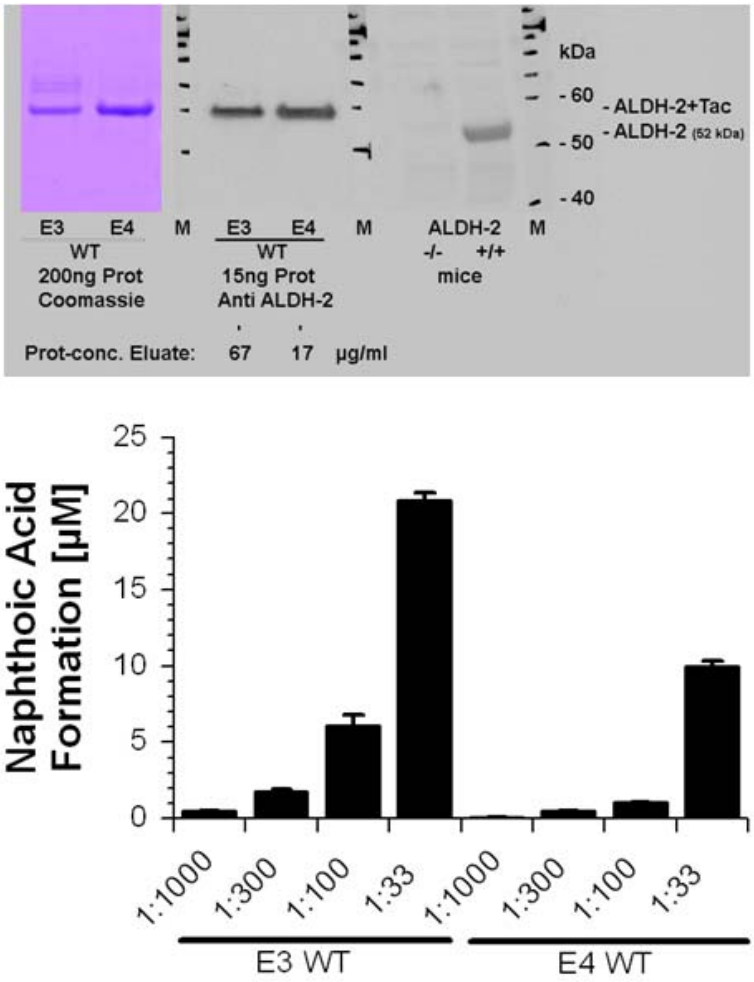
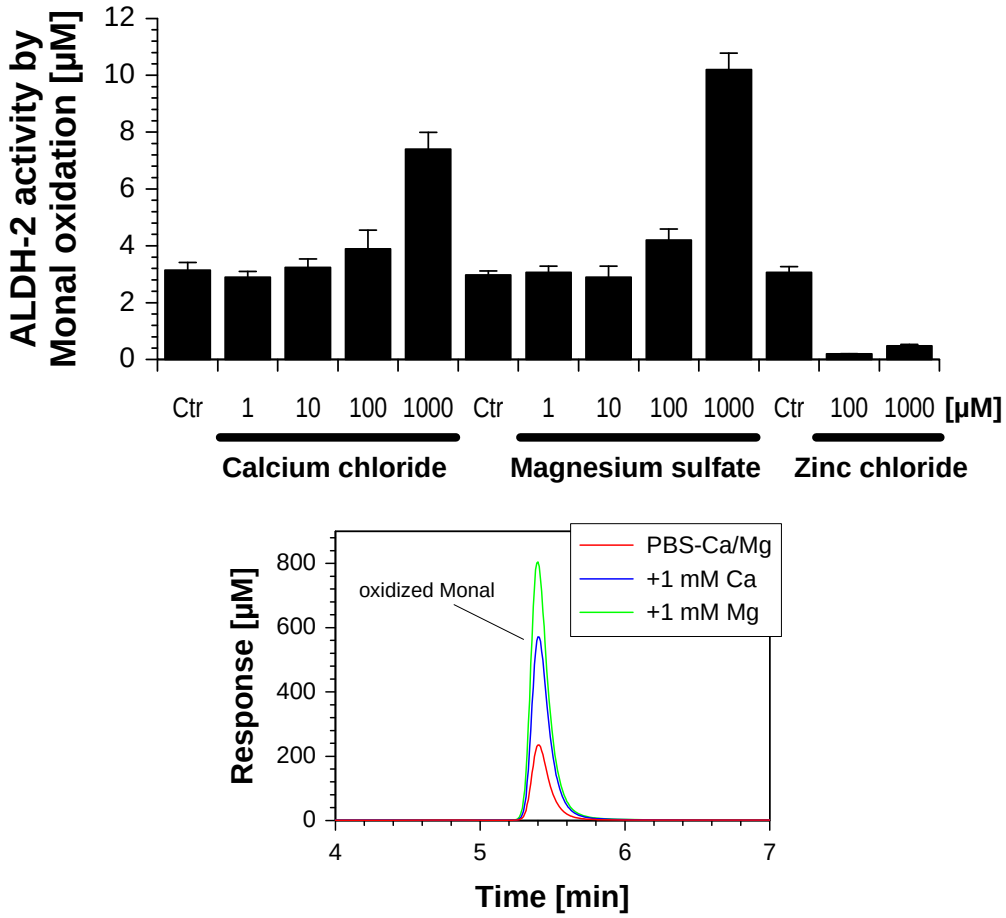
ONLINE SUPPLEMENT

REGULATION OF HUMAN ALDEHYDE DEHYDROGENASE (ALDH-2) ACTIVITY BY ELECTROPHILES IN VITRO

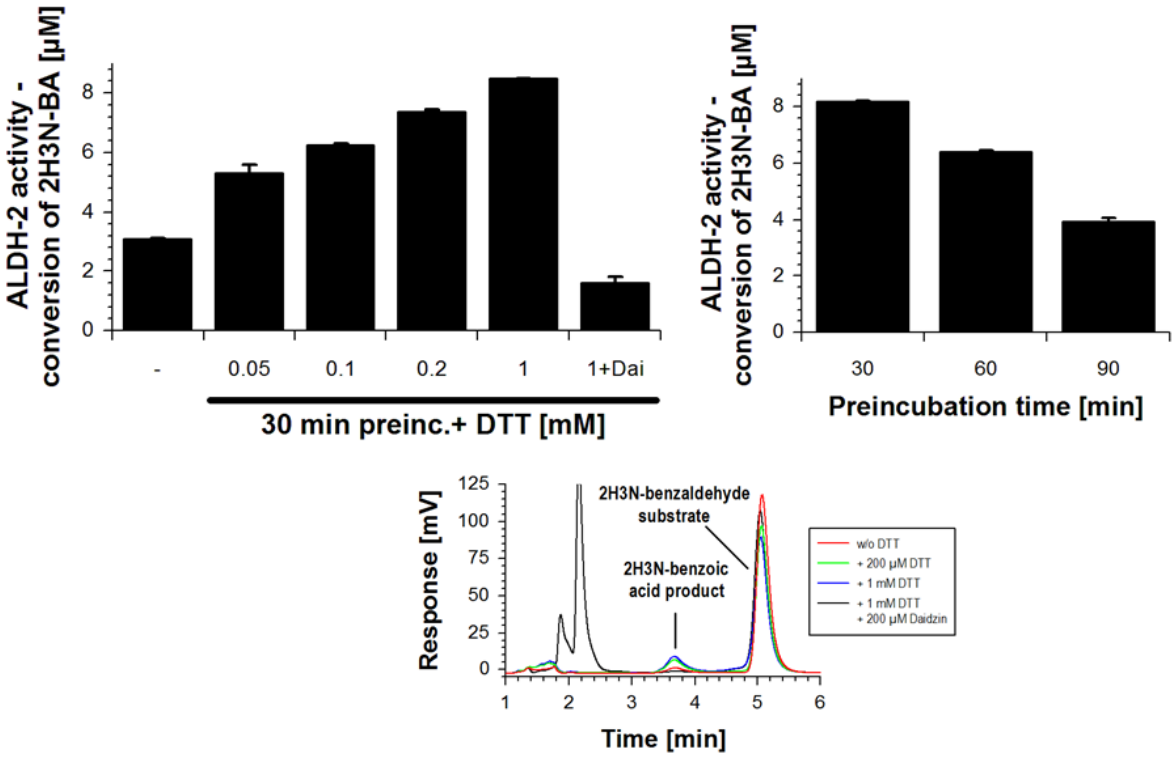
Matthias Oelze[1$], Maike Knorr[1$], Richard Schell[1], Jens Kamuf[l], Andrea Pautz[2], Julia Art[2], Philip Wenzel[1], Thomas Münzel[1], Hartmut Kleinert[2] and Andreas Daiber[1¶]



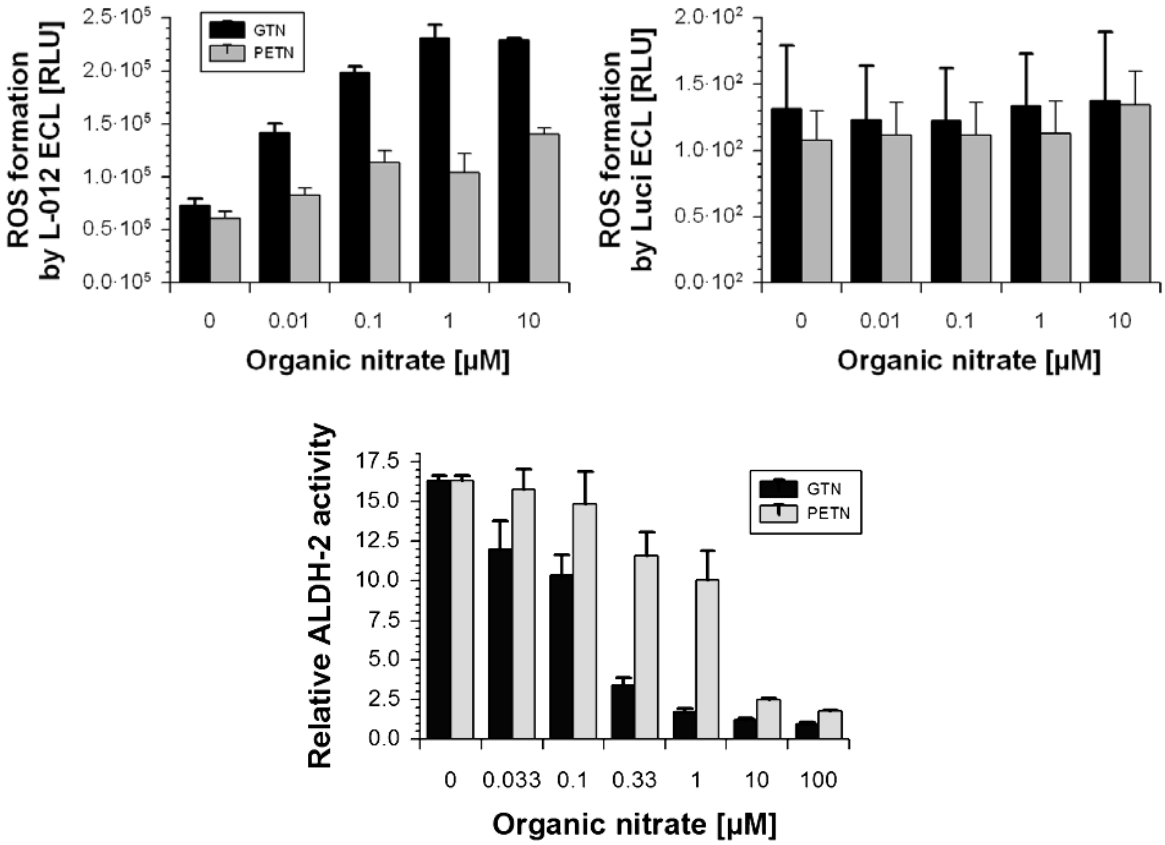
Supplemental Fig. S1. Characterization of ALDH-2 purity and activity in eluates E3 and E4. Upper panel: Coomassie staining upon SDS-PAGE and subsequent Western blot using a specific antibody against ALDH-2. For Western blot procedure and antibody incubations see (1). A rabbit polyclonal antibody to ALDH-2 was used (dilution 1:2,500; kindly provided from K.K. Ho and H. Weiner, Purdue University, West Lafayette, Indiana (2)). Total protein content was determined by the Quick Start Bradford assay (Biorad, Hercules, CA) and purity was estimated from Coomassie-stained gels. It should be noted that equal protein amounts were loaded to the gel and E4 (despite lower total protein content) contained ALDH-2 at a higher purity than E3 (82 vs. 52 %). **Lower panel:** Activity measurements in E3 and E4 revealed that eluate E3 contained at least 2-fold higher ALDH-2 activity as compared to E4.



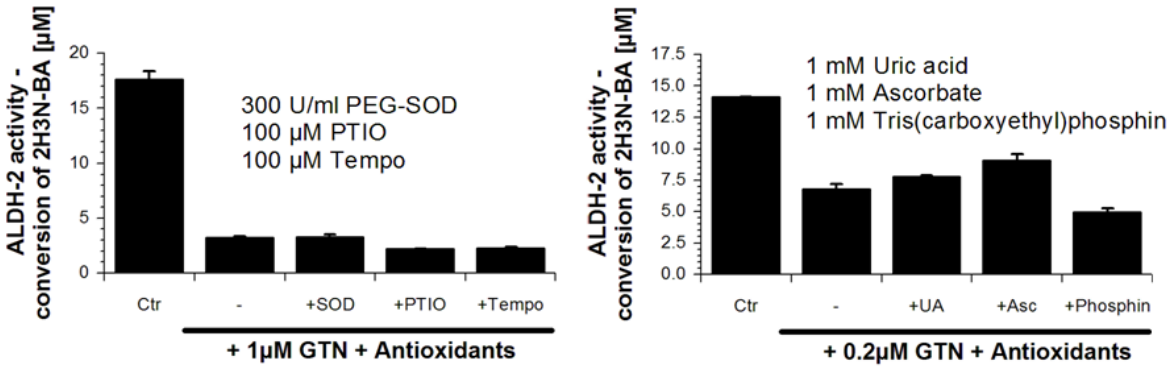
Supplemental Fig. S2. Effect of Ca^{2+}, Mg^{2+} and Zn^{2+} ions on ALDH-2 activity during longer incubation times. Upper panel: ALDH-2 (0.94 µg/ml) was incubated with the indicated concentrations of $CaCl_2$, $MgSO_4$ or $ZnCl_2$ for 45 min at 37 °C in PBS (w/o Mg^{2+}/Ca^{2+}) containing NAD^+ (200 µM) and DTT (400 µM). Activity was measured upon addition of 20 µM Monal by its conversion to naphthoic acid product using HPLC analysis (3). Calcium and magnesium ions increased the ALDH-2 activity at higher concentrations, whereas zinc ions completely abolished naphthoic acid formation. **Lower panel**: Representative chromatograms for ALDH-2 incubations in the absence and presence of Mg^{2+} or Ca^{2+} (conditions as described above).



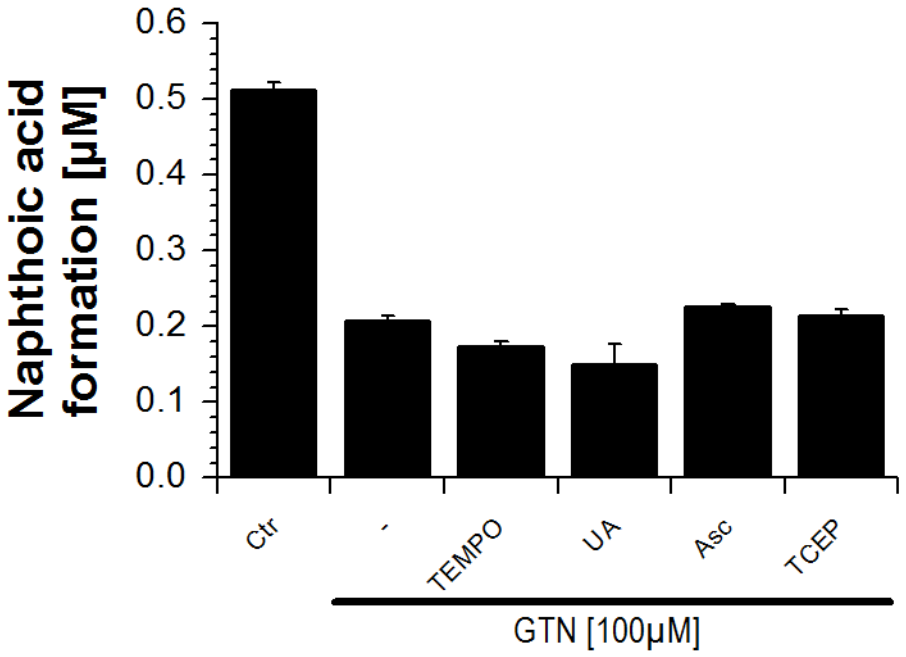
Supplemental Fig. S3. Effect of the dithiol DTT on ALDH-2 activity during longer incubation times. Left upper panel: ALDH-2 (0.11 µg/ml) was preincubated with the indicated DTT concentrations for 30 min at 37 °C in PBS (w/o Mg^{2+}/Ca^{2+}) containing 1 mM NAD^+ and activity was measured upon addition of 100 µM 2-hydroxy-3-nitrobenzaldehyde (2H3N-BA) and incubation for another 30 min by its conversion to 2-hydroxy-3-nitrobenzoic acid using HPLC analysis (4). The specific ALDH-2 inhibitor daidzin even suppressed ALDH-2 activity in the presence of the highest DTT concentration. **Right upper panel**: ALDH-2 (0.11 µg/ml) was preincubated for 30, 60 or 90 min at 37 °C in PBS (w/o Mg^{2+}/Ca^{2+}) containing 200 µM DTT and 1 mM NAD^+. The activity was measured upon addition of 100 µM 2H3N-BA and incubation for another 30 min by conversion to its benzoic acid product. **Lower panel**: Representative chromatograms for ALDH-2 incubations in the absence and presence of DTT and daidzin (conditions as described above).



Supplemental Fig. S4. Peroxynitrite formation from purified ALDH-2 with increasing concentrations of organic nitrates and their effect on ALDH-2 activity. Upper panels: ALDH-2 (1.3 µg/ml) was incubated for 5 min with NAD^+ (100 µM) and either L-012 (100 µM, **left panel**) or lucigenin (100 µM, **right panel**) in PBS (Dulbeccos phosphate-buffered saline, w/o DTT, Ca^{2+}/Mg^{2+}) at room temperature. GTN or PETN were added at increasing concentrations. RONS formation was detected over 5 min and expressed as counts/min (RLU) at 5 min. **Lower panel:** ALDH-2 (0.22 µg/ml) was preincubated with 200 µM DTT and the indicated concentrations of the organic nitrates for 30 min at 37 °C in PBS (w/o Mg^{2+}/Ca^{2+}) containing 1 mM NAD^+. Activity was measured upon addition of 100 µM 2-hydroxy-3-nitrobenzaldehyde (2H3N-BA) and incubation for another 30 min by its conversion to 2-hydroxy-3-nitrobenzoic acid using HPLC analysis (4). Data are mean ± SEM of 3 independent experiments.



Supplemental Fig. S5. Effect of nitroglycerin on ALDH-2 activity during longer incubation times and rescue by antioxidants. ALDH-2 (0.22 µg/ml) was preincubated with 200 µM DTT and GTN (0.2 or 1 µM) in the presence of antioxidants as indicated for 30 min at 37 °C in PBS (w/o Mg^{2+}/Ca^{2+}) containing 1 mM NAD^{+}. Activity was measured upon addition of 100 µM 2-hydroxy-3-nitrobenzaldehyde (2H3N-BA) and incubation for another 30 min by its conversion to 2-hydroxy-3-nitrobenzoic acid using HPLC analysis (4).



Supplemental Fig. S6. Effect of GTN on ALDH-2 activity during short incubation times and rescue by antioxidants. ALDH-2 (0.04 µg/ml) was preincubated with GTN (100 µM) for 45 min at 37 °C in PBS (with 1 mM Mg^{2+}/Ca^{2+}) containing 0.1 mM NAD^{+}, 200 µM DTT and 20 µM Monal 62. Effects of the antioxidants TEMPO (100 µM), uric acid (UA, 1 mM), ascorbate (Asc, 1 mM) and tris(carboxyethyl)phosphine (TCEP, 1 mM) on ALDH-2 activity was determined by formation of the fluorescent naphthoic acid product using HPLC analysis (3).

EXTENDED EXPERIMENTAL PROCEDURES

Chemicals– GTN was used from a Nitrolingual infusion solution (1 mg/ml) from G. Pohl-Boskamp (Hohenlockstedt, Germany) and PETN from a solution of moistured PETN (30 w/w% water) from Dottikon Exclusive Synthesis AG (Dottikon, Switzerland) in DMSO. Ethanolic stocks (10 mM) of 15-deoxy-Δ12,14-prostaglandin J_2 (dDPGJ_2), 10-nitrooleate (10NOA) and 9-nitrooleate (9NOA) were purchased from Cayman Europe (Tallinn, Estonia). 6-Methoxy-2-naphthaldehyde (Monal 62, 98%), dihydroethidine (DHE), malondialdehyde enolate (MDA), 4-hydroxynonenal (4HNE), polyethylene-glycolated superoxide dismutase (PEG-SOD), thioredoxin (Trx, E.coli) and thioredoxin reductase (TrxR, E.coli) were obtained from Sigma-Aldrich (Steinheim, Germany). Trx (human) and TrxR (rat) were obtained from IMCO corporation (Stockholm, Sweden). Restriction enzymes and calf intestine alkaline phosphatase were purchased from NEB (Frankfurt, Germany). The Sequenase Version 2.0 DNA Sequencing Kit was purchased from GE Healthcare (Freiburg, Germany), the Ni-NTA-Agarose from Qiagen (Hilden, Germany) and the procaryotic expression pET16B vector for the expression of His-tagged proteins from Merck/Novagen (Schwalbach, Germany). Free MDA was prepared by hydrolysis of the enolate in hydrochloric acid and the pH was neutralized prior to usage by equimolar amounts of sodium hydroxide. The luminol analogue L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4-(2H,3H)dione sodium salt) was from Wako Pure Chemicals (Osaka, Japan) and daidzin (7-glucoside of 40,7-dihydroxy-isoflavone) from Indofine Chemical Company Inc. (Hillsborough, NJ, USA). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich, Fluka, or Merck.

Overexpression and purification of ALDH-2- Recombinant human wildtype ALDH-2 was overexpressed in E. coli using the plasmid pET16B-hALDH2_wt. To generate pET16B-hALDH2_wt, the bacterial expression plasmid pT7-7-hALDH2.1 (coding for a His-tagged human wildtype ALDH-2 protein; His-hALDH2_wt; kindly provided by K.K. Ho and H. Weiner, Purdue University, West Lafayette, USA; (5)) was digested with *Nde*I and *Hin*d III, the fragment containing the His-ALDH2_wt coding region was isolated and ligated into pET16B restricted with *Nde* I and *Hin*d III, treated with CIAP and purified. The DNA sequence of the resulting construct pET16B-hALDH2_wt was controlled using the Sequenase Version 2.0 DNA Sequencing Kit. For purification of His-tagged human wildtype ALDH2 the plasmid pET16B-hALDH2_wt were transformed into Escherichia coli strain BL21 (DE3) and the protein was purified using Ni-NTA Agarose from Qiagen (Hilden, Germany) as described by the manufacturer. The yield of the purification procedure was estimated by comparison to a BSA standard on Coomassie blue-stained SDS-PAGE.

Characterization of purified ALDH-2- The purity of the wildtype His-ALDH-2 from different eluates was tested by SDS-PAGE with Coomassie staining and Western blotting using a specific ALDH-2 antibody (kindly provided by K.K. Ho and H. Weiner, Purdue University, West Lafayette, USA). As a positive control aortic protein from ALDH-$2^{+/+}$ mice was used and the negative control was a protein sample from ALDH-$2^{-/-}$ (an example is shown in supplemental figure S1 along with the conditions for Western blotting). An HPLC-based activity assay was used for determination of ALDH-2 activity in different eluates (see supplemental **Fig. S1**).

Determination of ALDH-2 activity- All ALDH-2 activity measurements were done at steady state rates. Conversion of 2-hydroxy-3-nitrobenzaldehyde to its benzoic acid product was followed by HPLC-based analysis by its absorbance at 340 nm as previously described (4). The oxidation of 6-methoxy-2-naphthylaldehyde (Monal 62) to the

fluorescent naphthoic acid product (3) was traced by HPLC analysis. The system consisted of a control unit, two pumps, mixer, detectors, column oven, degasser, and an autosampler (AS-2057 plus) from Jasco (Groß-Umstadt, Germany) and a C_{18}-Nucleosil 100-3 (125×4) column from Macherey & Nagel (Düren, Germany). A high pressure gradient was employed with acetonitrile/water (90/10 v/v%) and 25 mM citrate buffer, pH 2.2, as mobile phases with the following percentages of the organic solvent: 0 min, 40%; 8 min, 60%; 8–10 min, 100%; 11 min, 40%. The flow was 1 ml/min and naphthoic acid was detected by fluorescence (Ex. 310 nm/Em. 360 nm). A 6-methoxy-2-naphtoic acid standard was generated by incubation of 6-methoxy-2-naphthylaldehyde (20 µM) with 10 U/ml yeast-ALDH (Roche Diagnostics, Mannheim, Germany), 100 µM NAD^+ and 200 µM DTT in PBS for 120 min at 37 °C. The complete conversion of the substrate to the product was confirmed by HPLC analysis.

EXTENDED DISCUSSION

Mechanism of action of magnesium an calcium ions – Based on a report by Weiner and Takahashi (6), calcium and magnesium ions activated mitochondrial aldehyde dehydrogenases from different species, whereas the cytosolic isoforms were inhibited. According to a report by Bedino and Testore (7), the tetrameric ALDH-2 enzyme is in equilibrium between two conformational states R and T which display comparable affinities for capronaldehyde (the dissociation constants are 0.17 and 0.3 microM, respectively), but different catalytic power (VT = 2VR). The T state can bind with lower affinity a second molecule of aldehyde (K = 2.5 microM). Mg^{2+} stabilizes the T state (the dissociation constants for the R and T states are 2.2 and 0.12 mM, respectively) and acts as a strong activator of the R state, but as a weak inhibitor of the T state.

Clinical implications - Recent data revealed that ALDH-2/PKC_ε conferred anti-ischemic protection involves Hsp90-based import mechanisms and activation of the A2b/A3 receptor (8,9). The cardioprotective role of ALDH-2 is well known from studies in knockout mice (10) and supported by recent data (11,12). Moreover, these data are in good accordance with our previous observations that ALDH-2 deficiency renders mice more susceptible to exogenously triggered oxidative stress. ALDH-$2^{-/-}$ mice were more susceptible to age-related vascular disorders as well as nitroglycerin, acetaldehyde and doxorubicin induced cardiovascular damage (13,14). In their recent study, Ma et al. showed that ALDH-$2^{-/-}$ mice display a larger infarct area in response to MI, which was significantly improved by ALDH-2 overexpression (11). But also cultured cells displayed increased oxidative damage in the absence of ALDH-2 (15). These findings might be of great clinical importance since mitochondria obviously play an important role in the development and progression of cardiovascular disease (16) and even could act as an amplifier of cellular oxidative stress triggering vicious circles by a crosstalk between mitochondrial RONS and NADPH oxidase-derived ROS (17).

EXTENDED REFERENCES

1. Hink, U., Daiber, A., Kayhan, N., Trischler, J., Kraatz, C., Oelze, M., Mollnau, H., Wenzel, P., Vahl, C. F., Ho, K. K., Weiner, H., and Munzel, T. (2007) *J Am Coll Cardiol* **50**, 2226-2232
2. McMichael, M., Hellstrom-Lindahl, E., and Weiner, H. (1986) *Alcohol Clin Exp Res* **10**, 323-329
3. Beretta, M., Sottler, A., Schmidt, K., Mayer, B., and Gorren, A. C. (2008) *J Biol Chem* **283**, 30735-30744
4. Wenzel, P., Hink, U., Oelze, M., Seeling, A., Isse, T., Bruns, K., Steinhoff, L., Brandt, M., Kleschyov, A. L., Schulz, E., Lange, K., Weiner, H., Lehmann, J., Lackner, K. J., Kawamoto, T., Munzel, T., and Daiber, A. (2007) *Brit J Pharmacol* **150**, 526-533
5. Xiao, Q., Weiner, H., Johnston, T., and Crabb, D. W. (1995) *J Clin Invest* **96**, 2180-2186
6. Weiner, H., and Takahashi, K. (1983) *Pharmacol Biochem Behav* **18 Suppl 1**, 109-112
7. Bedino, S., and Testore, G. (1992) *Int J Biochem* **24**, 1697-1704
8. Budas, G. R., Churchill, E. N., Disatnik, M. H., Sun, L., and Mochly-Rosen, D. (2010) *Cardiovasc Res*
9. Koda, K., Salazar-Rodriguez, M., Corti, F., Chan, N. Y., Estephan, R., Silver, R. B., Mochly-Rosen, D., and Levi, R. (2010) *Circulation*
10. Ren, J. (2007) *Novartis Found Symp* **285**, 69-76; discussion 76-69, 198-199
11. Ma, H., Guo, R., Yu, L., Zhang, Y., and Ren, J. (2010) *Eur Heart J* **doi: 10.1093/eurheartj/ehq253**
12. Budas, G. R., Disatnik, M. H., Chen, C. H., and Mochly-Rosen, D. (2010) *J Mol Cell Cardiol* **48**, 757-764
13. Wenzel, P., Schuhmacher, S., Kienhofer, J., Muller, J., Hortmann, M., Oelze, M., Schulz, E., Treiber, N., Kawamoto, T., Scharffetter-Kochanek, K., Munzel, T., Burkle, A., Bachschmid, M. M., and Daiber, A. (2008) *Cardiovasc Res* **80**, 280-289
14. Wenzel, P., Muller, J., Zurmeyer, S., Schuhmacher, S., Schulz, E., Oelze, M., Pautz, A., Kawamoto, T., Wojnowski, L., Kleinert, H., Munzel, T., and Daiber, A. (2008) *Biochem Biophys Res Commun* **367**, 137-143
15. Szocs, K., Lassegue, B., Wenzel, P., Wendt, M., Daiber, A., Oelze, M., Meinertz, T., Munzel, T., and Baldus, S. (2007) *J Mol Cell Cardiol* **42**, 1111-1118
16. Di Lisa, F., and Bernardi, P. (2006) *Cardiovasc Res* **70**, 191-199
17. Daiber, A. (2010) *Biochim Biophys Acta*