
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416

Genotoxicity of dopamine *in vitro*
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The neurotransmitter dopamine has been described to cause apoptosis, oxidative stress and genomic damage in *in vitro* systems. Since dopamine levels are altered in certain diseases and treatments, the biological relevance of these *in vitro* findings needs to be determined. We are investigating the genotoxicity of dopamine in cell lines from different tissues upon characterization of these cell lines for the expression of dopamine-receptors and the dopamine transporter. Human lymphoblastoid TK6 cells, which are used in routine substance testing, express D1 and D5, rat neuronal PC12 cells express D1, D2, D4, D5 and the dopamine transporter, and rat kidney NRK cells express D1, D2, D4, D5. In all 3 cells lines, genomic damage was detected with the micronucleus test and the comet assay after treatment with dopamine. The lowest tested dose, 6.25 µM, was still positive in the micronucleus test in PC12 cells. The effect was reduced after blocking the D1 receptor with the antagonist SCH 23390 (in TK6, PC12 and NRK) or the D2 receptor with the antagonist sulpirid (in PC12 and NRK) or by unspecific dopamine receptor blocking with haloperidol (NRK). To test whether reduction of the effect is really due to receptor antagonism or some other property of the antagonists, the antioxidant capacities of the antagonists are investigated. In support of an antioxidative mechanism, addition of a radical scavenger (TEMPOL) to dopamine treatment abolished the genotoxic effect. Although physiological plasma levels are orders of magnitude lower than the dopamine concentrations applied here, the serum dopamine levels in the treatment of Parkinson´s disease can reach this order of magnitude.
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417

Induction of micronuclei by a cholesterol-rich diet: role of cholesterol oxidation products and prevention by phytosterols

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Phytosterols (PS) such as β-sitosterol and campesterol are constituents of plant membranes and are found at high concentrations in functional foods designed to reduce serum cholesterol (ChOL) levels. In order to investigate the genotoxicity of ChOL and PS *in vivo*, female guinea pigs were exposed to a diet either low in ChOL (8 animals, negative control, 0.05% (w/w) ChOL) or rich in ChOL (16 animals, ChOL group, 0.2% (w/w)). 8 animals of the ChOL group additionally received PS (PS intervention group, 0.5% (w/w) β-sitosterol). After two weeks, animals were killed and the number of micronucleated normochromatic erythrocytes (MNE) was determined in the peripheral blood, revealing a significant increase in MNE in the ChOL-only group. Furthermore, the hepatic levels of sterols and their oxidation products were determined by GC/MS. Significant higher amounts of ChOL and its oxidation products 7α- and β-hydroxycholesterol, 7-ketocholesterol, 5,6α- and 5,6β-epoxycholesterol were determined in the cholesterol group than in the negative control or PS intervention groups. In order to identify the genotoxic agent(s), the genotoxicity of 7-hydroxy- and 5,6-epoxy oxidation products was determined *in vitro*. However, neither oxidation product induced micronuclei in cultured Chinese hamster V79 cells. Interestingly, 7-HO-sterols were one order of magnitude more cytotoxic in human breast adenocarcinoma (MCF-7) cells expressing several sulfotransferases isozymes (SULTs) than in V79 cells which lack SULT activity. Moreover, cytotoxicity in MCF-7 cells was significantly decreased by inhibition of SULT activity, suggesting metabolic activation of 7-hydroxysterols. In conclusion, a ChOL-rich diet induced DNA damage *in vivo*, which may be due to metabolic activation of ChOL oxidation products and PS were able to prevent the genotoxicity of ChOL possibly by reducing ChOL absorption and thus reducing the exposure to reactive ChOL oxidation products. The possible activation of 7-hydroxysterols by SULTs might have implications for ChOL-associated pathologies and requires further investigation.

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418

A polymorphism in the vascular endothelial growth factor receptor-1 (Flt-1) promoter provides synergy between p53 and estrogen receptor under environmental stress

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Flt-1 is important for development, tissue regeneration, as well as proliferation and vascularization of tumors. Thus, addressing mechanisms that control Flt-1 regulation in response to environmental stress is relevant to understanding several diseases. Previously, we found that the tumor suppressor p53 could stimulate transcription at an Flt-1 promoter variant where a single nucleotide polymorphism (SNP) created a half-site p53 response element (RE; FLT1-T). However, informations about p53 gene responsiveness mediated by half-site REs in different promoters is missing. Thus, we addressed the question how the half-site RE influences Flt-1 expression. In addition to the p53 half-site RE, we found a second regulatory sequence comprising a half-site RE for estrogen receptors (ERE) which provides for synergistic stimulation of transcription at the FLT1-T allele through the combined action of genotoxic stress-activated p53 and ligand-bound estrogen receptor. We are also able to demonstrate how different environmental estrogenic chemicals are regulating this synergistic stimulation of transcription. Here, our study has established that the Flt-1 gene can be the target of cooperative interaction between stress-activated p53 and ligand-bound ER, mediated by two sub-optimal response elements, one of which contains a SNP. Since there is a dramatic increase in transactivation only when both environmentally responsive factors are expressed, this is an important example of environmentally distinct agents creating a synergistic response through a common, biologically important gene whose changes in expression may affect the risks associated with angiogenesis-driven diseases.

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419

Knock-down of the desmosomal cadherin desmoglein 2 (DSG2) in the intestine predisposes to colon cancer after treatment with azoxymethan

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Desmogleins are essential components of the desmosome and are assumed to be responsible for cell-cell adhesions. Recently, clinical data established that down-regulation of Desmoglein 2 (Dsg2) is associated with intestinal cancer and that low expression of Dsg2 correlates with poor survival rates in patients with gastric carcinomas. However, it is unknown whether, and if so, how Dsg2 contributes to the pathology of colon cancer. In this study, we addressed the consequences of Dsg2 down-regulation using a conditional RNAi knock-down mouse model. To functionally characterize the effect of decreased Dsg2 expression, transgenic mice carrying a Cre-inducible Dsg2-specific shRNA knock-down cassette were crossed to a colon-specific vilin-CreERT2 deleter mouse. The resulting bi-transgenic Dsg2 knock-down/vilin-CreERT2 mice were intraperitoneally injected with a single dose of the mutagenic agent AOM followed by one cycle of 3% DSS. Subsequent high resolution colonoscopy in live mice showed rapid hyperplasia and increased proliferation of epithelial cells, followed by colitis and tumour development only in conditionally activated knock-down mice, but not in the non-induced control group. Furthermore, histology of the colon of induced knock-down mice revealed the influx of immune cells into the highly proliferating tumour. In summary, RNAi-mediated conditional knock-down of Dsg2 in the colon demonstrated a causative relationship between Dsg2 loss-of-function and colon cancer.

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420

Tumorpromotion in liver of conditional c-Met knockout mice

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c-Met is the receptor for the hepatocyte growth factor / scatter factor (HGF/SF). Signalling through c-Met appears to affect tumor promotion, progression and metastasis. In this study we analysed the role of the receptor on mouse hepatocarcinogenesis using mice conditionally deficient of functional c-Met. Control and c-Met-deficient mice were injected with a single dose of N-nitrosodiethylamine (DEN, 90µg/g b.wt.) and groups of mice were subsequently kept on a phenobarbital (PB, 0.05%) containing or on control diet for 35 weeks. At the end of the experiment, the carcinogenic response in the liver of the animals was monitored. Surprisingly, DEN treated c-Met-deficient mice showed an increased incidence of macroscopically observable liver tumors and the number of liver lesions positive for glutamine synthetase (GS) and/or negative for glucose-6-phosphatase was elevated when compared to their wildtype counterparts. However, c-Met-deficient and wildtype mice showed a similar response to tumor promotion by PB. Interestingly, c-Met-deficient mice not treated with PB showed a comparatively large number of GS-positive lesions bearing mutations in the *ctnnb1*-gene encoding β-catenin. While a selection for *ctnnb1*-mutated liver lesions is typical for PB-mediated liver tumor promotion, lesions of this genotype are normally very infrequent in mice not treated with the tumor promoter. The absence of signalling through the Met-receptor in hepatocytes from Met-deficient mice may therefore be of advantage for *ctnnb1*-mutated hepatocytes with constitutively activated ß-catenin.

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421

Alterations in amylase activity in the mammary gland of female Fischer 344 rats after exposure to 50 Hertz magnetic fields

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Epidemiological data have raised concerns about the relationship between exposure to power-frequency (50 Hz) magnetic fields (MF) and breast cancer. We have shown previously that the effect of MF exposure on the rat mammary gland differs depending on the rat strain used. Comparison of different rat strains indicated that the genetic background plays a pivotal role in the MF effects. Among several rat strains, only Fischer 344 (F344) rats showed an enhanced proliferative activity in the mammary epithelium exposed to MF for 2 weeks. Prolonged MF exposure also significantly increased tumor development and growth in the dimethylbenz[a]anthracene (DMBA) breast cancer model in F344 rats. These results indicate that the F344 inbred rat serves as a MF-sensitive rat strain. Recently, we investigated the gene expression in the breast tissue of F344 rats and compared the results with Lewis rats (Lew) that are considered as MF-insensitive. Unexpectedly, the most striking result was a marked decrease of amylase gene expression in MF-exposed F344, but not in Lew. Because of this finding, we now determined amylase enzyme activity in the breast tissue of juvenile F344 rats that were exposed to MFs (100 µT) or to the synthetic estrogen diethylstilbestrol (DES). F344 were MF-exposed over different periods. The grade of differentiation of the breast tissue was checked up by whole mount analysis. DES application increased the appearance of more differentiated structures in the breast tissue in a dose-dependent manner. No alteration was observed in MF-exposed whole mounts of mammary glands. DES significantly increased amylase activity at the highest dosage (30 µg, 6 times). MF exposure also significantly increased enzyme activity in F344 and in Lewis rats.These data demonstrate that MF exposure and DES altered amylase activity in the rat mammary gland tissue. In literature, associations between amylase and tumor development are described, but the underlying mechanisms are not known. Future cell culture experiments from breast tissue might be able to reveal the amylase effect in the tissue.

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422

Effects of AICA riboside on ATP production at different stages of the cell cycle

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Aggressive cancer cells are typically characterized by a high metabolic activity. Therefore, one way to treat cancer cells seems to be the modulation of the cellular energy sensoric AMP activated protein kinase (AMPK) machinery because transformed cells are affected in this approach selectively. A modulation in this manner could be mediated by 5-aminoimidazole-4-carboxamide (AICA) riboside, which mimics intracellular AMP and thereby activates AMPK. Up to now the deactivation of energy consuming processes has primarily been analyzed. It seemed to us that an AICA riboside-mediated enhanced ATP production could also occur and this would lead to a decrease in cancer cell growth. In the presented study the above-mentioned hypothesis was tested experimentally. The well characterized mamma cancer cells MDA-MB-231 were synchronized by treatment with the cell cycle blockers mimosine or nocodazole, leading to the arrest of the cells in G1 and G2 phase, respectively. The subsequent application of AICA riboside surprisingly led to a decline in the ATP-concentration of cells arrested in G1. Not synchronized (untreated) cells show no differences in the ATP level. Hence we postulate that the effect of AICA riboside on cellular ATP production is

dependent on the phase of the cell cycle. This observation is an important aspect in the case of using AMPK modulators in cancer therapy.

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423

MGMT activity and promoter methylation in brain tumors

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The DNA repair enzyme O^6-methylguanine-DNA methyltransferase (MGMT) removes methyl groups from the O^6-position of guanine. If not repaired, O^6-methylguanine leads to the induction of cell death. Several anticancer drug, such as temozolomide, procarbazine, ACNU and CCNU utilize the cytotoxic properties of O^6-alkylguanine adducts to kill cancer cells. The therapeutic outcome of therapy with so-called O^6-alkylating agents is decisively determined by the amount of active MGMT molecules present in tumour cells. For brain tumours, notably glioblastoma multiforme, a correlation between the MGMT status and the therapeutical outcome of therapy with alkylating drugs was observed. Besides direct measurement of MGMT activity, determination of the methylation status of the MGMT promoter has been established for the evaluation of the MGMT status. To determine whether MGMT promoter methylation correlates with MGMT activity, and to elucidate which method correlates best with the therapeutic outcome, we determined the MGMT promoter methylation status by methylation specific PCR (MSP) using two different world-wide used primer combinations. We also determined MGMT activity in 41 glioblastomas (glioblastoma multiforme) and 48 brain and other tumour cell lines. Overall we found a correlation between MGMT activity and MGMT promoter methylation using both primer combinations. However, only MGMT activity correlated with overall survival (OS) and progression free interval (PFI). MSP using primer combination 1 correlates only with OS, and MSP using primer combination 2 correlates neither with OS nor PFI. The data indicate that determination of MGMT activity and MGMT promoter methylation using primer combination 1 are predictive tools in brain tumour therapy.

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424

Generation of reactive oxygen species - a major operating mechanism for fly ash particles

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Acute exposure to elevated levels of environmental particulate matter (PM) is associated with increasing morbidity and mortality rates. These adverse health effects, e.g. culminating in respiratory and cardiovascular diseases, have been demonstrated by a multitude of epidemiological studies. In this respect, particles derived from combustion processes appear to be especially potent environmental hazards. However, the underlying mechanisms relevant for toxicity are not yet completely understood. In this context the role of particle-induced reactive oxygen species (ROS), oxidative stress and inflammatory responses is of particular interest. The main focus of the present *in vitro* study was to understand the underlying mechanisms of ROS generation and how this is linked to oxidative stress- and inflammatory-responses. Incinerator fly ash (IFA) was used as a model for combustion derived particulate matter. As macrophages, besides epithelial cells, are the major targets of particle actions in the lung murine RAW264.7 and primary human macrophages were investigated. The interaction of IFA with macrophages induced oxidative stress, indicated by ROS-induced H_2DCF oxidation, increased intracellular glutathione contents and elevated amounts of the antioxidative protein hemeoxygenase-1 (HO-1) and the transcription factor Nrf-2. As part of cellular inflammatory responses we could observe an increase in the amount of free arachidonic acid, which depends on the activation of ERK1/2 mitogen activated protein kinases (MAPKs). Interestingly, ERK1/2 phosphorylation and mobilisation of arachidonic acid together with the induction of COX-2 and HO-1 are linked to the generation of ROS. Pre-treatment of macrophages with *N*-acetyl cysteine (NAC) blocked the IFA-induced ERK1/2 activation, the increase of free arachidonic acid as well as up-regulation of HO-1 and COX-2. Taken together, one of the primary mechanism initiating inflammatory processes by IFA particles seems to be the generation of ROS, which trigger the activation of downstream signalling and gene expression.

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425

ROS-mediated signal transduction pathways in lung epithelial cells activated by model environmental nanoparticles

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Environmental particles consist of a number of components, three of which are seen as toxicologically relevant: particle core, organic compounds and (transition) metals attached to it. These may cause the generation of extra- and intracellular reactive oxygen species (ROS) in the lung epithelium. The consequent oxidative stress is likely to be a regulation mechanism of signaling cascades leading to apoptosis and proliferation. For *in vitro* studies of additive/synergistic effects between particle components, a model particle system was used. This consists of pure ultrafine carbon particles, uncoated and coated with benzo[*a*]pyrene and/or $Fe_2(SO_4)_3$. Particle-treated rat lung epithelial cells (RLE 6-TN) were assessed for the induction of proliferative signaling involving phosphorylation of the MAP-kinase ERK1/2 as well as AKT. Particle-induced ROS generation was assessed both in cell-free systems (DCF-fluorescence and electron spin resonance) and within the cell by means of FACS-analyses of DCF-fluorescence. ROS-scavengers like catalase, superoxide dismutase, diphenyleneiodonium, and *N*-acetyl cysteine served to identify and characterize particle-induced ROS, and their influence on ERK1/2 and AKT signaling. Specific inhibitors were used for studying the involvement of the EGF receptor and ß1-integrins in these signaling pathways. Carbon nanoparticles induce phosphorylation of ERK1/2 and AKT. This effect is additively enhanced in the prescence of iron sulfate, whereas benzo[*a*]pyren shows no significant influence. Iron sulfate alone is also able to activate both signaling pathways, particularly at earlier time points. Inhibition studies indicate that particle-induced signaling is ROS dependent and receptor mediated. Our studies show that model particles are able to generate extra- and intracellular ROS, the resulting oxidative stress being likewise involved in the activation of proliferative signaling cascades via cell membrane receptors. Coating of the particle core modulates the particle-induced endpoints, depending on the nature of the bound substances, transition metals acting in this context as enhancers.

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426

The role of macrophage mediators in respirable quartz-elicited inflammation

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The instigation and persistence of an inflammatory response is widely considered to be critically important in quartz-induced lung cancer and fibrosis. Macrophages have been long recognised as a crucial player in pulmonary inflammation, but evidence for the role of type II epithelial cells is accumulating. For the current study, quantitative RT-PCR and immunohistochemistry were performed in lung tissue from rats after intratracheal instillation with DQ12 quartz. Increases in pro-inflammatory (iNOS, COX-2) and cellular stress (HO-1) genes were found on the mRNA level, while activation of the transcription factor NF-κB, know for its pivotal role in inflammation, was shown in alveolar epithelial type II cells and alveolar macrophages. To elucidate on the mechanism, *in vitro* investigations were performed in the rat lung type II cell line RLE and the rat alveolar macrophage cell line NR8383 using Western blotting, NF-κB binding ELISA, immunohistochemistry and qRT-PCR. The direct effect of quartz on pro-inflammatory signalling cascades and gene expression in RLE cells was compared to the effect of conditioned media derived from quartz-treated NR8383 cells. Conditioned media activated the NF-κB signalling pathway and induced a far stronger upregulation of iNOS mRNA than quartz itself. Quartz elicited a stronger, progressive induction of COX-2 and HO-1 mRNA. The effects of the macrophage media could not be inhibited by TNFα and/or IL-1β neutralising antibodies or *N*-acetyl cysteine. However, inhibition of the surface reactivity of quartz by polyvinyl-pyridine-*N*-oxide (PVNO) coating fully abrogated its effect. Our results suggest a differentially mediated inflammatory response, in which reactive particles themselves induce oxidative stress and activation of COX-2, while mediators released from particle-activated macrophages trigger NF-κB activation and iNOS expression in type II cells.

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427

Carbon nanoparticle-induced intracellular signaling: the role of membrane structures and receptors

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Environmental nanoparticles have been reported to induce adverse health effects in humans. Our previous studies showed that the interaction of nanoparticles (NP) with lung epithelial cells mediates cell proliferation via the activation of MAP kinases ERK1/2. Interestingly, the membrane receptors EGFR and ß1-integrins both are involved in this NP-specific signaling. The initial events, by which nanoparticles trigger this receptor-dependent signaling as well as the way how these receptors mediate the signal response, are not understood. In order to study these early signaling events on the level of membrane receptors, possible mediators of receptor crosstalk and membrane signaling platforms have been investigated in RLE-6TN (rat lung epithelial cells) treated with carbonaceous NP (Printex 90). Cells were preincubated with src family kinase inhibitor PP2 followed by treatment with NP. The induction of proliferative signal cascades involving phosphorylation of ERK1/2, Akt and src family kinases were studied by Western blot analyses. Furthermore, the consequence of inhibiting EGFR and ß1-integrins on the phosphorylation of src kinases was analysed using specific inhibitors/antibodies. Lipid rafts as membrane structures relevant for cell signaling were investigated using density gradient centrifugation.Treatment of cells with NPs resulted in an activation of src kinases at time points after 5min up to 8h. The src inhibitor dose-dependently prevented the activation of the NP-specific proliferative signaling pathway via ERK1/2 and Akt, demonstrating the relevance of src kinases for NP-specific signaling. Blocking of the receptors EGFR and ß1-integrins both resulted in a reduction of src phosphorylation. First investigations on lipid rafts, membrane microdomains in which the described receptors as well as src kinases are located, showed an impact of NP on raft protein composition. These results indicate an important role for src family kinases in the crosstalk of the identified membrane receptors in proliferative signaling. Moreover, NP-induced changes in lipid raft composition may be an early event in particle-cell interaction triggering NP-specific endpoints.

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428

Fibrous particles induced signaling in rat pleural mesothelial cells is ß1-integrin dependent

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Signaling events induced by fibrous particles have been described to be strongly dependent on the long and thin shape of these pathogenic materials leading to endpoints like fibrosis, apoptosis or proliferation. However, the molecular mechanisms triggering this shape specific signaling are not understood. In earlier studies we were

able to demonstrate that asbestos induced proliferative signaling via the ERK1/2 MAP-kinases and the anti-apoptotic Akt signaling cascade is mediated by ß1-integrins.Molecular signaling events linking ß1-integrin receptors with the proliferative signaling were investigated in the pleural mesothelilal cell line 4/4RM-4 with non-cytotoxic doses of UICC crocodolite.The role of integrins was confirmed using ß1-integrin specific shRNA which reduced the fiber induced Akt and ERK1/2 activation. As intracellular receptor coupled kinase, ILK (integrin linked kinase) was identified by experiments using dominant negative constructs. ILK dominant negative overexpression resulted in a reduced activation of Akt and ERK1/2 upon fiber exposure. As this kinase has been shown not to be activated by other particle types, we consider this signaling step as fiber specific. Using dominant negative constructs as well as specific pharmacological inhibitors additional steps connecting ß1-integrins and ILK to subsequent signaling steps were identified.With these results, we suggest a central role for integrins and ILK in the fiber specific signaling relevant for pathogenic endpoints. As ILK is known to link the cytoskeleton to focal contacts, cell responses induced by interaction with extremely long materials may be triggered at this site of signal transduction.

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429

PI3K/AKT signaling and FoxO4-mediated apoptosis in doxorubicin treated human colon carcinoma cells

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The PI3K/Akt signaling pathway is a well known cellular survival pathway which regulates cell growth and proliferation. Activated Akt (PKB) facilitates phosphorylation of multiple downstream targets on serine and threonine residues. One possible Akt downstream target is the FoxO4 forkhead transcription factor which is involved in cell cycle regulation and cell death. FoxO4 carries three Akt-specific phosphorylation sites whereby its cellular localization is regulated. Activation of PI3K/Akt signaling results in phosphorylation, nuclear exclusion and inactivation of FoxO4 and thereby cellular survival, whereas its nuclear localization and activation is associated with cell death and apoptosis. In this study we investigated the hypothesis that FoxO4 activation downstream of Akt is a crucial factor for doxorubicin (DOX) mediated cytotoxicity. Treatment of Hct-116 cells with DOX resulted in ROS burst, induction of cytotoxicity and apoptosis. As expected, activation of Akt was accompanied by phosphorylation and consequentially inactivation of FoxO4. In order to examine the effect of FoxO4 on DOX induced apoptosis, we transfected Hct-116 cells with wildtype and constitutive active FoxO4 which lacks Akt phosphorylation sites. Transient overexpression of wildtype FoxO4 increased DOX mediated cytotoxicity. In addition, overexpression of constitutively active FoxO4 even further increased DOX mediated cytotoxicity and apoptosis. To assess the role of Akt in DOX mediated FoxO4 signaling, inhibition of PI3K/Akt signaling by overexpression of dominant negative Akt was used. As expected overexpression of dominant negative Akt resulted in increased DOX mediated cytotoxicity and apoptosis. Altogether, the data emphasizes the crucial role of FoxO4 and Akt in DOX induced apoptosis in Hct-116 colon carcinoma cells.

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430

A cell-based assay for identification of natural compounds as modulators of the NF-κB signaling pathway

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The nuclear factor kappa B (NF-κB) transcription factors are known to play a key role in several physiological processes like inflammation and oxidative stress response, cell proliferation and apoptosis. As a consequence deregulated NF-κB activity contributes to human diseases like cancer, chronic inflammation or autoimmune disorders. It is therefor of great interest to identify specific and potent compounds that are capable of modifying this NF-κB pathway and to clarify their mechanism of action at the molecular level. For this purpose we stably transfected hepatoma cells with a pNF-κB-SEAP reporter construct where the expression of the secreted embryonic alkaline phosphatase (SEAP) is controlled by a synthetic promoter containing NF-κB binding sequences as enhancer elements. By that, it is possible to quantify modulation of NF-κB-activity simply by measuring the activity of SEAP in the medium. Stably transfected cell clones (H4IIE-SEAP) were selected and analyzed for NF-κB dependent SEAP activity after stimulation with known NF-κB activators like tumor necrosis factor-alpha (TNF-α) and phorbol 12-myristate 13-acetate (PMA). Treatment of H4IIE-SEAP cells with TNF-α or PMA increased NF-κB-dependent SEAP activity in a time and dose-dependent manner. Next we used two known inhibitors, resveratrol and caffeic acid phenethyl ester (CAPE), to determine whether the H4IIE-SEAP clones can be used to screen for NF-κB inhibitors. Resveratrol is a polyphenolic compound found in grapes and red wine, while CAPE is a caffeic acid derivative and compound of propolis (honeybee resin). Pretreatment of H4IIE-SEAP cells with resveratrol or CAPE inhibited TNF-induced NF-κB activation in a dose-dependent manner. These results show that the established reporter cell line H4IIE-SEAP responds as expected to known stimuli and therefore has the potential to identify new lead compounds that act as modulators of the NF-κB signaling pathway.

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431

Influence of GSK-3 inhibitors on phosphorylation of the NFkappaB subunit p65 in pancreatic cancer cells

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The nuclear transcription factor NFκB is constitutively activated in pancreatic cancer cells but not in healthy pancreatic tissues [1]. Several studies point out that glycogen synthase kinase 3β (GSK-3β) is involved in the regulation of NFκB mediated gene transcription. The GSK-3β inhibitor LiCl has been shown to inhibit transactivation of NFκB in HEK293 cells. GSK-3β inhibition in pancreatic carcinoma cells leads to an inhibition of cancer cell proliferation, followed by an induction of apoptosis [2]. GSK-3β also has been reported to influence NFκB transcriptional activation in HeLa cells by phosphorylation of p65 at Ser468 [3]. For this reason we investigated if inhibition of GSK-3 in the pancreatic tumor cell line Paca-44 leads to a decrease in phosphorylation of p65 at Ser468. Decrease of GSK-3 activity was studied using the specific GSK-3 inhibitors AR-A014418 and SB216763. Moreover, the influence of the novel indirubin derivate E671 on GSK-3 activity was investigated. The state of phosphorylation at Ser468 was measured by western blot analysis after 24 h treatment of Paca-44 cells with LiCl, AR-A014418 and SB216763. Furthermore, growth inhibitory properties of AR-A014418 and E671 were determined by treatment of Paca-44 cells using the sulforhodamine B assay (SRB). First results show that incubation of Paca-44 cells with SB216763, AR-A014418 and E671 induced a significant inhibition of GSK-3 activity. Whereas SB216763 and LiCl lead to a decrease in phosphorylation of p65 at Ser468, incubation of Paca-44 cells with AR-A014418 had no effect to the state of phosphorylation at Ser468. This indicates that phosphorylation of p65 at Ser468 is probably not only mediated by GSK-3 since the two similarly potent GSK-3 inhibitors, AR-A014418 and E671 show markedly different antiproliferate effects on Paca-44 cell growth. GSK-3 inhibition alone cannot explain the outstanding activity of E671. This bisindol has been shown earlier by us to be a multipotent kinase inhibitor, simultaneously inhibiting an array of aberrantly expressed tumor kinases some of which might be driving Paca-44 cell proliferation.

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432

DNA damage-dependent late activation of stress kinases (SAPK/JNK) by DNA cross-linking agents

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Activation of c-Jun-N-terminal kinases/stress-activated protein kinases (SAPK/JNK) is part of the cellular response to genotoxic insult, controlling gene expression, DNA repair, cell cycle progression and cell death. The contribution of DNA damage to genotoxin-triggered signaling to SAPK/JNK is fairly unknown. In the present study, we aimed to elucidate the contribution of genotoxin (i.e. UV-C light, cisplatin and transplatin) induced DNA damage (i.e. DNA-cross-links) in signaling to SAPK/JNK. Using rodent and human cell lines impaired in DNA damage processing (e.g. ATM-, DNA-PK-, CSB-, XPA- and XPC-defective cell lines), we could figure out that DNA damage impacts on signaling to SAPK/JNK. The data show that DNA cross-links are able to provoke a dual phosphorylation of SAPK/JNK. For example, after cisplatin and transplatin treatment cells harboring defects in nucleotid excision repair (NER) show stronger activation of SAPK/JNK as the corresponding wild-type. In contrast, UV-C treatment of CSB (Cockayne Syndrome B) cells leads to a reduced activation of SAPK/JNK. Ataxia telengiectasia (ATM) cells show strong differences in SAPK/JNK phosphorylation after cisplatin and transplatin treatment, but not after UV-C exposure. Based on these data, we hypothesize that DNA damage dependent activation of SAPK/JNK is agent and lesion specific. Cisplatin and transplatin induced initial DNA platination levels of wild-type and mutant cells were measured by ICP-MS and showed no differences. Also, formation of intrastrand-crosslinks produced by cisplatin was investigated by South Western analysis. As expected, mutant cells showed higher levels of residual DNA cross-links than the wildtype as analyzed 24h after exposure. To conlcude, late signaling to SAPK/JNK provoked by DNA-cross-linking agents (UV-light, cisplatin, transplatin) is affected by DNA repair factors. The type of repair factor involved depends on the nature of the genotoxin used.

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433

Role of stress-activated-protein-kinases p38 and JNK in benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) induced cytotoxicity

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Polycyclic aromatic hydrocarbons (PAH) are ubiquitous environmental pollutants formed during incomplete combustion of organic material e.g. coal and crude oil. For example benzo[*a*]pyrene (B[*a*]P) is a constituent and contaminant of cigarette smoke, automobile exhaust, industrial waste and even food products. B[a]P is carcinogenic to rodents and humans. B[a]P binds to the intracellular aryl hydrocarbon receptor thereby inducing its own metabolism by cytochrome P450s. Amongst several different metabolites the B[a]P-7,8-epoxide is considered as one of the most critical since, due to its metabolism, it generates the precursor for the highly reactive electrophilic genotoxin and ultimal carcinogen B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE). BPDE can bind to nucleophilic macromolecules such as proteins and DNA and causes mutations. Multiple defense mechanisms have evolved to protect the cell from DNA damage. Specific signaling pathways operate to detect and repair different kinds of lesions. In case the damage is poorly removed expansion of damaged cells can be counteracted by inhibition of proliferation or triggering apoptosis. Examples of damage sensors and transducers are stress-activated-protein-kinases (SAPKs). Previously, we reported rapid and sustained activation of SAPKs by BPDE in various mammalian cell lines. In this study we analyzed the role of SAPK activity in BPDE induced cytotoxicity. Compared to wild-type cells, knock-out fibroblasts deficient for the SAPKs p38alpha and JNK1/2 are hypersensitive to BPDE treatment. Similar results were obtained for p53 deficient cells. Closer examination of the modes of BPDE triggered toxicity revealed increases in necrosis, apoptosis and reduced proliferation rates in murine fibroblasts. Preliminary results indicate a critical role of p38alpha in the control of cell proliferation and in the balance of apoptosis versus necrosis in response to BPDE.

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434

Polybrominated diphenyl ethers and arylhydrocarbon receptor agonists: different toxicity and target gene expression

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Background: Polybrominated diphenyl ethers (PBDEs) accumulate in the environment and in humans. PBDEs are developmental neurotoxicants, disturb the endocrine system and induce tumors in rodents. However, underlying mechanisms of PBDE toxicity are still insufficiently understood. Some reports demonstrated activation of the aryl hydrocarbon receptor (AhR) by PBDEs based on induction of its target gene *cyp1A1*. In contrast, also inhibition of AhR activation by PBDEs has been observed. Objectives: In the present study, we used different PBDE congeners (BDE47, 99, 153 and 209) and analyzed their effects on AhR signaling. In addition to *cyp1A1* induction we monitored several known downstream consequences of AhR activation in various cell lines and zebrafish. Furthermore, we studied PBDE toxicity in zebrafish embryos to identify novel PBDE target genes. Methods: AhR activity was studied in hepatoma cells by investigation of multiple AhR target genes (microarray and real time PCR analysis), nuclear translocation and inhibition of proliferation. Zebrafish embryos were examined for developmental toxicity and identification of novel target genes. Results: PBDEs did not activate but rather inhibited AhR signaling. Moreover, BDE47 specifically induced malformations in zebrafish embryos. BDE47 induced toxicity and changes in gene expression were clearly different from those provoked by AhR agonists. Conclusions: PBDEs do not induce AhR signaling in higher vertebrates suggesting that chronic AhR activation is not involved in PBDE induced toxicity. Hence, activation of other signaling pathways are more likely to mediate adverse effects of PBDEs.

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435

Heavy metal stress as a modulator of insulin-like signaling

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Insulin/IGF1 signaling is impaired in several long-lived nonvertebrate mutants, with longevity being related to the activity of transcription factors of the FoxO family in these organisms. We have previously demonstrated that insulin-like signaling is elicited in various mammalian cell types exposed to stressful stimuli, such as reactive oxygen species and UV radiation. Exposure of human and rat hepatoma cells as well as human skin fibroblasts to stressful stimuli such as redox-active or thiol-reactive metal ions (Cu^{2+}, Zn^{2+} and Cd^{2+}) resulted in phosphoinositide 3'-kinase (PI3K)-dependent activation of the Ser/Thr kinase Akt, followed by a PI3K/Akt-dependent phosphorylation of several Akt substrates, including transcription factors of the FoxO family. As a consequence of metal-induced phosphorylation the inactivation and nuclear exclusion of FoxO1a was observed in hepatoma cells. Furthermore, activity of a known FoxO-responsive promoter (the glucose 6-phosphatase promoter) was strongly impaired in cells exposed to Cu^{2+}. Stimulation of the PI3K/Akt cascade by heavy metal ions neither required the cellular generation of reactive oxygen species to propagate signals nor did it depend on the activation of a receptor tyrosine kinase (RTK), pointing to a direct interference of Cu^{2+} or Zn^{2+} with regulators of PI3K/Akt signaling downstream of RTK. Different from copper and zinc, the exposure of cells to Mn^{2+}, Fe^{2+}, Co^{2+}, Pb^{2+} did not affect phosphorylation of Akt. Exposure of hepatoma cells to Ni^{2+} ions, however, resulted in a PI3K-dependent activation of Akt. Interestingly, nickel-induced activation of Akt – although coincident with a phosphorylation of Akt substrates, such as glycogen synthase kinase-3 – did not result in a nuclear exclusion of FoxO1a. In line with this finding, no significant modulation of the activity of a FoxO-responsive promoter construct was observed. As with mammalian cells, the FoxO ortholog DAF-16 was inactivated in *C. elegans* worms exposed to Cu^{2+}, pointing to a conserved stress response machinery. In summary, exposure of cells to stressful stimuli imitates insulin signaling independent of a physiological stimulator (such as insulin) and independent of the generation of reactive oxygen species. These data point to an interdependence between stress and senescence-related processes not only at the level of accumulation of (e.g. oxidant-induced) damage, but also at the level of signal transduction as senescence-related signaling cascades are modulated by stressful stimuli.

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436

Effect of TEGDMA on the activation of p38 and ERK

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Triethylene glycol dimethacrylate (TEGDMA) is a genotoxic monomer of dental resins that causes induction of reactive oxygen species (ROS), cell cycle arrest and apoptosis in oral cells. In previous studies we could show that the combination of TEGDMA with LPS resulted in a decrease in cytokine secretion compared to LPS. Since the phosphorylation of mitogen activated kinases (MAPK) like p38 and ERK is of crucial importance for the onset of cytokine production, the aim of this study was to investigate the influence of TEGDMA on the phosphorylation of the stress and DNA damage responsive kinases p38 and ERK in the human monocyte cell line THP1. To investigate the effects of the radical scavenger *N*-acetyl cysteine (NAC) on the expression of MAPK the cells were also co-incubated with TEGDMA and NAC. Monocytes were exposed to TEGDMA (1, 3 and 5mM) for 2, 24, and 48 hours with and without addition of 5mM NAC. The expression of phospho-p38 (pp38) and phospho-ERK (pERK) in THP-1 was detected by staining with specific antibodies and quantitated by flow cytometry (FACS). LPS from E. coli (25 µg/ml) was used as a positive control for pp38 and pERK induction. The data were statistically analyzed (Mann-Whitney-U test). The expression of pp38 was increased after 24 hours by 23 to 163% and after 48 hours by 21 to 262% in the presence of 1 to 5mM TEGDMA. Co-incubation with TEGDMA and 5 mM NAC resulted in reduced expression of pp38 at all time points and with all TEGDMA concentrations. In contrast, stimulation with LPS lead to a significant increase after 2h but not after 24h and 48 h. The stimulatory effect of TEGDMA on pERK was also concentration-dependent but even stronger than the effect on pp38. After 24h we measured an increase ranging from 31% (1 mM) to more than 500% (5 mM). Similar results were obtained after 48 h, except for a slight but insignificant decrease with 1mM TEGDMA. As with pp38 no significant effect of NAC on pERK expression was detected. These results indicate that TEGDMA influences intracellular signalling pathways mediated by p38 and ERK after long exposure periods.

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437

Improved characterization and interpretation of toxicological findings by combining omics technologies with conventional endpoints

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Elucidation of molecular mechanisms of toxicity gains increasingly importance in drug development. To explore the potential of omics technologies like transcriptomics and metabonomics to advance this field, the PredTox (Predictive Toxicology) project was initiated as part of the 6th Framework Program. For this project, rats were treated daily for 1, 3 or 14 days with the former drug candidate ZK 226830, which was discontinued due to severe hepatotoxicity. Animals were monitored by haematology, clinical chemistry and histopathology. In addition, transcriptional and metabolic profiles of liver tissue, serum or urine were investigated. Marked hepatocellular necrosis was present on day 2, with time-dependent decline in severity. Marked regeneration was present on day 4. Hepatocellular hypertrophy occurred from day 4 but was most prominent on day 14 where also mild cholestasis occured. ALT and AST increased on day 2, bilirubin and γGT on days 3 and 14, respectively. Gene expression changes in the liver indicated cell death (proteolysis, apoptosis, inflammation) and disturbance of energy metabolism (e.g. impaired glucose utilization) on day 2. Extensive transcriptional changes related to proliferation were present on day 4, suggesting peak regeneration. Induction of transcripts linked to biotransformation (e.g. GST Yc2) may contribute to the evidently enhancing tolerance towards ZK 226830. Changes on days 4 and 14 also indicated an increased de-novo synthesis and a decreased amino acid conjugation of bile acids which might be linked to the observed cholestasis. ^{1}H-NMR metabolic profiling of urine identified an increased excretion of taurine and creatine on day 2 which is indicative of liver toxicity. Changes on days 4 and 14 included a reduced excretion of creatinine and dimethylamine which has previously been liked to cholestasis. Together, this demonstrates the potential to monitor hepatotoxicity non-invasively by analysing urinary samples. In conclusion, integration of omics technologies into systemic toxicity studies supports the characterization of conventional toxicity findings and additionally provides valuable insights into the underlying molecular mechanisms.

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438

Characterisation of primary hepatocyte cell cultures using global gene expression profiles

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Primary hepatocytes are widely used for short time studies including enzyme induction, xenobiotic metabolism or mechanistic studies; however there is still a lack of understanding the behaviour and fate of primary hepatocytes in culture over time. Many different approaches are carried out to conserve liver specific functions in culture for a prolonged time to eventually be able to test subchronical or even chronical effects of compounds *in vitro*. We therefore analysed global gene expression changes in rat and human hepatocytes in monolayer and sandwich cultures incubated with different medium compositions and compared them to different short term cultures (i.e. liver slices and suspension culture). The isolation procedure of hepatocytes caused changes in gene expression related to cellular stress and cell adhesion. Most severe changes occurred during the first 24h after perfusion, including lipid and energy metabolism, immune response and cytoskeletal remodelling and can be assigned to stress during the procedure of isolating the cells, lack of other cell types in culture, removal of the extra cellular environment and the adaptation to the cell culture conditions. For short term cultures, a rapid decline of liver specific gene expression including phase I enzymes could be detected while phase II enzymes showed a more variable response. In longer time cultures, dedifferentiation was triggered by the flattened morphology in monolayer and the addition of serum to the culture media. Only in serum-free sandwich culture cells acquired hepatocyte specific morphology and maintained liver specific gene expression and functions for up to 10 days. Overall, gene expression in hepatocytes cultured in serum free sandwich culture remained stable over time and closer to that of liver than all other culture systems tested, making it a promising approach for repeated dose toxicity testing.

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439

Toxicogenomic analysis of chemically induced hepatotoxicity in primary rat hepatocytes

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Primary hepatocytes are a popular *in vitro* system for drug-induced hepatotoxicity research due to their strong resemblance to *in vivo* liver functions. Furthermore, it has been shown that hepatocytes cultured in a collagen sandwich formation maintain morphology, viability, and drug metabolizing activities for several weeks.Here, primary rat hepatocytes from male Wistar rats were cultivated in a collagen I sandwich with a serum-free medium formulation up to 12 days post isolation.The aim of this study was to analyse chemically induced hepatotoxicity by exposing hepatocytes to several chemical

compounds of two different categories (genotoxic carcinogens and hepatotoxicants). These substances have also been tested in a 28-day subacute toxicity study in male Wistar rats (OECD TG 407). In this BMBF-funded project, the prediction of carcinogenicity of chemical substances was studied. In our study, the cells were treated for up to 9 days with low and high doses of test compounds, determined by LDH- and ATP-based cytotoxicity assays. Global gene expression analysis was performed at different timepoints. We determined differential gene expression using a bead–based Illumina oligonucleotide microarray (whole-genome array) containing over 22,000 probes selected primarily from the NCBI Refseq database. The gene expression analysis showed that a variety of genes and pathways involved in cell differentiation and cell cycle progression along with typical stress marker genes were regulated. We are currently investigating whether marker genes can be identified that are predictive for the tested genotoxic carcinogens and hepatotoxicants. These expression profiles are then compared with the available *in vivo* data of the used model toxicants. By this study we try to pre-validate our *in vitro* model as predictive screening system for carcinogenic and hepatotoxic compounds.

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440

Gene expression profiling in toxicological studies: a comparison between FFPE and fresh frozen liver tissue

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Large numbers of Formalin-fixed Paraffin-embedded (FFPE) tissues from toxicological studies have been accumulated over the years. Although RNA extracted from FFPE tissue is significantly degraded, new microarray technologies are allowing their use for gene expression analysis. In this study, the DASL^{TM} (cDNA-mediated annealing, selection, extension and ligation) assay from Illumina Inc. was used. This method, compared to standard technologies, does not need intact poly-A tails of the mRNA for priming. The major aim of this study was to assess if gene expression data from FFPE tissue are comparable to those from fresh frozen samples. Livers from rats treated with a former drug candidate (EMD335823) against diabetic complications, which was positive for hepatotoxicity, and control animals were used. The DASL^{TM} assay was performed to reverse transcribe, amplify, label and hybridize samples derived from FFPE tissues onto a custom BeadChip (512 liver toxicity relevant genes).A dose and time dependant clustering was observed from the gene expression data. Focusing on the high dose treated animals it was clearly shown that two animals behaved differently than the others, which confirms the fresh frozen data and also the histopathological findings where these two animals showed the most severe hepatocellular hypertrophy, bile duct hyperplasia and bile duct/hepatocyte necrosis. Several deregulated pathways indicative of hepatotoxicity, including bile acid synthesis, lipid / fatty acid metabolism and cholesterol synthesis, were identified after high dose treatment.The data clearly demonstrate that robust and accurate gene expression data can be obtained from heavily degraded RNA using the DASL assay.

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441

Toxicoproteomics: C3 exoenzyme effects protein profile of a human neuroblastoma cell line

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Bacterial C3 exoenzymes inactivate the GTPases RhoA, B and C by ADP-ribosylation. Recently, it has been shown that C3 of *Clostridium botulinum* (C3*bot*) exhibits neurotrophic effects on neuronal cells. These effects are independent of the catalytic activity of the exoenzyme since an enzymatically inactive C3 protein mutant (E174A) promotes axon outgrowth even at low concentrations [1]. To further investigate this observation a comparative proteomic approach has been chosen to elucidate the protein expression profile of C3*bot* treated versus untreated neuroblastoma SHSY-5Y cells.For relative quantification proteins of C3*bot* treated and untreated cells were labelled using the ICPL technique [2]. Different stable isotopes are present in these labels which facilitate the quantitative comparison of proteins from different samples by MS analysis. These two protein extracts were labeled, combined, and separated using 1-D SDS-PAGE. The resulting gel lane was split into several pieces and the proteins were in-gel digested by trypsin. Resulting peptides were extracted and separated by means of reversed phase HPLC. Fractions were directly spotted onto a MALDI target plate and protein identification and quantification was done with a MALDI-TOF/TOF mass spectrometer. About 1500 different proteins were identified to be expressed in SHSY-5Y cells. Out of these nearly 75% were accessible for quantification. About 15% out of the identified proteins were upregulated, 20% of them were downregulated and 40% remained unchanged. The identified and regulated proteins include several proteins involved in growth factor signalling, cell cycle and actin cytoskeleton regulation. These results provide new starting-points for the investigation of the C3*bot* effected neuronal outgrowth.

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442

Comparative proteomics of *H-ras* and *B-raf* mutated mouse liver tumours

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The mitogen-activated protein kinase (MAPK) signalling pathway is frequently activated in mouse liver tumours. Constitutive activation of this pathway is often caused by activating point mutations in the *Ha-ras* or *B-raf* genes which are observed in a high percentage of hepatocellular tumours that were induced by chemical carcinogens such as *N*-Nitrosodiethylamine. This is in contrast to human liver tumours, which very infrequently harbour mutations in either of these two genes. In a previous microarray analysis we observed that *Ha-ras* and *B-raf* mutated tumors show strong similarities in their mRNA expression patterns. In extension of these studies, we now analyzed the global protein expression patterns of *Ha-ras* and *B-raf* mutated liver tumors. Our results did not reveal any striking differences in the protein expression patterns of the two tumour types, underlining our previous conclusion that mutational activation of the two oncogenes results in activation of a common set of transcriptional regulators. Metabolic pathways showing multiple expression changes in tumour cells are cholesterol biosynthesis (upregulation of several enzymes), bile acid synthesis (decreased expression of several enzymes), and other pathways in lipid metabolism, including upregulation of apolipoproteins. As a consequence, tumour cells preserve their cholesterol pool which may be important for the increased plasma membrane demand in proliferating cells. Other highly deregulated pathways relate to serine biosynthesis and pathways that utilize serine as a metabolite, for example for shingolipid metabolism, gluconeogenesis and tetrahydrofolate metabolism. This may have implications on membrane synthesis, energy utilization and reactions using tetrahydrofolate as a co-substrate. Furthermore, there was an increase in proteins belonging to the heat shock protein families 60, 70 and 90 which function as chaperons binding intracellular proteins and also have important implications in the regulation of cell survival and cell death.

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443

Host cell peptidyl prolyl cis/trans isomerases are required for translocation of the *Clostridium botulinum* C2 toxin into the cytosol of mammalian cells

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Among other toxins *Clostridium botulinum* produces the binary C2 toxin, consisting of the C2IIa component, responsible for binding and translocation and the enzyme component C2I which mono-ADP-ribosylates G-actin of eukaryotic cells. Following receptor-mediated endocytosis the C2 toxin is taken up into the cell, only being able to escape from early endosomes into the cytosol after acidification. Thereto, C2IIa forms pores in the endosomal membranes, and C2I translocates into the cytosol of the eukaryotic cell. Previously we showed that C2I requires unfolding to pass the C2IIa pore and has to be refolded in the cytosol to fully display its enzymatic activity. It was verified that this step is dependent on the activity of the host cell chaperone Hsp90. In our recent experiments we used the specific pharmacological inhibitors ciclosporine A and FK506 (Tacrolimus) to investigate whether peptidyl prolyl cis/trans isomerases (PPIases) are involved in the cellular uptake of the C2 toxin. Ciclosporine A, an inhibitor of cyclophilins, and FK506, which inhibits FK-binding proteins, delayed significantly the C2 toxin effects on HeLa and Vero cells. This was shown by observation of the toxin-induced morphological changes of the cells. Cells treated with C2 toxin in the presence of one of the inhibitors showed less ADP-ribosylated actin in the cytosol than those without inhibitor. An influence of the inhibitors on the ADP-ribosyl-transferase activity of C2I could be excluded as well as interference during the binding of the toxin to the cell surface. External acidification of cells in the presence of ciclosporine A or FK506 inhibited the direct translocation of cell-bound C2I into the cytosol across the plasma membrane, implying that PPIases are required for translocation of C2I across endosomal membranes. We used biotin-labelled C2I to co-precipitate cyclophilin A together with Hsp90 from C2 toxin treated cells. These findings give us proof of the direct interaction of C2I with cyclophilin A and Hsp90. As far as we know, this is the first report describing the involvement of peptidyl prolyl cis/trans isomerases on the uptake of a bacterial toxin into the cytosol of eukaryotic cells.

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444

Role of phenylalanine-428 in up-take of *Clostridium botulinum* C2 toxin

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The actin-ADP-ribosylating *Clostridium botulinum* C2 toxin belongs to the family of binary toxins. It consists of the enzyme component C2I and the binding and translocation domain C2II. The toxin ADP-ribosylates β/γ-actin at arginine-177 thereby inhibiting the polymerization of actin. Here we studied the role of phenylalanine-428 of C2II, which is structurally equivalent to phenylalanine-427 of the protective antigen (PA), the binding component of anthrax toxin. This residue is involved in toxin up-take in PA and has been suggested to act as a phi-clamp in a chaperone-like manner to translocate the enzyme components of anthrax toxin into the cytosol. Whereas exchange of phenylalanine-428 of C2II to tryptophan or tyrosine reduced cytotoxicity, rounding-up of cells by C2I was completely blocked with the F428A mutant of C2II. The F428A mutant increased potassium conductance caused by C2II in artificial membranes by about 2-3 fold. C2II caused pore formation and release of $^{86}Rb^+$ ions from preloaded cells after a short-term pH-shift of the medium. In contrast to the effects in artificial membranes, F428A inhibited $^{86}Rb^+$ release from preloaded intact cells. Our findings indicate that the role of phenylalanine-428 in C2 toxin is not restricted to a chaperone-like role in toxin translocation but has additional functions, which are essential for the up-take of C2I into target cells.

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445

Inhibition of *Clostridium difficile* toxin B by human alpha-defensins

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Clostridium difficile cytototoxins A and B are major pathogenicity factors implicated in pseudomembranous colitis and antibiotics-associated diarrhea. The inactivation of Rho-signalling by mono-O-glucosylation of various small GTPases is made responsible for the underlying severe inflammatory processes in the intestine. Human α-defensins are modulatory constituents of the innate immune response known to inactivate a broad spectrum of pathogens as well as specific bacterial exotoxins. Here, we studied the effects of the antimicrobial peptides human alpha-defensins HNP-1 and -3 (human neutrophil protein-1 and -3) and enteric HD-5 (human defensin-5) on action and activity of *C. difficile* toxins A and B. Inactivation of *C. difficile* toxin B, but not toxin A, by α-

defensins *in vivo* was verified by cytotoxicity assays, including the determination of the transepithelial resistance of CaCo-2 monolayers and analysis of the glucosylation status of Rac1 in toxin-treated cells. *In vitro*-glucosylation was utilized to determine the corresponding IC_{50}-values. HNP-1, HNP-3 and HD-5, but not β-defensin-1 or LL-37, inhibited toxin B-catalyzed *in vitro*-glucosylation of Rho GTPases in a time- and concentration dependent manner. The IC_{50} values range from 0.6 – 1.5 µM, depending on the defensin, the GTPase and the utilization of either holotoxin B or the N-terminal catalytic glucosyltransferase domain. Precipitation and turbidity assays demonstrated a concentration-dependant complex formation, comparable to *Bacillus anthracis* protective antigen (PA) and lethal factor (LF). The formation of aggregates was found not to be responsible for the inhibitory potential. Our data indicate that toxin B but not toxin A interacts with high affinity with defensins HNP-1, HNP-3 and HD-5 and suggest that defensins may provide a defense mechanism against some types of clostridial glucosylating cytotoxins.
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446

Cyotoxic action of the ADP-ribosyltransferase SpvB from *Salmonella enterica*
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Salmonella enterica, a Gram-negative food-born pathogen, causes human diseases ranging from mild gastroenteritis to severe systemic infections. The virulence of *Salmonella* is closely associated with its intracellular replication in macrophages and the bacterial ADP-ribosyltransferase SpvB is absolutely crucial for the intracellular growth. We cloned and expressed the catalytic domain of SpvB (C/SpvB) and demonstrated that SpvB mono-ADP-ribosylates G-actin at position Arg-177. In infected cells, SpvB is directly secreted from intracellular growing *Salmonella* into the host cell cytosol, most likely by type-III-secretion through a bacterial protein needle. Thus SpvB is not taken up into cells when applied to the medium and therefore, we chose two different approaches to investigate the effect of SpvB on intact mammalian cells. At first, we took advance of a recombinant fusion toxin (C2IN-C/SpvB) to deliver C/SpvB into the cytosol via the binary *Clostridium botulinum* C2 toxin. Treatment of various mammalian cell lines with C2IN-C/SpvB resulted in the depolymerization of actin filaments and cell rounding. However, the cytopathic effect of C2IN-C/SpvB was transient due to degradation of the toxin in the cytosol. Intoxicated cells regained a flat morphology but failed to divide, resulting in enlarged binuclear cells. Currently, we investigate whether the observed effects reflect the situation in the infection model. To this end, we infect cultured J774.A1 macrophages with a genetically modified *S. enterica* strain, over-expressing SpvB. Following infection, the SpvB protein is detected in the cytosol of J774.A1 cells by a specific antibody raised against SpvB and the degradation of SpvB is monitored.
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447

Organic anion transporter 1 (OAT1) as mediator of nephrotoxicity of 5-sulphooxymethylfurfural (SMF)
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5-Hydroxymethylfurfural (HMF) is formed when sugars are acidified or heated. It is present at high levels in numerous foods. HMF itself was inactive in various genotoxicity tests. However, it has been shown that HMF can be metabolised in animal models *in vivo* as well as by human enzymes *in vitro* into a chemically reactive metabolite, 5-sulphooxymethylfurfural (SMF), which is mutagenic and carcinogenic. In a recent *in vivo* study conducted in our department SMF was highly toxic in mice: most animals died on the 5-11th day after the treatment with a single dose of 250 mg/kg body mass. The most severe effects were seen in the kidneys with abundant acute necrosis and proteinaceous casts in the proximal tubules. Since proximal tubule cells of the kidney are the main site of the active organic anion secretion we hypothesised that the transporter-mediated uptake of the SMF in to the cells could be the reason for the selective organotoxicity. To test this hypothesis, we studied whether renal basolateral transporter OAT1, primary responsible for the basolateral concentrative uptake of organic anions into the proximal tubule cells, could use SMF as substrate. Human OAT1 was stably expressed in the Chinese hamster V79 cell line, a suitable model for genotoxicity testing. SMF inhibited the uptake of the model substrate *p*-aminohippurate in OAT1-expressing V79 cells, suggesting that it is a competing substrate. The K_i was calculated to be around 300 µM. Moreover, the expression of OAT1 significantly enhanced SMF-induced mutagenicity in the *hprt* gene mutation assay. Addition of probenecid, a known inhibitor of OAT, to the incubation medium reduced the SMF-induced mutation level to that observed in control cells. Taken together, these results indicate that OAT1 mediates the transport of SMF into the proximal tubule cells of the kidney and thus could play role in SMF-induced nephrotoxicity.
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448

Establishment of an insect cell expression system for rat Abcb6, an ATP-binding cassette transporter involved in copper tolerance
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Several transporters of the ATP-binding cassette (ABC) superfamiliy are involved in cellular protection by extruding potentially toxic substances through biological membranes. Rat Abcb6 (rAbcb6) is a half-transporter that is expected to achieve functionality as part of a dimer. We have previously shown rAbcb6 expression to confer tolerance to copper. Fluorescence microscopy revealed that rAbcb6 was distributed intracellularly, displaying co-localization with lysosomal/endosomal markers. Although rAbcb6 is related to the multidrug/xenobiotic resistance transporter MDR1, its substrate spectrum remains to be defined. To establish procedures for systematic screening of substrate preferences, systems enabling high levels of transporter expression are required. Thus, baculovirus-dependent expression of rAbcb6 in the SF9 (*Spodoptera frugiperda*) insect cell line was attempted. The cDNA sequence for rAbcb6, extended by a C-terminal foreign V5-epitope, was integrated into the baculovirus (BaculoDirect^{TM}) genome. SF9 cells were transfected with the recombinant viral DNA to produce infectious virus particles that were released into the culture medium. To scale up the viral stock, several rounds of infection with culture supernatants were performed. Viral titers were evaluated by detecting virus producing foci in infected cultures with a baculovirus antibody. Finally, high baculovirus expression vector titers were used for SF9 infection, and viral concentration- and time-dependent rAbcb6 expression within cells was examined by immunoblot analyses of cell lysates. Rat Abcb6-V5 was probed for with a primary antibody against its V5-tag. Substantial rAbcb6-V5 expression was observed within two days of infection. In mammalian expression systems, rAbcb6 molecules were shown by immunoprecipitation to interact with each other, supporting the conclusion that homodimers are formed and that co-expression in SF9 cells of a different half-transporter is not required to achieve a functional status. In summary, the presented results demonstrate that the baculovirus/SF9 system is suitable to obtain high rAbcb6 expression, which provides an important basis for systematic functional analyses.
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449

Effect of liposomes on the uptake of catechols into V79 cells
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Compounds containing a catechol structure, i.e. an ortho-diphenolic group, are often encountered among plant constituents or are formed as metabolites of aromatic substances. In general, catechols are chemically and metabolically unstable, and numerous reactions of catechols are known leading to toxic effects, e.g. oxidation to semiquinone/quinone intermediates which generate reactive oxygen species through redox-cycling, or complex formation with metal ions. Due to their instability and also to their rather low lipophilicity, which hampers diffusion through cell membranes, the concentration of catechols in cells after *in vitro* exposure is usually low. The aim of the present study was to increase the uptake of quercetin, a model catechol, into cultured male Chinese hamster V79 lung fibroblasts by packing it into liposomes. The use of this vehicle was expected to increase the concentration and also the induction of DNA strand breaks by quercetin in these cells. When the stability of free quercetin was studied in cell culture medium, a fast decline was observed, probably due to chemical degradation; this degradation was much slower when quercetin was packed in liposomes. Incubation of V79 cells with liposomes containing quercetin also exhibited a slower degradation of quercetin in the incubation medium, and an increased amount of quercetin was determined in these cells as compared to cells incubated with free quercetin. Surprisingly however, no elevated level of DNA strand breaks was observed in the liposome-exposed cells. These results show that packing a catechol into liposomes leads to a higher stability and increased cellular uptake of the catechol. The failure of the elevated amount of catechol to increase DNA strand breaks suggests that the catechol is not or only partly released from the liposomes.
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450

Mechanism of TCDD-induced oxidative stress, role of estrogen receptor, and modulation by estradiol in liver cells
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2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic member of a large family of halogenated aromatic hydrocarbons present as contaminants in food, mother's milk and environmental samples. TCDD was classified as a group I human carcinogen by the International Agency for Research on Cancer (IARC). Its effects are mediated by the arylhydrocarbon receptor (AhR) that regulates transcription of target genes including cytochromes P450 (CYP) 1A1, 1A2, and 1B1. TCDD acts as a liver carcinogen in female but, to a much lower extent, in male rats. There is good evidence for a role of estrogens in the mechanism of TCDD-induced hepatocarcinogenesis in the rat. Estrogens may act as genotoxic procarcinogens. Particularly, the 17β-estradiol (E2) catechol metabolite 4-hydroxyestradiol formed mainly by CYP1B1 can undergo redox cycling and thus generate DNA damaging reactive oxygen species (ROS). As previously seen, induction of CYPs by TCDD led to increased formation of ROS and increased DNA levels of 8-oxo-2'-deoxyguanosine (8-oxo-dG) in rat H4IIE hepatoma cells and in rat primary hepatocytes but not in human HepG2 hepatoma cells. E2 alone increased ROS formation only in hepatocytes while the combination with TCDD had no additive effect. Real-time RT PCR analysis revealed that TCDD induced CYP1B1 mRNA level to a slightly higher extent in rat hepatocytes than in hepatoma cells, while E2 had no clear effect. Since estrogen receptor alpha (ER)α mRNA was present in hepatocytes but not in hepatoma cells, it can be speculated that the presence of ERα may be responsible for the elevated ROS formation after E2 treatment.
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451

Protection by indolo[3,2-*b*]carbazole against DNA-damage by benzo[*a*]pyrene and hydrogen peroxide in Caco2-cells
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Epidemiological studies suggest that high dietary intake of fruit and vegetables protects against tumour development in many organs including colon. Especially compounds derived from the Brassica genus, e.g. broccoli, cauliflower, brussels spout or chinese cabbage, appear to be protective against colorectal cancer. Brassica vegetables are rich

in glucosinolates which – after physical damage to the plant material - are hydrolysed to a variety of compounds. For instance, glucobrassicin is hydrolysed during digestion to sulforaphane and indole-3-carbinol, which further condensates in the organism to diindolylmethane and indolo[3,2-*b*]carbazole. In animal studies, a protective effect of indole-3-carbinol against carcinogen-induced tumour development has been demonstrated in various organs including colon. In line with the high instability of indole-3-carbinol, these protective effects are probably mediated by the condensation products, e.g. indolo[3,2-*b*]carbazole. However, the underlying molecular mechanisms are not yet known. Interestingly, indolo[3,2-*b*]carbazole is known to be a potent agonist of the aryl hydrocarbon receptor (AhR). In accordance, we could show a strong indolo[3,2-*b*]carbazole-dependent induction of CYP1A1 which could be blocked by downregulation of the AhR by siRNA. Since it is further suggested that the AhR plays a tumour-protective role in the gastrointestinal tract, we are interested in the molecular effects of indolo[3,2-*b*]carbazole in the colonic epithelial cell line Caco-2. Using the alkaline Comet assay we could show that pre-incubation with indolo[3,2-*b*]carbazole (1 μM) protects against DNA-damage induced by benzo[*a*]pyrene (1 and 10 μM) or hydrogen peroxide (30 μM). This suggests that indolo[3,2-*b*]carbazole protects against oxidative stress. The possible involvement of both the AhR, and the transcription factor Nrf2, which plays a pivotal role in anti-oxidant defence mechanisms, is currently under investigation.

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452

Production rates of endogenous low molecular weight alkanes in rats under normoxia and short-term hyperoxia

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Exhalation of endogenous low molecular weight alkanes is used as a measure of oxidative stress in animals and humans. Ethane and *n*-pentane result from lipid peroxidation, propane, isobutane, and *n*-butane from protein peroxidation. Exhalation reflects only partly the endogenous production because metabolic elimination is not accounted for. Knowledge of clearances of exhalation and of metabolism is required for quantifying endogenous production. The aim of the study was to determine the production of endogenous alkanes at normoxia (20% O_2) and at short-term hyperoxia (100% O_2) in male Wistar rats. Using gas chromatography, alkane specific toxicokinetic parameters were obtained from gas uptake studies and from monitoring concentration-time courses of the endogenous alkanes, exhaled up to 8 h into the atmosphere of closed exposure chambers. Derived clearances of inhalation, exhalation, and metabolism fitted excellently to a prediction considering alkane-specific physicochemical properties, cardiac output, and alveolar ventilation. Hyperoxia did not influence the clearances of metabolism. Alveolar retention (%), reflecting the metabolic elimination at steady state, was for ethane 2.8, propane 9.3, isobutene 4.3, *n*-butane 7.8, and *n*-pentane 11.4. Production of ethane (nmol/h/kg) increased significantly ($p<0.05$) from 0.8 at normoxia to 1.5 at hyperoxia. Production of *n*-pentane (nmol/h/kg) was 5.1 at hyperoxia, differing not significantly from the value of 3.8 at normoxia. Production (nmol/h/kg) of the proteinogenic alkanes was for propane 1.8, isobutene 0.3, and *n*-butane 0.6. These rates were independent of the oxygen concentration. Considering short-term hyperoxia, we conclude: Membrane lipids are the predominant targets of reactive oxygen species. Of the investigated alkanes, ethane is the only suitable marker of oxidative stress.

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453

Spin adducts formed from different carbamoyl-substituted EMPO derivatives

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The detection of superoxide and other oxygen-centered radicals is an important aspect of detecting oxidative stress in biological systems. In continuation of our previous studies we developed a series of amide-type spin traps and tested their spin trapping behaviour towards different oxygen and carbon centered radicals. Five carbamoyl-substituted EMPO derivatives were investigated, namely 5-carbamoyl-5-methyl-pyrroline *N*-oxide (CAMPO), 5-carbamoyl-5-ethyl-pyrroline *N*-oxide (CAEPO), 5-carbamoyl-5-propyl-pyrroline *N*-oxide (CAPPO), 5-carbamoyl-5-butyl-pyrroline *N*-oxide (CABPO), and 5-carbamoyl-5-pentyl-pyrroline *N*-oxide (CAPtPO). The spin trapping behaviour towards superoxide strongly depended on the length of the alkyl chain, the butyl and pentyl derivatives being comparable to EMPO while the others formed a persistent secondary product. The structure of all compounds was confirmed by ^{1}H and ^{13}C-NMR. Furthermore, spin adducts obtained from different carbon-centered radicals derived from methanol, ethanol, and formic acid have also been characterized.

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454

The COLIPA Skin Metabolism Project – towards the inclusion of appropriate metabolism in *in vitro* alternatives for skin sensitisation and genotoxicity

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The 7th amendment of the European Union Cosmetics Directive will demand a stop of animal testing for toxicity testing of cosmetic products and the development of alternative testing strategies for topically applied cosmetic/toiletry ingredients. New *in vitro* models for assessing skin sensitization and genotoxicity potential should be metabolically active in order to interpret data in the context of risk assessment. Therefore it is vital to understand how metabolism of ingredients in *in vitro* models compares to metabolism in human skin. To determine the question whether an ingredient is metabolized by skin cells, compounds will be applied to *ex vivo* human skin, as well as more 'simple' *in vitro* models (e.g. 3D skin model or a monolayer of a keratinocyte like cell line) to metabolically profile the ingredient. Cell culture models are primary keratinocytes, keratinocyte derived cell lines like HaCaT and NCTC cells and primary fibroblasts. The expression of different phase I and II enzymes will be characterized by western blot analysis, proteomics techniques and functional assays. First data indicate that most model systems express several P450 family members. Activities of CYP1A1, CYP1A2 and Glutathione *S*-transferases are detectable in all tested skin models. But maximum specific activity of the enzymes differs in the different models. This approach will provide insight in the 'mechanism of action' useful for risk assessment.

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455

Human neurospheres for DNT testing *in vitro*

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Because developmental neurotoxicity testing (DNT) requires large amounts of animals, alternative methods are needed to reduce animal consumption. Therefore, we have established a human *in vitro* model for DNT based on normal human neural progenitor (NHNP) cells, which are cultured as proliferating neurospheres. On appropriate extracellular matrices, NHNP cells migrate radially out of the sphere and thereby differentiate into the major neural celltypes of the brain. During brain development, cell proliferation, apoptosis, differentiation and migration are fundamental processes. Therefore, we developed *in vitro* methods, which reflect these endpoints *in vitro*. Neurosphere proliferation is measured by FACS analyses or using the Cell Titer blue assay in a high throughput 96-well format. The same assay is used to test for cytotoxicity of chemicals and can be multiplexed with a caspase-3/-7 assay which assesses apoptosis-induced effector-caspase acitvity. Migration is measured by evaluation of the distance which cells wander from the edge of the sphere over time. For differentiation analyses, immunucytochemical stainings are performed. Applying chemicals to this cell system, we show here that indirubin produces cell cycle arrest and mercuric compounds inhibit proliferation, while staurosporin decreases viability and increases apoptosis in neurospheres. Cell differentiation analyses revealed that cAMP and retinoic acid induce neuronal differentiation, while mercury causes an increased formation of astrocytes. Oligodendrocyte development was promoted by thyroid hormone and inhibited by exposure to lead in these cultures. Migration analyses revealed that exposure to mercury or carbaryl inhibited cell migration. Investigations of the underlying mechanisms indicated that cell migration is regulated via the MAP kinase ERK1/2-dependent and ERK1/2-independent pathways, which are regulated by PKC, EGFR and src family kinases.In summary, we set up NHNP cells as a human *in vitro* model for DNT testing by establishing endpoints which detect alterations in basic processes involved in brain development.

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456

Neural development in mice and men: Same but different?

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For detection of disturbances of neuronal development by chemicals, animal studies are the gold standard in toxicology. However, the predictability of animal experiments for humans is often unsatisfactory due to species differences. The European REACH legislation demands that alternative human *in vitro* models are needed to identify chemicals that are hazardous to human health. Therefore, we and others are developing human cell culture models which are suitable to identify hazardous chemicals. One problem of these human-based *in vitro* models is the interpretation of data. When *in vitro* results differ from animal data it is important to distinguish if there are species or an *in vitro-in vivo* difference. One way to address this problem is a comparison of the same *in vitro* system from animals and humans. We have developed a human (GW 16) and a mouse (E14) neurosphere-based *in vitro* system for brain development. In both systems, basal processes like proliferation, differentiation and migration were investigated. First results show that in both species, cells start to migrate out of the sphere after mitogen withdrawal, but there is a remarkable difference regarding the organization of the migrated cells. This is probably due to the lack of cells with radial glia-like morphology which are only found in human cultures. In both species, the three major types of brain cells (neuron-, astrocyte- and oligodendrocyte-like cells) were present. However, the composition of differentiated cells is very different. While after 2 days, 15% of differentiated cells are pre-oligocytes in the murine system, it takes approx. 4 days for the human cells to occur in the migration area. Furthermore, maturation of mouse oligodendrocytes is quicker than in the human system. This could be due to differences in gene expression of the oligodendrocyte marker O4. Our results indicate that mouse and human *in vitro* systems reflect the differences in neurodevelopment of the respective species. Therefore, it might be helpful to include respective animal *in vitro* models for extrapolation and interpretation of human *in vitro* data for risk assessment.

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457

Precision cut lung slices: metabolic characterization of an alternative model for lung toxicity

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Precision cut lung slices (PCLS) offer the opportunity to gain insight into lung physiology under *in vitro* cell culture conditions. PCLS facilitate to address cellular and functional responses in laboratory species and humans. Using this technique, interspecies

comparisons and comparisons of large numbers of test substances can easily be performed thereby offering a way of reducing the number of experimental animals used. We aim to develop a strategy to employ PCLS as pre-study test system for *in vivo* inhalation toxicity studies. Many *in vitro* systems such as cell cultures lack xenobiotic metabolism. Metabolic capacity is however an important feature of *in vitro* systems to predict *in vivo* toxicities.The aim of this study was to characterize the metabolic capacity of cultured PCLS. The activities of xenobiotic metabolizing enzymes were probed with diagnostic substrates and metabolic profiles were investigated. Rat PCLS were prepared using a Krumdieck tissue slicer and cultured under conventional cell culture conditions. After different times of incubation sub-cellular fractions of PCLS were analyzed for their CYP-, glutathione *S*-transferase- and uridine-glucuronic-acid-transferase-activity. The metabolic competence of lung slices was investigated by the incubation with the model compound testosterone and the assessment of its resulting metabolic profile. From the above results we conclude that the characterization of PCLS is an important step towards the utilization of this *in vitro* technique to screen potential lung toxicity of substances.

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458

HEPAC^2: Serum-free, standardized, validated and re-usable primary human hepatocytes for the analysis of xenobiotics

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Human hepatocytes are the *in-vitro* system of choice to study drug-induced processes in man. Here, we present HEPAC^2: A standardized and validated culture system in which functional human hepatocytes can be maintained serum-free for several weeks. Anabolic and catabolic hepatocellular functions and cellular vitality are monitored daily. Albumin and urea are produced on a relatively constant level for up to 2-3 weeks, while the cells remain viable. An extensive analysis of RNA-expression profiles has been performed using Affymetrix microarrays. Based on this, a standard protocol was established that allows repeated exposure of hepatocytes to test substances for studying drug metabolism. In this protocol hepatocytes are exposed to test pieces for 24 hrs. Subsequently, the culture medium is replaced by medium without the test substance and the same exposure scenario is repeated in intervals of 4 days. As a first model substance we used acetaminophen (APAP) to assay the feasibility of this system. High doses of AAP (2815 mg/l) diminished urea production by 15-30% and albumin secretion by 70-80% and led to a complete loss of glycogen. These effects were reversible. After removal of AAP, secretion of urea and albumin returned to control levels, the glycogen stores were refilled. Within one cell culture this exposure scenario could be repeated 4-5 times without loss of reproducibility. A second protocol has been initiated to assay the cytochrome P450 (CYP) induction potential of xenobiotics. Here, the hepatocytes are cultured in the presence of prototypical inducers like rifampicin or ß-naphthoflavone or the substance(s) of interest for 48 hrs to induce CYP activities. After 48 hrs, CYP activities are detected by conventional assays, e.g. testosterone-6ß-hydroxylase or ethoxyresorufin-*O*-deethylase. Then, in the absence of inducers CYP activities are allowed to return to basal levels. At least 2-3 induction cycles can be initiated that lead to CYP activities at identical levels as determined during the first induction. In conclusion, these data demonstrate the suitability of our long-term culture system to serve as a tool for repetitive screening of drug-mediated changes on hepatocellular functions. It may be used to assay long-term effects of drugs and thereby become an alternative to animal testing. In addition, HEPAC^2 may be combined with artificial devices as a support system during liver failure.

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459

Optimization of culture conditions for gene expression studies with primary rat hepatocytes

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Primary hepatocytes are a well established *in vitro* system for the study of drug metabolism. But up till now, the advances as regards the optimal condition which allows the precise study for *in vitro* gene expression in the toxicogenomic field using also cultured rat hepatocytes were not so much. In the present study we compared hepatocytes cultured under three different conditions: (i) on collagen coated dishes (CC), (ii) in Matrigel (M), (iii) in collagen-sandwich culture (CS). We studied the influence of methapyrilene (MPy) on expression of four genes which are known to be influenced by MPy: 4-aminobutyrate aminotransferase (Abat), glycogen synthase kinase 3-beta (Gsk3β), myeloid differentiation primary response gene 116 (Myd116) and sulfotransferase family 1a (Sult1a1). In all three culture systems Gsk3β and Myd116 were induced, whereas Abat and Sult1a1 were down regulated. Gsk3β was induced by median factors of 1.1, 2.5 and 3.5 for CC, M and CS. Induction factors of Myd 116 were 4-, 6.2- and 6.9-fold. A 5.6-, 6.5- and 2.4-fold down regulation of Abat was observed for CC, M and CS, respectively. Also Sult1a1 was down regulated by MPy with factors of 19.6, 8.2 and 9.5. In conclusion, qualitative similar results were obtained for MPy induced gene expression alterations in CC, M and CS.

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460

Characterization of embryonic stem cell differentiation into cardyomyocytes under serum reduced culture conditions

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Since the differentiation of embryonic stem cells (ESC) is a dynamic process of continuously changing cell characteristics, it is necessary to have valid information on the “actual status” of these cells when they are used for research in developmental toxicology. Especially, in case molecular endpoints are used, a highly standardized culture of ESC is needed. Therefore, a serum free culture medium for the differentiation of mouse embryonic stem cells (line D3) into cardiomyocytes was established. To verify the suitability of a serum free culture medium, the gene expression of the ESC was investigated. The new serum replacement is based on BSA, fetuin, chemical defined lipid concentrate, transferrin, insulin, EGF, BMP-2, bFGF and a complex mixture of trace elements. It allowed a serum reduction from 15 to 0.5% in the culture medium, which still supported the ESC to differentiate into spontaneously contracting cardiomyocytes. During a differentiation period of up to 13 days the expressions of the heart muscle specific genes (alpha MHC, MLC-1 and cardiac actin) and pluripotency (Oct-4) were studied by quantitative RT-PCR. During the differentiation of ESC under standard culture conditions, the pattern of gene transcripts were comparable to *in vivo*. The expression of MHC, MLC-1 and cardiac actin increased (100-1000x) and Oct-4 decreased (~10x) during the differentiation days 4 to 10. After up to 13 days in gradually serum reduced culture conditions the TaqMan analysis revealed that the supplementation mixture facilitated the serum reduction down to 0% without a significant change of investigated gene expression. The described results confirm our former studies that the serum replacement provides a possibility to increase the standardization of the murine embryonic stem cell test (EST) by decreasing the necessity of serum supplementation from 15 to 0.5%.

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461

Development of an *in vitro*-assay for the assessment of teratogenic effects

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Developmental defects leading to grave malformations and dysfunctions can result from alterations in the differentiating embryo caused by exogenous embryotoxic compounds. During early embryonic development five highly conserved signaling pathways are very important for the regulation of differentiation, e.g. the Wnt-, TGF-β-, Notch-, Hedgehog and Ras-signaling pathway. Teratogenic noxa have major impacts on these pathways implying that signaling pathway-specific reporter systems could be used for the assessment of teratogenic effects. Signaling pathway-specific reporter constructs for the Wnt- and TGF-β-pathway comprising DNA binding elements coupled to a luciferase reporter gene were transiently transfected into a murine embryonic stem (ES) cell line and their proper function was demonstrated with pathway-specific agonists and antagonists. In stable transfectants the activity of the respective signaling pathway was screened during the first ten days in embryonic development. A combination of a resazurin-based cell viability assay with the luciferase reporter system was used for the assessment of effects onto the particular pathway caused by various embryotoxicants. Both, the Wnt- and TGF-β pathway were active in undifferentiated murine ES cells and their activity was down-regulated during differentiation. Treatment of undifferentiated ES cells for 24 hours with all-trans retinoic acid, as an embryotoxic model compound, caused an up-regulation in Wnt-signaling, while the TGF-β pathway was down-regulated as compared to the pathway activity of untreated ES cells. In the context of REACH and in the effort to reduce animal experiments, such new *in vitro*-assays are needed and promising. The results demonstrate that this assay can help to elucidate the complexity of signaling pathways during ES differentiation and embryonic development and detect the influence of embryotoxic compounds.

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462

Evaluation of the chicken embryotoxicity screening test (CHEST) as *in vitro* test system for teratogenicity / embryotoxicity

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The chicken embryotoxicity screening test (CHEST) is performed using fertilized hen eggs during early developmental stages. Literature gives evidence that this test system could be useful for the determination of teratogenic effects. Compared to other embryotoxicity assays (e.g. whole embryo culture) the CHEST is easier to conduct and does not require the use of embryonic stages from mammalian species. The chicken embryo is considered by legislation not to be an animal at the developmental stages used in this assay. Therefore, the CHEST can be considered as a replacement method. The CHEST could either be a potential replacement for *in vivo* testing or could be used as part of testing strategies as an early tier test. Consequently this test has the potential to significantly reduce the number of animals used for teratogenicity / embryotoxicity testing.In our laboratory, we have obtained results for ten substances, representing both, strong teratogens (e.g. retinoic acid, hydroxyurea, valproic acid, thalidomide) and non-teratogens. The results show a good correlation with the teratogenic potential of these substances *in vivo*. Therefore, the CHEST assay has confirmed its potential to serve as a replacement study on teratogenicity / embryotoxicity.

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463

Disturbance of microtubule assembly *in vitro* by organic chemicals based on hydrophobic interactions

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A concept connecting the lipophilicity of organic chemicals with their genotoxicity on a chromosomal level has been put forward by Schultz and Önfelt (Chem. Biol. Interact. 126:97-123, 2000). Aneuploidy in Chinese hamster V79 cells was elicited by lipophilic chemicals at concentrations related to their lipophilicity (log P). Hence, the lipophilic character of organic chemicals determines a background of chromosomal genotoxicity that can be addressed as “non-specific”. In contrast, toxicants with a “specific” mode of action act at effective concentrations consistently lower than predicted based on their

lipophilicity. Such specific processes involve karyokinesis and cytokinesis. Furthermore, the dynamics of the assembly and disassembly of microtubules is a partial process critical for the chromosomal segregation.In order to broaden the database for hydrophobic interactions that lead to chromosomal genotoxicity, a set of chemicals was selected for which existing information points to possible interactions with the tubulin-microtubule system. Acetamide, acrylamide, methylmethane sulfonate, acetonitrile, acrylonitrile and cyclohexanone were assessed as to their potencies to influence the dynamic processes of microtubule assembly and disassembly in a cell-free system *in vitro*. These compounds covered a lipophilicity range of log P between -1.5 and +1.0, complementary to compounds assessed earlier. The disturbance of microtubule assembly (no-observed-effect-concentrations; NOEC) was related to the compounds` lipophilicity. For non-specific compounds, a clear dependence of microtubule assembly on the lipophilicity of xenobiotics added to the test system was seen. This contrasts to the effects of the known specific agents, which are much higher than predicted from their log P values.The obvious dependence of the microtubular interaction on lipophilicity corroborates the general concept that hydrophobic interactions are connected with non-specific processes, which contribute to a background chromosomal genotoxicity. It also points to the dynamics of assembly and disassembly of microtubules as a crucial partial process for the integrity of chromosomal segregation.

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464

The HET-MN assay as an alternative approach to *in vivo* clastogenicity assays with rodents

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For regulatory purposes several *in vitro* assays are currently available to analyze the genotoxic/mutagenic potential of raw materials. However, a considerable fraction of *in vitro* positives may not be biologically relevant. For safety evaluations it is therefore often required to additionally perform *in vivo* assays. One important and widely used endpoint of *in vivo* genotoxicity testing is the formation of micronuclei (MN). The determination of micronucleus frequencies *in vivo* is part of a battery of genotoxicity tests and is accepted and recommended by regulatory agencies around the world to be conducted as part of safety assessments (OECD TG 474, Mammalian Erythrocyte Micronucleus Test, MNT). The HET-MN (Hen’s Egg Test for the induction of MicroNuclei) assay [1, 2, 3, 4] combines the use of the genetic endpoint ‘‘formation of micronuclei’’ with the well-characterized and complex model of the incubated hen’s egg, which enables metabolic activation, elimination and excretion of xenobiotics including mutagens and promutagens. Our results indicate that this alternative approach may be an option to replace the *in vivo* MNT in the future to verify the relevance of *in vitro* results. Here we present new data of substances tested in the HET-MN assay.

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465

Development of a roboter-supported softagar assay for the high-throughput screening of substances

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The assessment of anchorage-independent cell growth is usually performed by using the softagar assay *in vitro*. The classical method is time-consuming and therefore only suited to a limited extent for the evaluation of a high number of substances. Ke *et al.* (Biotechniques 36: 826-833, 2004) had previously established an one-week 96-well softagar growth assay by using the AlamarBlue™ staining. Here we show that the softagar assay can be performed by using the automated pipetting system epMotion (Eppendorf AG). The experimental design is highly variable and in 96-well plates it is possible to test up to 21 different compounds or several compounds in decreasing concentrations. Alternatively, it is possible to perform the assay with 3 different cell lines and 7 compounds in one plate. At the beginning of the assay the culture medium constituents pre-heated agar, stock solutions of the test compounds and the cell suspension(s) are needed. All pipetting steps are performed by the epMotion, so that a high reproducibility is warranted. Within a week the assay can be completed with the AlamarBlue™ staining by using the epMotion in a 96-well followed by readout of photometric absorbance or fluorimetric emission in a 96-well plate reader. The poster shows the results of incubating three colon cancer cell lines (DLD-2, HT-29 and MIP-101) with the widely used chemotherapeutic agent 5-fluorouracil (5-FU) in different concentrations. Taken together, the results with the roboter support the concept that the softagar assay performed in 96-well plates is obviously a powerful tool to test the anchorage-independent growth of cells of a high number of substances in a short period of time.

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466

Analysis of the antioxidative potential of natural compounds

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An increasing number of compounds isolated from a variety of edible products are thought to have a positive influence on normal intestine physiology and pathologic disorders such as intestinal inflammation, artherosclerosis and cancer. Whereas in the case of a single compound it is feasible to use various assays with different degrees of complexity to test its health-promoting activity, in the case one wants to analyze many (up to a few thousand) compounds the number of assays to be performed has to be reduced to a minimum. The aim of the present study was to establish a combination of assays with which one can analyze the antioxidative potential of an extremely high number of compounds in a relatively short period of time. The free radical scavenging potential of natural compounds was measured by using the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical cation decolorization assay. The formation of thiobarbituric acid-reactive substances (TBARS) was chosen as the parameter to measure the capacity of natural compounds to interfere with lipid peroxidation. In order to test whether natural compounds are able to induce cellular antioxidative enzymes, luciferase reporter gene assays were established. Concentrations of the compounds were assessed by the lowest non-cytotoxic concentration tested in V79-4 cells. Examples of how the test platform was successfully applied to analyse a number of compounds with antioxidative activities present in vegetables and fruits and further substances will be shown.

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467

Biomonitoring of polychlorinated biphenyls and organochlorine pesticides in human milk from mothers living in Northern Germany

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Polychlorinated biphenyls (PCBs) and organochlorine pesticides are persistent organic pollutants that have a widespread distribution in the environment. Human biomonitoring is a suitable tool to assess the burden of humans with these substances. Over a time span of eight years, a free analysis of their milk was offered to lactating mothers residing in the state of Lower Saxony, Germany. The human milk was analysed for levels of PCBs, DDT (dichlorodiphenyltrichloroethane), hexachlorobenzene (HCB) and beta-HCH (beta-hexachlorocyclohexane) and a number of other chemicals. Factors that may influence these levels were investigated using a questionnaire to obtain information regarding personal characteristics and different life style factors. In total 4314 samples were collected in the years 1999 to 2006 and analyzed for their content of these persistent organic pollutants (POPs). A clear downward trend of median total PCB, DDT, beta-HCH and HCB values in all participants and also in different selected subgroups could be observed. The median value of calculated total PCB in the year 2006 including all participants was 0.1825 mg/kg lipid, that of DDT 0.0815 mg/kg lipid, beta-HCH 0.0116 mg/kg lipid and of HCB 0.0229 mg/kg lipid. There were reductions between 40.9% and 47.1% compared to the year 1999. Among other influencing factors, median concentrations of total PCB, DDT, beta-HCH and HCB showed a clear rise with increasing age of mothers whereas an increasing number of breastfed infants per mother lead to a decrease. The proportions of other measured substances exceeding detection limits were as follows: Dieldrin 68.6%, alpha-HCH 1.3%, gamma-HCH 60.1%, heptachlor epoxide 41.5%, musk xylene 15.6%, musk ambrette 0.4%.

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468

Relation between genotoxic effects and urinary metabolites of naphthalene, phenanthrene and pyrene in mastic asphalt workers

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A cross-shift study with 202 bitumen-exposed workers and 55 construction workers without exposure to bitumen was conducted to determine the associations between external and internal exposure and genotoxic effects. Exposure to fumes of bitumen during the shift was measured by personal air monitoring. Hydroxylated metabolites of naphthalene [1- and 2-naphthol, OHNaph], phenanthrene [1-,2+9-,3- and 4-hydroxyphenanthrene, OHPhe] and pyrene [1-hydroxypyrene, 1-OHP] were measured in pre- and post-shift urine. 8-Oxo-7,8-dihydro-2’-deoxyguanosine (8-oxodGuo) and DNA strand breaks and alkali-labile sites were determined as biomarkers of genotoxic effects in white blood cells (WBC). Mixed linear models were applied for the different outcomes regarding exposure to bitumen, time of measurement, current smoking, German nationality and age as fixed factors and subjects as a random factor. Median concentration of fumes of bitumen during the shift was 3.7 mg/m^3 (range: 0.1- 41.7 mg/m^3). After shift significantly increased concentrations of OHNaph (29.7 µg/g crea), OHPhe (1490 ng/g crea) and 1-OHP (459 ng/g crea) could be determined in exposed workers compared with the referents (11.3 µg/g crea, 992 ng/g crea, and 191 ng/g crea, respectively). Post-shift levels of urinary OHNaph (r_s = 0.18, P<0.01), OHPhe (r_s = 0.36, P<0.001) and 1-OHP (r_s = 0.25, P<0.001) showed a moderate association with the concentrations of fumes of bitumen. Significantly higher levels of oxidative DNA damage (8-oxodGuo) and DNA strand breaks were found in exposed workers compared with reference subjects. After shift DNA strand break frequencies were moderately associated with urinary OHNaph in exposed workers (r_s = 0.20, P = 0.01) and 1-OHP (r_s = 0.19, P = 0.01) but not with OHPhe. No association between internal exposure and 8-oxodGuo could be observed. In conclusion, our results show that mastic asphalt workers are exposed to polycyclic aromatic hydrocarbons. Exposed workers also show increased DNA damage in WBC. However, no dose-response between exposure and DNA damage could be observed on an individual level.

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469

Assessment of long-term health effects by means of haemoglobin adducts of 1-chloro-2,3-epoxypropane (ECH) after accidental exposure

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On September 9th, 2002, two freight trains crashed on the outskirts of Bad Münder, Lower Saxony (LS), and caused the release of 1-chloro-2,3-epoxypropane (ECH, CAS 106-89-8) into the environment. From the originally 49.4 tons of ECH transported in a tank wagon about 5 tons drained away into the subsurface of the close-up range and the proximate area of the combustion following the crash. Further 29 to 33 tons of burnt or vaporised ECH were released into the atmosphere. In the early phase of the accident systematic measurements of ECH were not performed and thus especially the ambient air concentrations of ECH were virtually unknown.ECH is an alkylating agent that can react with nucleophilic functionalities of DNA and proteins to form specific adducts that can be used in monitoring chronic or accidental exposure. The EU has classified ECH as 'toxic', 'corrosive', 'skin sensitising' and as 'animal carcinogen'.To evaluate the accidental internal dose and to assess possible long-term effects, haemoglobin adducts (HbAs) of ECH were determined. *N*-(3-Chloro-2-hydroxypropyl)valine (CHPV) and *N*-(2,3-dihydroxypropyl)valine (DHPV) were used as biomarkers. Globin samples were analysed by the *N*-alkyl Edman method. The LOQ was 25 pmol/g globin (CHPV) and 20 pmol/g globin (DHPV), respectively. A total of 54 haemolysates of LS state policemen and 172 haemolysates of the federal police have been investigated. Further 328 subjects were part of a '*Public Health Service*' program (PHSP). CHPV adduct levels of 31 and 30 pmol/g globin were analysed in 2/54 samples and 80 pmol/g globin in 1/172 samples, respectively. In the PHSP, CHPV levels in the range of 35 - 45 pmol/g globin were found in 3/328 samples. DHPV adducts were not observed in any of the globin samples analysed.Internal doses were extrapolated to airborne ECH with the presumption that 0.1 % of the body dose has bound to globin and 20 % of this fraction are *N*-terminal adducts. Based on an inhalative unit risk of 1.2×10^{-6} $[\mu g/m^3]^{-1}$ and considering the removal of HbAs from the erythrocyte population from the time of exposure to time of sampling, the estimated cumulative additional carcinogenic risks range from 2.6×10^{-8} - 1.07×10^{-7}.

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470

Acrylamide: Impact of food matrices on bioavailability and biological effects in rats

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Acrylamide (AA) which is classified as genotoxic carcinogen is formed in food during the Maillard reaction from precursors, primarily asparagine. Significant AA formation occurs during heating of carbohydrate-rich food with low water content, such as French fries and cookies. Moreover, AA has also been found in other major food items such as coffee and bread. Up to now, only few data are available to assess to which extent AA is bioavailable from food matrices. Animal studies on toxicokinetics and biological effects of AA up to now were mainly carried out by application of AA via drinking water. In this study we investigate whether the matrix of representative foods, including French fries, gingerbread and bread crust affect bioavailability of AA by comparison to AA uptake via drinking water. Mercapturic acids (LC-MS/MS) in urine as well as hemoglobin (Hb) adducts of AA and its genotoxic metabolite glycidamide (GA) (GC-MS) were determined as biomarkers for internal exposure, bioactivation and detoxification. Genotoxicity in lymphocytes and hepatocytes was determined by alkaline single cell gel electrophoresis (Comet Assay). Foods were given over 1-9 days to male Sprague-Dawley rats (daily uptake of 100 µg AA/kg bw). Urine was collected for 24 h after the last feeding. Blood and liver samples were collected 24 h after the last dietary administration. In the first set of experiments AA was administered at the same dosage in drinking water, French fries (common and reconstituted from dry potatoe granules) and in gingerbread over 3 or 7 days. Levels of AA-Hb adducts after 7 days were nearly 2 fold those after 3 days, indicating cumulation of AA-Hb adducts. GA-Hb adducts were comparable at both time points. In contrast urinary Mercapturic acid excretion of days 3 and 7 remained fairly constant. AA ingestion in water, French fries or gingerbread induces comparable biomarker response (Hb adducts, mercapturic acid excretion). Moreover, irrespective of the type of diet the comet assay did not as yet show significant induction of DNA damage in blood cells or liver tissue. Taken together, thus far, the selected food matrices do not seem to influence bioavailability of AA as compared to drinking water.

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471

Are supplemented dietary flavonoids a hazard for human brain development?

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Flavonoids are a large family of phytochemicals which are proposed to protect against a multitude of diseases. Due to these beneficial effects, these compounds are increasingly used as dietary supplements in functional food. Thus, their daily intake can exceed 1 g and plasma levels of 0.3-7.5 µM were measured in humans. Many of the biological actions of flavonoids have been attributed to their antioxidant properties. Yet, there is emerging evidence that flavonoids do not only act as conventional hydrogen-donating antioxidants but also interfere with a large number of cell signaling pathways which are involved in normal development. However, the hazard of flavonoids in high concentrations for human health is an understudied field of research. We investigated the effects of different flavonoids (epigallocatechingallate [EGCG], epigallocatechin [EGC], epicatechin [EC], quercetin, hesperitin, kaempferol) on normal human neural progenitor (NHNP) cells, which are a neurosphere-based model system for human brain development *in vitro*. Basic processes of brain development – proliferation, migration and differentiation – can be assessed with this 3D cell culture system. Cytotoxicity was measured with the CellTiterBlue Assay (Promega). Proliferation was examined by measuring increases in (i) neurosphere diameter and (ii) enzymatic activity of single neurospheres (CellTiterBlue Assay) over two weeks. Migration distances were measured from the edge of the neurosphere to the furthest outgrowth of cells. Treatment of NHNP cells with different flavonoids from 1 to 10 µM caused no cytotoxicity. Neurosphere proliferation monitored over two weeks was not affected by flavonoids either. However, migration of differentiating cells out of the neurosphere was disrupted or even completely inhibited when cells were exposed to EGCG, EGC or EC in the lower micromolar range. First experiments show, that EGCG disrupts the interaction between NHNP cells and the extracellular matrix indicating that EGCG disturbs rather cell adhesion than migration. These results show that flavonoids affect neural development in a human *in vitro* model. The underlying mechanisms are currently elucidated.

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472

Microcystins in alga products as dietary supplement

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Algae and cyanobacteria, so called blue-green algae, gain more and more importance in Europe as dietary supplements. Often assigned are positive nutritional effects and/or numerous medical effects, but the evidence is lacking. Some alga species, e. g. *Spirulina* and *Chlorella* are cultured commercially in aquaculture while *Aphanizomenon flos aquae* (*AFA*) are mainly received form natural resources. Therefore, AFA might be contamined with microcystins (MC), a biotoxin produced by various cyanobacteria genera. More than 80 different structure variants of microcystins are known, all are cyclical heptapeptides with varying toxicity. MCs are hepatotoxic and classified as tumor promotors. The hepatotoxicity is based on a nonreversible inhibition of proteinphosphatases, as a result of covalent binding to the active centre. Currently, a content of 1 µg MC-LR/g is accepted for *AFA* products used as dietary nutritional supplement. In the present study 87 commercially available dietary supplements based on algae products (*Chlorella* (15), *Spirulina* (14), and *AFA* (58)) were examined. The investigation was performed with a screening method based on direct competitive enzyme linked immunoassay (ELISA) with polyclonal antibodies against MC –LR with cross reactivity proved for four other MCs. In all 29 tests concerning *Spirulina* and *Chlorella* no MC could be found, but in 21 of the 58 probes containing *AFA* products up to 0.5 µg MC-LR/g were detectable. These positive *AFA* products were examined in detail by high pressure liquid chromatography (HPLC). With the latter method higher MC contents were found as compared to the results obtained by ELISA. A verification of this by mass spectroscopy is under way. It is concluded that other MCs than MC-LR are poorly detectable by the ELISA due to lack in cross reactivity therefore HPLC gives much higher values. The present results demonstrate the requirement of regulary examinations of dietary supplements containing algae.

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473

Uptake of nanoparticles in the food by gut-associated dendritic cells

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Nanoparticles are defined as particles with a diameter smaller than 0.1 µm. They are constituents of food supplements and so called nanofood. The regular intake of inorganic particles by humans is estimated as 10^{12} particles per day. Since nanoparticles are already known to induce severe inflammatory reactions and cancer in the lung, and contribute to the establishment of cardiovascular diseases, there is an urgent need for further studies on the toxic potential of nanoparticles in the intestine. It has been suggested that a low nanoparticle diet might be associated with a better prognosis of inflammatory bowel disease (IBD), even though contradicting data exist. In ex vivo studies an amplifying effect of ultrafine TiO_2 on LPS-induced gut inflammation could be demonstrated. We are presently analysing the effect of orally administered nanoparticles on the induction of acute and chronic colitis in different transgenic mouse models. Our main focus lies on dendritic cells since they are the most potent inducers of immune reactions and known to be involved in the pathology of IBD. In *in vitro* studies we could show that murine bone marrow derived dendritic cells display differentiation defects if generated in the presence of nanoparticles. Differentiation was less efficient in the presence of ultrafine titanium dioxide and when cells were generated in the presence of ultrafine carbon black the effects were even more pronounced. Thus, the number of dendritic cells as well as the density of MHC- and costimulatory molecules on the cell surface was reduced in these cultures. If ultrafine carbon black was added to immature dendritic cells, the frequency of MHC-class-II molecules was enhanced on the cell surface suggesting a stimulatory effect of ultrafine carbon black on dendritic cell maturation.

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474

Oral exposure to inorganic arsenic: evaluation of non-carcinogenic effects

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Inorganic arsenic is a potent carcinogen, causing tumours of the skin, lung, urinary bladder and other locations. It also induces a number of non-carcinogenic effects like skin lesions (dyspigmentation and keratosis), peripheral vascular diseases, reproductive toxicity and neurological effects. Health effects caused by drinking water highly contaminated with arsenic are one of the most serious health problems in some countries in South-Eastern Asia and some regions of South America. However, due to its geogenic origin exposure to low levels of inorganic arsenic also occurs in North

America and Europe. Existing risk assessments are based on epidemiological studies from regions with high exposure concentrations (thousands of micrograms per litre drinking water). It is a matter of debate whether these findings are useful to predict arsenic-induced effects at low concentrations. In recent years, numerous epidemiological studies on cancer and non-cancer effects of inorganic arsenic have been published. It was the aim of this project to review recent toxicological data on inorganic arsenic with special emphasis on publications concerning effects in the low dose range. The results of the re-evaluation of the non-carcinogenic effects are presented. Induction of skin lesions is the most sensitive non-carcinogenic endpoint. Men are more susceptible than women and susceptibility is influenced by the nutritional status. A tolerable daily dose was derived based on an epidemiological study on arsenic-induced skin lesions in a population from Bangladesh. Influences of gender, nutritional status and background exposure from food were considered for the benchmark-dose modelling.

This work is part of a research project on the toxicological activity of arsenic in soil, groundwater and drinking-water sponsored by the Federal Environmental Agency (Umweltbundesamt), Berlin, Germany (grant no. FKZ 206 61 201).

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475

Drug residues in drinking water

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The Bavarian Drinking Water Study* ("Antibiotics in drinking water") was initiated in 2002 to monitor drug residues in drinking water samples all over the state of Bavaria, with a special focus on antiinfective agents. The study identified 11 wells (out of a total of 55 sampled sites) with traces (7-66 ng/L) of sulfamethoxazole (SMZ), a sulfonamide antimicrobial that has been available in Germany since 1968, but is now usually being prescribed in combination with trimethoprim. In order to distinguish between human and veterinary origin of SMZ, we also looked for other contaminants. Samples were drawn from household taps or drinking water reservoirs, concentrated by solid-phase extraction and analyzed using LC / electrospray tandem MS techniques with a limit of quantitation (LoQ) of 2-10 ng/L for most analytes. In the 11 SMZ-positive sites we also detected small amounts of carbamazepine (CBZ; 2-157 ng/L) and X-ray contrast media, e.g. diatrizoate (5-405 ng/L), iopamidol (5–144 ng/L), and iothalamate (5–69 ng/L). The co-occurrence of these drugs, which are only rarely being used in veterinary settings, strongly indicates that human use is the major source of contamination. As some of the sites had treatment processes installed, it became evident that passive treatment such as bank filtration, quartz fills, etc. were ineffective measures for drug removal. Oxidative processes, e.g. ozonation and chlorine dioxide treatment, reduced the concentrations of SMZ and CBZ almost below the LoQ, but X-ray contrast agents were rather unchanged. Unfortunately, little is known about the nature and the toxicology of the by-products of the oxidation processes. Thus, further research is needed before the countrywide installation of such drinking water treatment facilities can be recommended for effective drug removal.

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476

Pesticide poisoning in domestic and wild animals in Germany and Austria: a retrospective study

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Domestic and wild animals are exposed to a variety of commercial pesticide formulations or pesticide contaminated baits. In literature there is only few information concerning the incidence and causes of animal poisoning in Germany and Austria. This retrospective study summarizes the analytical results obtained in the department of pharmacology, toxicology, and pharmacy of the faculty of veterinary medicine in Munich between 2000 and 2007.The requests for toxicological analysis were submitted by veterinary practitioners, public health authorities, local police departments, pet owners, and animal protectionist groups from all over Germany, and by academic departments of veterinary pathology of Germany and Austria. Samples sent in for analysis included animal tissues (mainly liver of dog, cat, horse, and bird), stomach contents, blood, baits (meat, sausages, and other material), liquids, or undefined material. The samples were analysed using HPLC-techniques, thin layer chromatography, and combined gaschromatography/mass spectrometry.Organophosphate insecticides were the most frequently detected pesticides, followed by carbamate insecticides and rodenticide anticoagulants. Less common were findings of alkaloids and metaldehyde. Animals found positive for pesticide poisoning were mainly dogs, wild birds and cats. Inhibitors of cholinesterase, anticoagulants, and strychnine were the most usual toxic agents in baits prepared for intentional poisoning.The data emphasize the enduring significance of pesticide poisoning in domestic and wild animals although the use of many of the detected pesticides is restricted by law.

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477

Toxicity and elemental composition of PM_{10} in classrooms

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Background: Outdoor air PM_{10} pollution is associated with a wide range of health effects and a European threshold limit concentration of 50 µg/m^3 was established in 2005. Given the fact that people spend at least 85% of their time indoors, where particle concentrations are often higher than outdoors, we investigated the health effects of indoor air particles within the PAMINA (Particulate Matter in Indoor and Ambient Environments) project. *Methods:* PM_{10} was collected on Teflon filters using a medium volume sampler (2.3 m^3/h) in two schools with two classrooms per school in Munich during teaching hours. Particles were recovered by sonication, lyophilized and resuspended in water. Toxicity was assayed as a decline of cellular ATP concentration in human primary keratinocytes, human lung epithelial A549 cells and Chinese hamster V79 lung fibroblasts at concentrations from 3 ng/ml to 10 µg/ml. In addition, toxicity was assayed after metabolic activation in V79 cells expressing human cytochrome P450 1A1, 1A2, 1B1, 2A6, 2B6, 2C9, 2D6, 2E1, 3A4 or 3A5. Elemental composition of the particles of one school was analyzed via energy dispersive X-ray spectroscopy (EDX). *Results:* While in A549 and V79 cells no toxicity was observed, in human primary keratinocytes PM_{10} at a concentration of 10 µg/ml caused a slight, but statistically significant decrease in vitality. This cytotoxic effect was also found in V79 cells after metabolic activation by CYP1A1 (one classroom) or CYP2C9 (all four classrooms). As assessed by EDX analysis, most of the particles were silicates (42%) like aluminium silicate, feldspath and silicon dioxide, followed by organic (33%) and calcium carbonate particles (8%). The major elements were O> Si> Ca> Al. The particle composition did not differ in the two analyzed classrooms. *Conclusion:* The collected PM_{10} consisted mainly of silicates and organic particles. The direct cytotoxicity and metabolic activation by cytochrome P450 isoforms 1A1 and 2C9 were statistically significant at a PM_{10} concentration of 10µg/ml, which is about 10,000 times higher than exposure encountered in classrooms. We therefore expect no toxic effects of these particles in school children.

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478

New evidence for the lack of carcinogenic and sensitizing potential by pyrethrins

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Extracts from the chrysanthemum flower have been used since antiquity as efficient natural insecticides. Currently, the modern Pyrethrum extract (Pyrethrins) is being re-evaluated both as Plant Protection Product and Biocide in Europe under the Directives 91/414/EC and 98/8/EC, respectively. In connection with these applications new evidence has been presented regarding the toxicity or rather the safety of Pyrethrins for humans, namely the lack of carcinogenic hazard for humans and the lack of skin sensitizing properties. While Pyrethrins had been proven to be non-genotoxic in a standard test battery both *in vitro* and *in vivo*, increased incidences of liver and thyroid tumours were noted at high dose levels in rats in chronic dietary study. Mechanistic studies both *in vitro* and *in vivo* provided evidence for a rodent specific hepatotoxic tumour induction. Similar to phenobarbital hepatic enzyme induction was demonstrated and confined to high dose levels.Pyrethrins and insecticidal products containing Pyrethrins have been demonstrated to be devoid of skin sensitizing properties in Bühler- or Magnusson & Kligman-Tests with guinea pigs. These findings were confirmed in a preliminary murine local lymph node assays conducted with several batches of the Pyrethrum pale extract. A recently published literature survey gave supporting evidence that no data are published on contact dermatitis induced by the modern refined Pyrethrum extract. Pyrethrins do not pose a carcinogenic hazard to humans and there is no evidence for a skin sensitizing potential by the refined Pyrethrum extract.

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479

A new testing strategy for reproductive toxicology under REACH

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Under the new EU chemicals legislation REACH 30.000 existing chemicals should be evaluated within a period of 15 years. About 3,000 high production volume chemicals have to be assessed first. For most of these chemicals large data gaps must be closed by conducting various studies, repeated dose studies in particular. According to estimates of the European Commission, 70 percent of all experimental animals are used to test chemicals for effects on reproduction. This is also where 70 percent of costs are generated - particularly for testing substances on impairment of fertility and reproductive ability, because REACH prescribes a two-generation test in rats (OECD Test Guideline 416) as the standard procedure for chemicals with production volumes of more than 1,000 tonnes. Around 3,000 animals are needed to test one substance using OECD TG 416. If the standard testing requirement could be limited to a one-generation study, around 1,400 experimental animals for each substance tested would be saved. With an estimated 2,000 substances that have to be tested over the next three years under REACH, this would mean a 2.8 million reduction in the number of laboratory animals used. Another promissing approach to reduce animal numbers und costs under REACH would be the restriction to only one animal species instead of two for developmental toxicity testing. First results of a retrospective evaluation have shown that a second species does not contribute to decision making in terms of classification and labelling or risk assessment. Therefore, an improved testing strategy for reproductive toxicology is proposed.

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480

Clinical trial applications: requirements for the preclinical documentation

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After implementation of the European Clinical Trials Directive 20/2001/EG into German national law in August 2004, about 4000 applications for authorization of clinical trials have been submitted to the BfArM, including about 24% by non-commercial sponsors for so called investigator initiated trials (IITs). The assessment of the preclinical documentation of these IIT applications revealed a high rate of formal deficiencies and pharmacological and toxicological objections. Therefore, the requirements of preclinical documentation (i.e. pharmacodynamics, pharmacokinetics and toxicology) will be introduced for investigational medicinal products which are either tested before marketing approval (Phase I – III) or are licensed already, but are used outside the approval conditions in the intended clinical trial. The presentation considers the standard requirements, but also deals with specific demands for special groups of substances

such as anticancer medicinal products, topicals, biotechnology-derived pharmaceuticals and substances with the potential to cause severe adverse reactions in the proposed clinical trial.

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481

The mu-opioid receptor genetic variant N40D wipes out differences in receptor signalling efficacy between pain relevant human brain regions

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Aim: To investigate the consequences of the N40D human μ-opioid receptor variant (coded by the genetic variant 118A>G) on receptor expression, binding and signalling in human brain tissue from regions involved in transmission and sensory perception of pain, with the latter agreeing with the observed effects of this variant on opioid effects on pain-related brain activation. Methods: Mu-opioid receptor-expression (analyzed by [3H]-DAMGO saturation binding, OPRM1 mRNA RT-PCR) and -signalling (analyzed by [35S]-GTPγS binding) was assessed in human post mortem samples from the secondary somatosensory area SII, a cortical projection region coding for pain intensity, and the lateral thalamus, an important region for nociceptive transmission, obtained from 22 non-carriers, 21 heterozygous and 3 homozygous carriers of the μ-opioid receptor variant N40D. Results: In both brain regions, neither receptor expression nor affinity in carriers of μ-opioid receptor variant N40D differed from wild-type samples. However, in non-carriers of μ-opioid receptor variant N40D, μ-opioid receptors activated the coupled G-protein more efficiently in SII than in the lateral thalamus (Factor 1.55-2.27). This regional difference in signalling efficacy was absent in heterozygous (Factor 0.78-1.66) and homozygous (Factor 0.66-1.15) carriers of μ-opioid receptor variant N40D. Conclusion: These data demonstrate that a genetic human μ-opioid receptor variant exerts its effects by interfering with the interplay between brain components of the neural network processing pain and support the adoption of a brain-region specific analytical approach to the genetic modulation neuronal responses. Acknowledgement: European Research Training Group "Roles of eicosanoids in biology and medicine" (GRK 757)

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482

Moleculargenetic analyses of promoter variants within the human G protein-coupled receptor kinase 4 gene (GRK4)

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The G protein-coupled receptor kinase 4 (GRK4) is involved in renal sodium handling and genetic hypertension. Three genetic variants within coding regions (R65L, A142V, and A486V) are associated with hypertension, but no promoter studies have yet been published. Therefore, we focused on analyzing the GRK4 promoter and the impact of novel variants on its transcriptional regulation. Genomic DNA of 94 hypertensive Caucasians was scanned for genetic variants within the GRK4 gene. Promoter variants were analysed using three different cell lines, renal HEK293T and COS7, and non-renal SaOs-2, both under basal conditions and phorbol ester stimulation (10-8M PMA). Transient transfection assays were performed with wild type deletion promoter constructs to identify cis-active regions, as well as with variant full length constructs. In silico predicted (Alibaba2.1/Transfac7) transcription factor binding sites were analysed by EMSA experiments. Of the GRK4 promoter, 1851 bp of the 5'-flanking region and 275 bp of the 5'-UTR are sufficient for effective transcriptional activity in all cell lines tested. A distal portion of 293bp (from -1851 to -1558), comprises a powerful cis-active element. The 5'-UTR functions as an active part of the GRK4 promoter. We identified four novel genetic variants (C-1702T, G-1436C, G+2T, and G+268C) within the regulatory region, two of which result in a drastic reduction of promoter function (C-1702T, G+2T) in all cell types. The activating impact of G+268C is cell type-specific and not present in embryonic HEK293T cells. Variants resulted in allele-specific binding patterns of nuclear proteins. The GRK4 core promoter resides in the first 1851 bp upstream of its transcription start site. The identified functional genetic variants within this region show allele-specific impact on both cell type- and stimulation-dependent transcription and may play a role in renal sodium handling and hypertension by affecting the stoichiometry of renal GRK4.

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483

Effects of low-dose aspirin and *Helicobacter pylori* on the expression of the prostaglandin transporter (OATP2A1) in human stomach and duodenum

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Helicobacter pylori-induced changes of acid production and the reduced production of protective prostaglandins (PGs) due to the inhibition of cyclooxygenases by NSAIDs are recognized mechanisms in the pathogenesis of gastroduodenal ulcerations. However, the role of the prostaglandin transporter OATP2A1 for gastrointestinal ulcerations has not yet been elucidated. OATP2A1 mediates the uptake of prostaglandins into to cells. Therefore, the aim of our study was to investigate the effects of low-dose aspirin and *H. pylori* on the expression of OATP2A1 in the human stomach and duodenum. 20 healthy volunteers received 100 mg/day aspirin for 7 d. At days 0 and 7, the volunteers underwent a gastroduodenoscopy to obtain biopsies from antrum, corpus, and duodenal bulb which were used for gene expression analyses. *H. pylori* positive subjects repeated the protocol 3 months after successful *H. pylori* eradication therapy. OATP2A1 mRNA expression was quantified by real-time RT-PCR and expressed as arbitrary units (au.). OATP2A1 showed the highest mRNA expression in the antrum (A) followed by the corpus (C) and bulbus (B) independent from aspirin intake and *H. pylori* status (A: 8.2±5.6 a.u., C: 5.9±4.0 a.u., B: 2.5±1.4 a.u.; A vs C: p>0.05, A vs B and C vs B: p<0.001). Low-dose aspirin led to a relevant decrease of 58% in the OATP2A1 mRNA expression in the corpus of H. pylori-infected subjects (p=0.05). After the eradication of *H. pylori*, the level of OATP2A1 mRNA increased about 113% (p=0.05). Overall, low-dose aspirin and H. pylori infection did not significantly affect the OATP2A1 mRNA expression in stomach and duodenum. Whether OATP2A1 plays a pivotal role for the development of nonsteroidal anti-inflammatory drugs-induced ulcerations in context to *H. pylori* infection requires further investigations.

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484

Expression of endocannabinoid-system Genes in Human adipose tissue during high and low fat diets

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Introduction: Attempts to modulate endocannabinoid system (ECS) activity reach from enhancement in chronic inflammatory disorders to blockade in obesity and metabolic diseases. In human obesity, increased activity of the ECS was described in the circulation and in adipose tissue. High fat feeding also increased endocannabinoid levels in mouse liver by inhibition of the activity and expression of degrading enzymes. We hypothesized that a high fat diet in humans would also influence gene expression of cannabinoid type 1 receptors (CB1-R), and the degrading enzymes fatty acid amide hydrolase (FAAH) and monoglyceride lipase (MGL) in adipose tissue. Methods: 33 lean and obese healthy human subjects underwent a 4 x 2 weeks isocaloric dietary study with 4 periods of varying fat content (30% fat from calories - 15 or 45% - 30% - 45 or 15%). The sequence of low and high fat diet periods was randomly assigned in a cross-over fashion to each subject. At the end of the low and high fat periods, several parameters of glucose and lipid metabolism were determined in the fasting condition and subcutaneous abdominal adipose tissue biopsies were obtained to measure expression of CB1-R, FAAH and MGL by TaqMan Real-time RT-PCR. All variables were tested by 2-way ANOVA with the diets as intra-individual and the obese/lean phenotype as inter-individual factors. Results: Body mass index (21,7 kg/m2 vs. 33,0kg/m2) as well as body composition and fat distribution did not change with the diets in either group. Fasting glucose was similar between the groups whereas fasting insulin was increased 3x in the obese group. High or low fat intake did not change fasting glucose and insulin. Also, adipose tissue gene expression of CB1-R, FAAH and MGL was not different between groups and not influenced by the diet. Discussion: These data do not support dietary influence on genes of the ECS in human adipose tissue. Further analyses will show whether fat intake modulates endocannabinoid levels in the blood and adipose tissue. If bioavalibility of endocannabinoids is increased in human adipose tissue by high fat intake, other mechanisms than gene regulation may contribute.

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485

Influence of non-synonymous ABCC2 SNPs on protein expression and substrate transport

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Introduction: Multidrug resistance-associated protein 2 (MRP2/ABCC2) is expressed mainly on the bile canalicular membrane and plays an important role in the biliary excretion of various kinds of substrates, in particular glutathione, glucuronide and sulfate conjugates and also some unconjugated drugs, such as pravastatin and doxorubicin. Functional loss of MRP2 is found in patients suffering from the Dubin–Johnson syndrome (DJS), whereas less penetrant alleles alters sensitivity to certain drugs. Recently we identified new MRP2 protein variants by direct sequencing of African-American and Korean DNA samples. Little is known about the consequences on MRP2 function. We have started to undertake functional characterization of MRP2 protein variants found in different ethnic groups. Methods: 6 MRP2 missense genetic variants (non-synonymous SNPs), fused to green fluorescent protein (GFP), were examined in *Xenopus laevis* oocytes for their effect on expression, localization and function of the transporter. Radiolabeled bromosulfophthalein (BSP) was chosen as transport substrate. Results: All MRP2 protein variants investigated thus far were found to be expressed predominantly in the oocyte membrane. Three MRP2 protein variants (R1174H, R1181L, P1291L) exhibited a 20 to 50% reduced expression level, compared to wild-type. In addition, the MRP2 variants R1174H and R1181L showed a decreased BSP transport activity. Conclusions: The investigated MRP2 variants affect protein expression and transport, which may be clinically relevant in treatments with MRP2 drug substrates.

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486

Consideration of orthologous sequences improves the prediction of CYP3A4 and CYP3A5 regulatory elements

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A full understanding of the transcriptional regulation of CYP3A could lead to the reduction of clinical interactions involving induction of these promiscuous drug metabolizers. However, all existing software packages generate large numbers of false-positive predictions of regulatory elements. Differentiation between true and false-positives requires laborious functional characterization. We attempted to improve prediction accuracy of gene regulatory elements by the consideration of the available

phylogenetic information. Orthologous upstream CYP3A4 and CYP3A5 sequences from humans and several other primate species were analyzed using regulatory sites prediction packages MATCH, ClusterBuster, NUBISCAN, and NHR-SCAN. Taking into consideration only elements conserved in all species greatly reduced the number of predicted TFBS and increased the proportion of true-positives. Thus, the three binding sites for orphan nuclear receptors with highest scores found in upstream CYP3A4 sequences comprise the well characterized proximal ER6, the XREM, and the recently described distal ER6 at -11.4 kb. Analogous high score sites within CYP3A5 upstream sequences comprise the previously characterized proximal ER6 site, a DR2 site at -2.2kb, and a DR4 site at -4.4 kb. EMSA and transfections of LS174T cells with the CYP3A5 transcriptional regulators PXR and CAR and an appropriate reporter-gene constructs revealed the responsiveness of the DR4 site towards CAR, but not PXR. Functional characterization of the DR2 site is in progress. In conclusion, the consideration of orthologous sequences from other species greatly improves prediction accuracy of CYP3A gene regulatory elements. The CAR-selective DR4 site identified in CYP3A5 upstream sequences is consistent with the previously reported pronounced responsiveness of this gene towards CAR inducers such as phenobarbital.
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487

An in silico screen to identify SNPs causing variation in the splicing pattern
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In various internet-databases there is information about a huge number of polymorphisms in the human genome. There is much less information available on the phenotypic effects that go along with these variations. SNPs affecting the amino acid sequence and promoter SNPs which impact on the transcription efficiency are widely analysed, e.g. as cancer risk factors or in pharmacogenetic studies. Another potential phenotypic effect of SNPs, which is less apparent from the genomic data alone, manifests itself in a variation of the RNA splicing pattern. Splice-relevant SNPs are attractive for future molecular epidemiological studies as the phenotypic effect can be expected to be high.Using the computer program AASsites we performed in-silico analysis to identify such SNPs. AASsites was created using an analysis pipeline design. It calls two other programs, Geneid (Guigo, 1998) and Genscan (Burge and Karlin, 1997), which both predict the intron-exon structure of a gene's mRNA based on the DNA sequence data. Predictions based on the wild-type sequence and on the polymorphic sequence are generated and then compared, thereby identifying potential candidates for splicing-relevant SNPs. Additionally AASsites performs ORF (open reading frame) analysis and ESE (exonic splicing enhancers) analysis. 35 genes, mostly relevant to xenobiotic metabolism or transport, were selected for screening. Then within the sequences of these genes 6994 polymorphisms described in the NCBI SNP database were evaluated using AASsites. As a result 44 SNPs with a potential impact on splicing were identified. One of these SNPs, CYP21A2 A/C656G (rs6467), had previously been described by Olney et al. (2002) as leading to an intron 2 splice variation. 6 SNPs are not present in Caucasians and 3 have a frequency of >0.05 making them unsuitable for further analysis. 3 SNPs with an adequate frequency were rejected as putative candidate polymorphisms for other reasons. For the remaining 31 SNPs there is no information about the allelic frequency in Caucasians. In vitro validation of some of the most promising potential splice variants is still required to verify the applicability of these in silico results.
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488

Influence of polymorphisms in apoptotic genes on the outcome of chemotherapy in patients with lung cancer
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Individual differences in the function of certain proteins might influence the response of patients to chemotherapy. Identification of genetic polymorphisms may help towards predicting chemotherapy outcome and individually optimised therapy. Because resistance to chemotherapeutic agents can be due to a lower sensitivity of the tumor cells to apoptotic signals we investigated the influence of 5 SNPs in 4 apoptosis relevant genes on chemotherapy response in lung cancer patients. FAS: rs2234767, rs1800682, BAX: rs4645878, BCL-2: rs2279115 and BCL-x: rs6121172. All SNPs were chosen due to their known or suspected phenotypic effect. For lung cancer the variant allele of rs6121172 is hypothesised to upregulate apoptosis and thereby influence chemotherapy response positively, whereas the other four polymorphisms are expected to downregulate apoptosis. For 427 patients with primary lung cancer chemotherapy response was assessed after the 2nd or 3rd cycle of chemotherapy based on the recommended response evaluation criteria in solid tumors (RECIST). DNA isolated from peripheral blood lymphocytes was genotyped using fluorescence-based melting curve analysis for rs2279115, rs4645878, rs2234767, rs1800682 and by RFLP methods for rs6121172. Genotype frequencies were compared in responders (complete or partial remission) and non-responders (stable or progressive disease). Crude odds ratios (ORs) for the risk of being a non-responder were calculated for all lung cancer patients and in the subgroups SCLC and NSCLC. In the group of all lung cancer patients, none of the polymorphisms above modified response statistically significantly. In accordance with our hypothesis the risk of being a nonresponder was significantly increased for NSCLC patients (n=232) carrying the rs2234767 G allele (cOR 2.20; 95% confidence interval (CI) 1.14-4.24). Further statistical analysis including multiple logistic regression and cox regression adjusted for confounding factors will be performed. We conclude that the G-allele of the promoter polymorphism rs2234767 may modify response outcome in a histology-specific manner. Funded in part by the “Deutsche Krebshilfe“ and an DKFZ-intramural grant to L.F.K.
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489

Rapid screening of functional polymorphisms in the human 5-lipoxygenase gene using PyrosequencingTM
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Introduction: Leukotrienes, a group of lipid mediators derived from the arachidonic acid, are involved in the development of chronic inflammatory diseases, asthma, and the acute respiratory distress syndrome (ARDS). The first steps of their biosynthesis are catalyzed by the 5-lipoxygenase. Human genetic ALOX5 variants have been associated with asthma and atherosclerosis. Variants of the ALOX5 gene are also candidates for modulating the individual risk of developing ARDS. To facilitate further investigations towards the clinical role of ALOX5 variants, we designed reliable high-throughput screening assays for ALOX5 polymorphisms. Methods: Ten simplex PyrosequencingTM assays were developed for ALOX5 polymorphisms dbSNP rs4986832, rs4987105, rs2115819, rs3740107, rs156096, rs2291427, rs10751382, rs2242334, rs2229136 and rs3802548 covering the whole ALOX5 gene range organized into 14 exons and 13 introns. They have been associated with functional consequences or are frequent with a minor allele frequency > 0.1. Assays were established and validated in DNA samples from 180 healthy unrelated Caucasians. Results: In all 180 DNA samples the ten ALOX5 polymorphisms were identified correctly as verified by control samples obtained by conventional sequencing. The observed frequencies of homozygous, heterozygous and non-carriers of the minor alleles were in agreement with the Hardy-Weinberg equilibrium. Minor allelic frequencies were: rs4986832G>A = 0.15, rs4987105C>T = 0.06, rs2115819T>C = 0.45, rs3740107G>A = 0.25, rs156096A>G = 0.22, s2291427G>A = 0.31, rs10751382A>G = 0.31, rs2242334G>T = 0.34, rs2229136A>G = 0.07 and rs3802548A>T = 0.26. Conclusion: The presently developed PyrosequencingTM assays allow for quick and reliable detection of ALOX5 genotypes and may facilitate further investigations of ALOX5 genetic functional associations. Acknowledgement: German Research Association (DFG) “Roles of eicosanoids in biology and medicine” (GRK 757)
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490

Genome-wide analysis of gene expression in human placenta: influence of gestational age and cell differentiation
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The placenta of mammals represents the central auxiliary organ during gestation and is characterized by fast growth and rapid development. Cell proliferation and differentiation as well as accessing the host's vascular system for nutrient and substrate supply are physiological hallmarks of the growing placenta. High-throughput functional genomics have become powerful tools to identify functional networks in the investigation of physiological pathways and complex human pathologies. In the present study, we conducted a global mRNA profiling to detect transcripts whose amounts are changed in the human placenta during the third trimester compared to full-term pregnancy. Pooled RNA samples from women of different gestational ages, i.e. early preterms (<week 30), preterm (week 30-37) and term (>week 37), were analyzed by the GeneChip Human Genome 133 Plus 2.0 Arrays (Affymetrix). Using pathway analysis tools, we observed striking similarities to specific gene expression patterns typical for cancer development and neoplasia, including angiogenesis and vascularization. However, the self-limitation of placental growth suggests tight regulation of the pathways which are involved in placental development.
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491

Phenotypic characterization of polymorphic human metabolism by CYP2D6 and CYP2C9 with the probe drug combination dextromethorphan / flurbiprofen
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Up to now, there is no method to characterize the cytochrome P450 activity in humans simultaneously for CYP2D6 and CYP2C9 in a non-invasive manner. We developed a phenotyping procedure based on the analysis of urine metabolites two hours after administration of a combination of two OTC drugs. Dextromethorphan (DEX) and flurbiprofen (FLB) are established probes for in vivo CYP2D6 and CYP2C9 activity, respectively. For CYP2D6, an improved version of our published method was applied, based on the metabolic ratio MRDEX/DOR, i.e., the ratio of the urinary concentrations of dextromethorphan (DEX) and its O-demethylated metabolite dextrorphan (DOR) after ingestion of 10 mg DEX. For CYP2C9, the metabolic ratio MRFLB/OHF was based on the concentrations of FLB and its 4-hydroxylated metabolite (OHF) after a low dose of FLB (8.75 mg in the form of a lozenge [Dobendan Direkt®]). Urine was treated with alkali to cleave glucuronides and a liquid chromatography / tandem mass spectrometry analysis of FLB and OHF was developed for optimal sensitivity and specificity, using deuterated internal standards. Experiments performed at weekly intervals with either the single drugs or the mixture indicated that the MRs were not affected by the combined administration. In a group of 112 volunteers, the MRDEX/DOR showed the well-known separation of about 10% poor and 90% extensive metabolizers (PM and EM, respectively), with MR-values ranging from 0.01 to 100. The MRFLB/OHF for CYP2C9 varied only 5-fold (0.43–2.17) among 111 participants. One subject showed a metabolic ratio of 10.4. This indicates that this person is a PM for CYP2C9, probably a carrier of at least one variant allele (*2 and /or *3). Blood samples obtained from most participants will be used for genotyping. This will allow testing our hypothesis that the FLB metabolic phenotype of the PM is due to its presumed CYP2C9 genotype. In future studies we will investigate the predictive ability of the genotype for the phenotype and the question to

what extent fingerprints of networks of candidate genes involved in polymorphic pharmacokinetics are represented by individual metabolic profiles.

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492

Interaction of drugs with the human organic cation transporter, hOCT2: Influence of their molecular structure

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Organic cation transporters (OCTs) provide an essential pathway for the uptake of cationic compounds in the liver and the kidney, the first step in their elimination from the organism. Although many drugs have been identified which interact with human OCT2, structural elements required for an interaction with OCT2 are not well defined. To address this issue, HEK293 cells stably expressing hOCT2 were generated. [3H]MPP+ uptake in these cells was inhibited to varying extents by a diverse set of 48 structurally unrelated drugs. A subset of 25 of these molecules was used to determine IC50 values for inhibition of [3H]MPP+ uptake and to correlate these with physicochemical descriptors such as molecular weight, logP, pKa, volume, solvent accessible surface area, and topological polar surface area (TPSA). The most potent inhibitors were imipramine, fenfluramine, doxepine, amitryptiline, chlorpromazine, ipratropium, clonidine, diphenhydramine, propafenone, and sibutramine. The IC50 values for the uptake of 10 µM [3H]MPP+ were 6.0, 9.7, 13.3, 14.2, 14.4, 14.6, 16.4, 20.5, 25.1, 29.1 µM, respectively. Moreover, we found a significant correlation between observed IC50 and TPSA values (r=0.66, p=0.0004). Structural alignment of these compounds was used to construct a two point pharmacophore which consists of an ion pair interaction feature and a hydrophobic aromatic feature at a distance of 4.95 Å. We then tested phenylmethylamine and three derivatives which differed only in the distance between the aromatic ring (Ar) and the amine nitrogen atom (N). Most potent inhibition of OCT2-mediated MPP+-uptake was achieved with 2-phenylethylamine and 3-phenylpropylamine with Ar-N distances of 5.17 and 6.36 Å, respectively. In contrast, those compounds with a shorter (3.80 Å) or a longer Ar-N distance (7.76 Å) were significantly less potent OCT2 inhibitors. In conclusion, the following descriptors may be used to predict whether a compound is a potent inhibitor of OCT2: (1) amines that have a positive charge at physiological pH, (2) TPSA value <50, (3) a hydrophobic feature which contains an aromatic group, (4) and an Ar-N distance of about 5 Å.

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493

Acetaminophen (paracetamol) is a selective cyclooxygenase-2 inhibitor in man

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Objective: For more than three decades, acetaminophen (INN, paracetamol) has been claimed to be devoid of significant inhibition of peripheral prostanoids. Attempts to explain its action by inhibition of a central cyclooxygenase (COX)-3 have been meanwhile rejected. The fact that acetaminophen is acting functionally as a selective COX-2 inhibitor led us to investigate the hypothesis whether it works via preferential COX-2 blockade. Methods: Ex vivo COX inhibition and pharmacokinetics of acetaminophen were assessed in five volunteers receiving single oral 1000-mg doses. Coagulation-induced thromboxane B2 and lipopolysaccharide-induced prostaglandin E2 were measured ex vivo and in vitro in human whole blood as indices of COX-1 and COX-2 activity. Results: In vitro, acetaminophen elicited a 4.4-fold selectivity towards COX-2 inhibition (IC50 = 113.7 µmol/L for COX-1; IC50 = 25.8 µmol/L for COX-2). Following oral administration of the drug, maximal ex vivo inhibitions were 56% (COX-1) and 83% (COX-2). Acetaminophen plasma concentrations remained above the in vitro IC50 for COX-2 for at least 5 h post-administration. Ex vivo IC50 values (COX-1: 105.2 µmol/L; COX-2: 26.3 µmol/L) of acetaminophen compared favorably with its in vitro IC50 values. Conclusions: In contrast to previous concepts, acetaminophen inhibited COX-2 by more than 80%, i.e. to a degree comparable to non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors. On the other hand, a >95% COX-1 blockade relevant for suppression of platelet function was not achieved. Our data may explain acetaminophen´s analgesic and anti-inflammatory action as well as its superior overall gastrointestinal safety profile compared with NSAIDs. In view of its substantial COX-2 inhibition, recently defined cardiovascular warnings for use of COX-2 inhibitors should also be considered for acetaminophen.

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494

The 50-mg dose of lumiracoxib is sufficient to elicit complete cyclooxygenase-2 inhibition in man

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Objective: In several countries the selective cyclooxygenase-2 (COX-2) inhibitor lumiracoxib is approved at doses of 200 mg (osteoarthritis) and 400 mg (acute pain), both of them exceeding the dose necessary to inhibit COX-2 at the time of maximal plasma concentration. The interest in lower effective doses has been renewed in view of recent concerns over hepatic toxicity that led to lumiracoxib´s withdrawal (Australia, Canada) or suspension of marketing and sale (Great Britain, Germany). Regrettably, a detailed analysis of dose-dependent effects of lumiracoxib under different clinical settings is still missing. To stimulate more research in this respect, the present study provides a comparative analysis addressing dose-dependent extent and duration of COX-2 blockade by the drug. Methods: Ex vivo COX-2 inhibition and pharmacokinetics were assessed in four volunteers receiving lumiracoxib at single oral doses of 50, 100 and 200 mg. Pharmacodynamic parameters (mean inhibition, area within the effect versus time curve) were assessed for different time frames (0.25 to 5, 8, 12 or 24 h post-administration). Results: Lumiracoxib at either dose completely suppressed COX-2 within 1 to 1.5 h. Maximal plasma concentrations were 29- (50 mg), 65- (100 mg) and 131-fold (200 mg) above the concentration causing half maximal COX-2 inhibition ex vivo (i.e., 0.14 µmol/L). Mean COX-2 inhibitions within the first 5 hours were 89% (50 mg), 94% (100 mg) and 97% (200 mg). Although there was a trend of dose-dependent COX-2 inhibition at later time points (8, 12 h), pharmacodynamic parameters showed no significant difference between either dose. In each group COX-2 activity had returned to pre-dose levels at 24 h. Conclusion: Lumiracoxib at 50 mg elicits a complete and sustained COX-2 inhibition that does not significantly differ from the effect of the two higher doses. In light of current discussions regarding the apparently COX-2-independent but reactive metabolite-derived hepatic toxicity of the drug, lumiracoxib doses less than 100 mg should be considered for pain therapy.

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495

Influences of levodopa on metabolism in patients with idiopathic Parkinson´s disease

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The substantial weight loss in Parkinson's patients may be related to direct influences of levodopa treatment on fat mobilization / oxidation. We studied 10 Parkinson's disease patients and examined adipose tissue and skeletal muscle metabolism directly with microdialysis. We monitored dialysate [ethanol], [glucose], [lactate], [pyruvate], and [glycerol] to assess tissue blood flow and metabolism before and after levodopa/benserazide intake. We also conducted in vitro studies on adipocytes from healthy women. Levodopa/benserazide increased serum levodopa, 3,4-dihydroxyphenylacetic acid (DOPAC), and norepinephrine (p<0.01). Serum, adipose tissue and skeletal muscle glycerol did not change or decreased. Adipose tissue glycerol was inversely correlated with serum levodopa concentrations (p<0.05). In isolated adipocytes, levodopa attenuated isoproterenol-induced glycerol release (p<0.05). Even though plasma norepinephrine increased substantially with levodopa/benserazide ingestion, adipose tissue lipolysis failed to increase. The phenomenon suggests antilipolytic actions of levodopa. We suggest that levodopa/benserazide does not induce fat wasting through direct influences on adipose tissue metabolism.

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496

Effects of ghrelin alone or co-administered with GHRH or CRH on sleep EEG and nocturnal hormone secretion

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The neuropeptide ghrelin promotes sleep, growth hormone (GH) and cortisol secretion in young normal male subjects. Similarly GH-releasing hormone (GHRH) increases slow-wave sleep, rapid-eye-movement sleep (REMS) and GH in young males, whereas it inhibits cortisol release. In contrast corticotropin-releasing hormone (CRH) decreases SWS, REMS and GH, whereas it stimulates GH [1]. In order to elucidate the interaction of ghrelin, GHRH and CRH in sleep regulation we performed a sleep-endocrine study in 10 young normal male subjects. Sleep electroencephalogram (EEG) and the nocturnal concentrations of GH and cortisol were investigated simultaneously in a randomized cross-over study. Subjects underwent 4 sessions in the sleep laboratory, separated by at least 1 week with administration of placebo (PL) + PL (A), 50 µg ghrelin + PL (B), 50 µg ghrelin + 50 µg GHRH (C), 50 µg ghrelin + 50 µg CRH (D) at 2200, 2300, 0000 and 0100 h. After all verum conditions NonREMS increased (mean±SEM: B: 355.3±7.4; C: 365.4±8.1; D: 371.4±3.9 min) significantly compared to PL (336.3±6.8 min). The most distinct NonREMS promoting effect was found after ghrelin + CRH as the time spent in NonREMS was significantly more than after A and B. Furthermore ghrelin + CRH decreased wakefulness compared to PL. REMS decreased significantly after C and D, and by trend after B. After D REM latency was decreased compared to the other treatments. CRH enhanced the ghrelin-induced cortisol concentration, but had no effect on GH. In turn, GHRH enhanced the ghrelin induced GH secretion without affecting cortisol. Our study corroborates the view that ghrelin promotes sleep in young male subjects. It comes as a surprise, however that the combined administration of ghrelin and cortisol increases distinctly NonREMS and shortens REM latency, since previous studies showed impairing effects of CRH on spontaneous in humans [2]. Recently we showed a sleep promoting effect of CRH during recovery sleep after sleep deprivation [3]. The shortened REM latency after ghrelin + CRH points to a REMS permissive effect. Similarly REMS is elevated in CRH overexpressing mice [4]. It appears that the effect of CRH on sleep is influenced by other hormones and by sleep propensity. References: [1] Steiger, J Psychiatr Res 41 (2007) 537-52, [2] Schüssler et al, Am J Physiol Endocrinol Metab 291 (2006) E 549 - 56, [3] Holsboer et al, Neuroendocriniology 48 (1988) 32-36, [4] Kimura et al, J Sleep Res 15 (Suppl 1)(2006) 101Supported by a grant from the Deutsche Forschungsgemeinschaft Ste 486/5-4.

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497

L-arginine improves erectile dysfunction in patients

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Background: Erectile dysfunction (ED) is a common medical disorder often affecting the aging male. Nitric oxide (NO) is a physiological signal essential to penile erection. NO synthase (NOS) catalyzes the production of NO from L-arginine. ADMA, a competetive inhibitor of NO synthase increases with age and many disorders that reduce NO in the erectile tissue are commonly associated with ED. Although new pharmacological strategies have been used for medical treatment of ED, patients often seek alternative therapies for cost or side effect reasons. Thus, we determined the efficacy of orally administered L-arginine on ED not caused by established organic disease. Patients and Methods: 57 male patients, median age 58 years, with erectile dysfunction were entered into a prospective randomized, double-blind, placebo-controlled, crossover study. After a four week run-in peroid each patient received 12 g/d of L-arginine as effervescent

tablets (2g each, 2 tablets t.i.d.) for 4 weeks followed by a 4-week wash-out period and a second 4-week treatment phase. Main criterion was the International Index of Erectile Function (IIEF- questionnaire). -L-arginine was measured in plasma by gas-chromatography-mass-spectrometry. Results: L-arginine plasma levels increased significantly in the verum phase. ED improved significantly by IIEF. In particular there was significant improvement in erectile function, intercourse satisfaction and overall satisfaction. On questioning, there were no side effects reported. Conclusions: Oral l-arginine leads to a significant improvement in patients with erectile dysfunction.
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498

Effect of exercise on cardiovascular parameters, blood nitrate/nitrite levels and blood parameters indicating oxidative stress/antioxidant capacity: impact of training and endothelial nitric oxide synthase genotype in healthy male volunteers
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Nitric oxide (NO) is produced by endothelial cells and platelets via the endothelial NO synthase (eNOS) and plays an important role in the regulation of the cardiovascular system. The influence of eNOS gene polymorphisms on blood NO concentrations and on cardiovascular parameters, however, is still under debate. Thus, the aim of the present study was to investigate the impact of a 12-week moderate training and of three eNOS polymorphisms on exercise-induced changes in cardiovascular parameters, plasma nitrate/nitrite (NOx) levels and on parameters indicating oxidative stress/antioxidant capacity in a total of 71 healthy non-smoking male volunteers. The volunteers were stratified into three groups: (1) 24 young control subjects, aged 20-30 years; (2) 22 elderly control subjects, aged 40-60 years; (3) 25 elderly subjects, aged 40-60 years, receiving a 12-week moderate training. At the beginning of the experiment as well as after 12 weeks (with/without training) subjects had to perform a 1h-exercise at 80% of their individual anaerobic threshold (IAT) (young subjects at 100% of their IAT at the second session). 1h-exercise caused an elevation in plasma NOx concentrations and a decrease in the blood values of reduced glutathione with all groups of volunteers at both sessions. Plasma ascorbic acid concentrations were significantly diminished at the first session only. No exercise influence was seen on plasma uric acid concentrations, on trolox equivalent antioxidant capacity or ferric reducing ability of plasma and on blood levels of lipid peroxidation products. There was, however, a significant association between lipid peroxidation product and reduced or oxidized glutathione values. A 12-week moderate training displayed some influence on cardiovascular parameters. It did not affect basal values and exercise-induced changes of plasma NOx-concentrations and of blood/plasma parameters indicating oxidative stress/antioxidant capacity. There was no consistent impact of the polymorphisms in the promoter region (T-786C), in exon 7 (Glu298Asp) or in intron 4 (VNTR) of the eNOS gene both on basal values and on exercise-induced changes of all the parameters investigated.
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499

Functional characterization on the importance of lysine- and arginin-residues in the uptake transporter OATP1B3
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The importance of the hepatic uptake transporter OATP1B3 (organic anion transporting polypeptide) encoded by the SLCO1B3 gene for the disposition and elimination of drugs and endogenous compounds is well recognized. However, detailed insights regarding the structure, transport mechanisms and catalytic amino acids are still rare. Therefore, we investigated the role of several conserved Lys and Arg residues in OATP1B3 for the transport activity of the substrate sulfobromophthalein (BSP). First, Lys28 and Arg580 were mutated to Lys28>Gly (A82>G, A83>G), Lys28>Arg (A82>C, A83>G) and Arg580>Gly (A1738>G) and Arg580>Lys (G1739>A), respectively. These cDNAs were used for transient transfection of HEK293 cells in order to investigate the role of these Lys and Arg residues for protein expression, localization and transport activity using immunofluorescence, Western blot and cellular uptake assays. The Western blot analysis of all mutants showed at most a reduction in protein amount of ~25 %. Interestingly, as demonstrated by immuno-fluorescence, the changes of Lys28 to Arg and Gly as well the changes of Arg580 to Gly and Lys did not lead to an altered membrane localization of the mutated proteins. However, in contrast to the Lys28 mutants, Arg580>Gly and Arg580>Lys showed a significant decrease of the uptake of BSP indicating that this conserved amino acid might play an important role for the transport mechanism of OATP1B3 and other OATPs. Taken together, the significant reduction of the uptake activity could not be explained by altered membrane localization and the reduced protein expression of the mutants only contributes in part to the observed reduced transporter activity of the Arg580 mutants. Furthermore, these data indicate that the Lys28 residue in OATP1B3 is of minor importance for substrate recognition and transport of organic anions. In contrast, Arg residues might be important for the transport activity of OATP1B3.
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500

Osteopontin gene variation and cardio/cerebrovascular disease phenotypes
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Osteopontin (OPN) is a matrix glycoprotein which is involved in cardio- and cerebrovascular disease pathophysiology. We scanned the OPN gene in 190 chromosomes from myocardial infarction (MI) patients and identified five variants in the 5'-flanking region (G-655T, G-616T, T-443C, DelG-156InsG, T-66G), three synonymous variants in exon 6 (D94D [T/C], D147D (T/C)) and exon 7 (A250A [T/C]), and one non-synonymous variant in exon 6 (E122K [G/A]). All variants were investigated in case-control studies for MI (ECTIM: 990 cases and 900 controls) and brain infarction (BI) (GÉNIC: 466 cases and 444 controls).In both the ECTIM and GENIC studies, the D147D C allele was independently and significantly associated with lower apoB levels (P=0.044 and P=0.03, respectively). The D147D C allele frequency was significantly lower in BI cases compared to controls (OR [95% CI] 0.39 [0.20-0.74], P=0.004), and C allele carriers had a significantly lower frequency of carotid plaques (P=0.02). Band shift assays in HepG2 did not reveal any functionality of promoter variants, whereas western blot analyses in OPN-negative HEK293T showed a slightly decreased expression of the pOPN-441C carrying construct, which would comply with its protective effect on the phenotypes studied.In the present analysis, we show that a portion of the OPN locus is likely to associate with cardio-/cerebrovascular disease-related phenotypes. However, further experiments are warranted to clarify the functional role of OPN variants.
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501

Insulin-like effects after water drinking
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Recently, we found that after drinking 500 ml water, energy expenditure increased promptly by about 30% in both normal and overweight men and women. This effect mediated by an increase in sympathetic nervous system activity and activation of osmoregulatory processes. Possibly, skeletal muscle contributes to this thermogenic response to water. Therefore, we tested the hypothesis that water-induced thermogenesis is associated with a stimulation of aerobic glucose metabolism in muscle and this to a greater extent in non-smokers vs. smokers. Sixteen young healthy women (8 smokers) were studied. After an overnight fast (12h), a microdialysis probe was implanted into the right M. quadriceps femoris vastus lateralis and subsequently perfused with Ringer's solution (+50 mM ethanol). After 1h, volunteers were asked to drink 500 ml Water (22°C) followed by continuing microdialysis for another 90 min. Dialysates (15 min fractions) were analyzed for [ethanol], [glucose], [lactate], [pyruvate], and [glycerol] in order to assess changes in muscle tissue perfusion (ethanol dilution technique), glycolysis and lipolysis. At baseline, tissue perfusion and dialysate [glucose] were lower, whereas dialysate [lactate] / [pyruvate] ratio was higher in smokers vs. non-smokers. After water drinking, tissue perfusion decreased slightly in both groups whereas dialysate [glucose] decreased in non-smokers but not in smokers. There were also increases in dialysate [lactate] (non-smokers < smokers) and [pyruvate] (non-smokers > smokers). Dialysate [lactate] / [pyruvate] ratio was always higher in smokers vs. non-smokers both at baseline and after water drinking. Dialysate [glycerol] did not differ at baseline but decreased significantly after water drinking in non-smokers but not in smokers. Therefore, drinking of 500 ml water stimulates aerobic glucose metabolism and inhibits lipolysis in skeletal muscle and this to a greater extent in non-smoking than smoking women. These effects are similar to the effects of insulin. Therefore, this insulin-permissive of water drinking could possibly help to improve insulin sensitivity in patients with disturbed glucose tolerance or diabetes mellitus.
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502

In-depth investigation of the functional relevance of missense variants of ICAM-1
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Objective: Non-synonymous variants of intercellular adhesion molecule-1 (ICAM-1) are inconsistently associated with cardiovascular disease. Insight into the molecular functional relevance of these variants is still lacking. Methods and Results: After scanning 190 alleles of MI patients from the ECTIM Study, we identified eight coding variants, three in exon 4 (G241R [rs 1799969], R258K, Y308F), 2 in exon 5 (I316V, P352L [rs1801714]), 2 in exon 6 (T408T, K469E [rs 5498]) and 1 in exon 7 (R478W [rs5030400]). For molecular analyses, G241→R, I316→V, P352→L, K469→E, and R478→W were introduced as single or double variants into ICAM-1 cDNA by site-directed mutagenesis in the expression vector pcDNA3.1. To evaluate their impact at both the transcriptional and translational level, CV1 cells were transiently transfected. We quantified the amount of ICAM-1 mRNA by RT-PCR and expression at the protein level by PAGE and immunoblot, but no differences were detected. ELISA measurements of cell surface expression exclude an influence of amino-acid exchanges on ICAM-1 synthesis and translocation. The process of ICAM-1 dimerization was also not altered for the variants investigated as observed by chemical cross-linking experiments. Pulse-chase experiments to evaluate the variants' impact on ICAM-1 turnover revealed that turnover was influenced, leading to altered degradation rates compared to wild-type. In variants R478→W and K469→E an up to 30% prolonged half-life was observed, whereas in G241→R, P352→L and in the double variant P352→L/K469→E a higher degradation rate was estimated. The altered degradation rate could not be attributed to an increased release of membrane-bound ICAM-1 into the medium. Yet, the observed differences were not connected with a reduced expression level of ICAM-1 under steady-state conditions, as revealed by western blot and

metabolic labeling experiments.Conclusions: Our molecular biological analysis does not support a major molecular biological functional role of ICAM-1 missense variants, either in single or double mutant combination.
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503

Differential effects of FIIa inhibitors on the endogenous thrombin potential
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Blood coagulation takes place in three overlapping stages: (i) initiation on a tissue factor bearing cell, (ii) amplification, in which platelets and cofactors are activated to set the stage for large scale thrombin generation, and (iii) propagation, in which large amounts of thrombin are generated. Based on mathematical modeling (Nagashima, J Biol Chem 2002;277:50439-50444), it has been proposed that FXa inhibitors delay the initiation of coagulation, thus resulting in a prolongation of the lag time in thrombin generation assays. According to this hypothesis, FIIa inhibitors should not affect the lag time but inhibit the peak concentrations of the generated thrombin.This study compared the concentration-dependent effects of different FIIa inhibitors (melagatran, argatroban, lepirudin, bivalirudin) as compared to FXa inhibitors (fondaparinux, DX-9065a) on the endogenous thrombin potential (ETP, Hemker et al., Pathophysiol Haemost Thromb 2003;32:249-253). In accordance with the theoretical prediction, FXa inhibitors concentration-dependently prolonged the lag time of thrombin generation, while the monovalent FIIa inhibitors (melagatran, argatroban) reduced peak thrombin concentrations without affecting the lag time. However, both bivalent FIIa inhibitors (lepirudin, bivalirudin) very markedly prolonged the lag time of thrombin generation and, thus, behaved like “super” FXa inhibitors.It is concluded that the anticoagulatory effects of bivalent FIIa inhibitors cannot be simulated by this mathematical model working for monovalent FIIa inhibitors. This has important consequences for monitoring lepirudin or bivalirudin effects using the ETP.
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504

Correlation between the severity of coronary artery disease and the plasma levels of the soluble receptor of advanced glycation end products
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Receptor of advanced glycation end-products is a multi-ligand cell surface receptor whose signaling pathway has been implicated in atherosclerosis. Aim of our study was to investigate the association between plasma levels of soluble receptor of advanced glycation end products (sRAGE) and established cardiovascular risk factors in coronary artery disease (CAD) patients, with or without diabetes mellitus (DM) and also to determine the relationship between the sRAGE levels and the severity of CAD, according to the number of stenosed vessels. Forty eight patients (mean age 63.2±9.4, 39 males) who underwent coronary angiography were enrolled in the study. Angiography indications were either suspicion or evaluation of known CAD. CAD was defined as more than 70% luminal narrowing in at least one major coronary artery. The study population was divided into CAD patients having DM or impaired fasting glucose (Group 1, n=19) and CAD patients with normal fasting glucose levels (Group 2, n=29). Plasma sRAGE levels were determined using a commercially available enzyme-linked immunosorbent assay kit according to the manufacturer’s protocol. Group 1 had significantly higher levels of sRAGE than Group 2 (1294.9 pg/dl vs 1059 pg/dl, p=0.04). CAD patients with three-vessel disease had significantly higher levels of sRAGE than CAD patients with single or two-vessel disease (1352.8±307.9 pg/dl vs 1056.5±391.5 pg/dl, p=0.01). Plasma concentrations of sRAGE were inversely correlated with total cholesterol (r = -0.33, p=0.02) and LDL (r = -0.51, p<0.00001). CAD patients with DM or impaired fasting glucose had elevated sRAGE levels. Furthermore, sRAGE correlate positively with the severity of CAD. Thus, sRAGE may prove to be an important molecule involved in coronary atherosclerosis.
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505

Improvement of drug safety and dose optimization by the computerized physician order entry (CPOE) system TheraOpt®: A pilot study on an internal medicine ward
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Aims: Does TheraOpt® as a CPOE system help to prevent medication errors (overdosage/underdosage, drug interactions, allergies) on an internal medicine ward? Methods: We compared all medical drug orders on an internal medicine ward given routinely with the same orders entered by using TheraOpt®. 123 consecutive patients admitted during a period of 3 months were followed and documented. All drug interactions and allergies were analysed, categorized according to severity and stored. All dosages prescribed were compared to the recommended dosages by TheraOpt®. The CPOE system calculates the dosages based on the following individual patient characteristics: age, gender, height, weight, liver and kidney function. Results: During the study period 1811 orders (14,7 orders per patient) were observed. The average incidence of drug interactions was 3,9 per patient. Of all 474 detected drug interactions, 8 were classified as severe drug interactions, 135 as medium-severity and 321 of minor importance. For 10 drug interactions no classification was given due to lack of published information in the scientific literature. 9 drugs had been ordered even though the patients had known allergies to these drugs. Therefore, 12 of 123 patients were at high risk (severe drug-drug interaction or allergy).For 995 orders TheraOpt® made dosage calculations. 43% of the calculated dosages were equal to the actually prescribed dosages. 23% of the prescribed dosages were more than 50% higher than the calculated dosages, 19% were more than 50% lower. These excessively high dosages were to the largest part caused by the absence of dosage adjustments for age, weight and kidney function. Conclusions: The risks of drug therapy can be remarkably reduced by use of a CPOE system: In comparison to the current practical experience, such software can help to better recognize and prevent overdosage/underdosage, allergies, cross sensitivities and drug interactions.
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