Supplementary materials

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Oxidative stress-induced activation of Abl and Src kinases rapidly induces P-glycoprotein internalization via phosphorylation of caveolin-1 on tyrosine-14, decreasing cortisol efflux at the blood-brain barrier

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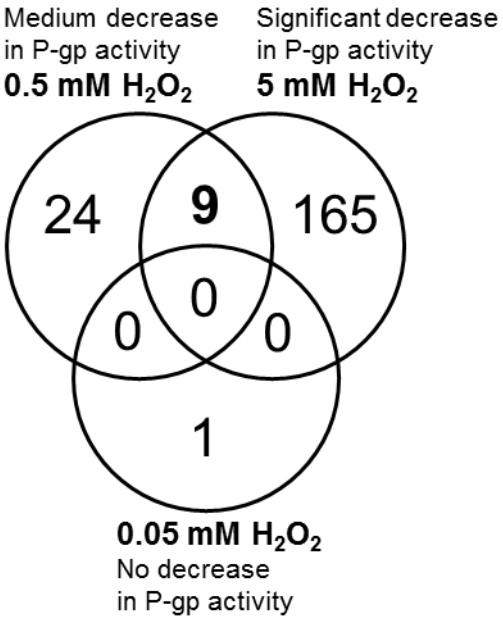

Supplementary figure 1

Peptides with altered phosphorylation level in H_2O_2-treated hCMEC/D3 cells. hCMEC/D3 cells were treated with 0.05 mM, 0.5 mM or 5 mM H_2O_2 for 20 min. The whole-cell lysate of hCMEC/D3 was digested with lysyl endopeptidase and trypsin. The phosphorylated peptides were enriched with the HAMMOC method and then dephosphorylated with hydrofluoric acid as described in Materials and Methods. The dephosphorylated peptides were subjected to nanoLC-MS/MS. Among the detected peptides, the peptides which showed over 3-fold difference between control and H_2O_2 treated cells with statistically significance ($p < 0.01$) were selected as peptides with altered phosphorylation level. Since P-gp efflux activity was decreased by both 0.5 mM and 5 mM H_2O_2 treatment (Figure 1(b)), we finally selected the commonly identified peptides from the 0.5 mM and 5 mM H_2O_2-treated cells (Supplementary table 1).

Supplementary table 1 The nine phosphoproteins showing significant changes in their phosphorylation levels in hCMEC/D3 cells treated with 0.5 mM and 5 mM H_2O_2

Description	Peptide sequence	H_2O_2 Conc.	Average peak intensity (cps)		Peak intensity ratio (H_2O_2/control)	Students *t*-test p-value
			H_2O_2	Control		
Caveolin-1	YVDSEGHLYTVPIR	0.5 mM	6221	319	19.5	6.28E-06
		5 mM	16817	878	19.2	2.43E-05
Stromal interaction molecule 1	AEQSLHDLQER	0.5 mM	5901	582	10.1	6.29E-05
		5 mM	13395	402	33.3	2.69E-06
Nuclear mitotic apparatus protein 1	ATSSTQSLAR	0.5 mM	7993	1708	4.68	7.70E-05
		5 mM	15812	2260	7.00	2.05E-03
DENN domain-containing protein 4C	VPSGIFDVNSR	0.5 mM	2151	523	4.11	6.01E-04
		5 mM	4721	535	8.83	9.61E-06
Microtubule-associated protein 4	LATNTSAPDLK	0.5 mM	14773	2730	5.41	1.36E-03
		5 mM	12494	3054	4.09	1.92E-04
Platelet endothelial cell adhesion molecule	DTETVYSEVR	0.5 mM	3312	849	3.9	9.57E-03
		5 mM	33236	1545	21.5	1.01E-06
Myelin protein zero-like protein 1	SESVVYADIR	0.5 mM	6495	1878	3.46	1.22E-04
		5 mM	15034	3492	4.31	2.94E-06
Heat shock protein beta-1	QLSSGVSEIR	0.5 mM	51592	15842	3.26	2.12E-04
		5 mM	129024	21109	6.11	7.66E-07
Proto-oncogene tyrosine-protein kinase Src	LIEDNEYTAR	0.5 mM	513	3022	0.17	5.60E-05
		5 mM	15848	5172	3.06	1.49E-02

hCMEC/D3 cells were treated with 0.5 mM or 5 mM H_2O_2 or vehicle (Control) for 20 min, and then whole-cell lysate (1800 μg protein) was digested with lysyl endopeptidase and trypsin. The phosphorylated peptides were enriched by the HAMMOC method. To increase the sensitivity of LC-MS/MS detection, the phosphorylated peptides were dephosphorylated by incubation with hydrofluoric acid. Finally, the dephosphorylated peptides were measured by nanoLC-MS/MS in the shotgun mode (information-dependent acquisition mode) in triplicate experiments. The peak intensities of the precursor ions of the dephosphorylated peptides were comprehensively compared between 0.5 mM or 5 mM H_2O_2- and vehicle-treated conditions using 2D-ICAL software. Among the identified peptides, the peptides that showed over 3-fold difference between control and H_2O_2-treated cells with statistical significance ($p < 0.01$) were selected and are listed in Supplementary table 2 (0.5 mM H_2O_2 treatment) and Supplementary table 3 (5 mM H_2O_2 treatment). In this experiment, it is assumed that the difference in the detected peak intensities of dephosphorylated peptides is consistent with the difference in the phosphorylation levels in the peptides between the two conditions before the dephosphorylation step with hydrofluoric acid (possible phosphorylation sites are indicated with underlines). Among the proteins listed in Supplementary tables 2 and 3, we selected the nine listed in this table as candidate proteins potentially involved in regulating P-gp efflux transport activity, because these proteins were commonly identified in both 0.5 mM H_2O_2 and 5 mM H_2O_2-treated cells.

Supplementary table 2 (0.5 mM) List of peptides phosphorylated by 0.5 mM H_2O_2 incubation for 20 min in hCMEC/D3 cells

Description	Peptide sequence	Average peak intensity (cps)		Peak intensity ratio (H_2O_2/control)	Student's *t*-test p-value
		0.5 mM H_2O_2	Control		
Caveolin-1	YVDSEGHLYTVPIR	6221	319	19.5	6.28E-06
Stromal interaction molecule 1	AEQSLHDLQER	5901	582	10.13	6.29E-05
Protein LYRIC	LSSQISAGEEK	4466	787	5.67	1.39E-04
Myosin-9	NLPIYSEEIVEMYK (12M:Oxidation)	1324	260	5.09	1.96E-05
	SMEAEMIQLQEELAAAER (2M:Oxidation|6M:Oxidation)	828	188	4.40	7.27E-05
	LQVELDNVTGLLSQSDSK	1061	284	3.73	1.03E-03
Transgelin-2	NFSDNQLQEGK	2106	419	5.03	3.48E-04
Nuclear mitotic apparatus protein 1	ATSSTQSLAR	7993	1708	4.68	7.70E-05
Myelin protein zero-like protein 1	SPSDTEGLVK	3846	886	4.34	2.83E-03
	SESVVYADIR	6495	1878	3.46	1.22E-04
Calnexin	APVPTGEVYFADSFDR	1637	383	4.28	2.85E-03
DENN domain-containing protein 4C	VPSGIFDVNSR	2151	523	4.11	6.01E-04
Microtubule-associated protein 4	LATNTSAPDLK	12494	3054	4.09	1.92E-04
Platelet endothelial cell adhesion molecule	DTETVYSEVR	3312	849	3.90	9.57E-03
Peptidyl-prolyl cis-trans isomerase A	VSFELFADK	3128	818	3.83	5.06E-05
Histone H4	TVTAMDVVYALK (5M:Oxidation)	4163	1125	3.70	3.73E-03
Proteasome subunit alpha type-4	LLDEVFFSEK	640	193	3.31	1.18E-03
Heat shock protein beta-1	QLSSGVSEIR	51592	15842	3.26	2.12E-04
Exocyst complex component 1	LTGSTSSLNK	1120	3417	0.330	6.75E-05
Nucleolar and coiled-body phosphoprotein 1	VADNSFDAK	608	2053	0.300	1.69E-04
Splicing factor 3B subunit 1	GSETPGATPGSK	497	1767	0.280	2.20E-04
Antigen KI-67	LDLLGNLPGSK	357	1824	0.200	1.81E-04
Tyrosine-protein kinase Src	LIEDNEYTAR	513	3022	0.170	5.60E-05
Arf-GAP domain and FG repeat-containing protein 1	GTPSQSPVVGR	288	2290	0.130	4.52E-05

hCMEC/D3 cells were treated with 0.5 mM H_2O_2 or vehicle (Control) for 20 min, and the whole-cell lysate (1800 µg protein) was digested with lysyl endopeptidase and trypsin. The phosphorylated peptides in the digests were enriched by the HAMMOC method. To increase the sensitivity of LC-MS/MS detection, the phosphorylated peptides were dephosphorylated by incubation with hydrofluoric acid. Finally, dephosphorylated peptides were measured by nanoLC-MS/MS in the shotgun mode (information-dependent acquisition mode) in triplicate experiments. The peak intensities of the precursor ions of the dephosphorylated peptides were comprehensively compared between 0.5 mM H_2O_2- and vehicle-treated conditions using 2D-ICAL software. Among the identified proteins, we focused only on peptides including either S, T or Y in this experiment, because serine (S), threonine (T) and tyrosine (Y) are generally considered potentially phosphorylatable. The average values of peak intensities from the three different measurements were calculated and used to calculate the ratio in the H_2O_2 and control groups. The identified proteins whose peptide(s) exhibited more than 3-fold differences with statistical significance ($p < 0.01$, Student's *t*-test) between the two groups were finally selected and are listed here (24 peptides; 21 proteins). In this experiment, it is assumed that the difference in the peak intensities of dephosphorylated peptides detected is consistent with the difference in the phosphorylation levels in the peptides between the two conditions before the dephosphorylation step by hydrofluoric acid (possible phosphorylation sites are indicated with underlines).

Supplementary table 3 (5 mM) List of peptides phosphorylated by 5 mM H_2O_2 incubation for 20 min in hCMEC/D3 cells (continued from page 6 - 13)

Description	Peptide sequence	Average peak intensity (cps)		Peak intensity ratio (H_2O_2/control)	Student's *t*-test p-value
		5 mM H_2O_2	Control		
Heterogeneous nuclear ribonucleoprotein D0	NEEDEGHSNSSPR	53294	403	132	5.00E-08
Band 4.1-like protein 1	HQASINELK	16724	359	46.6	0.0013
	SDSDTEGLLFSR	3361	10777	0.310	1.20E-04
Lupus La protein	FASDDEHDEHDENGATGPVK	23696	521	45.5	0.0057
Secretory carrier-associated membrane protein 2	AASSAAQGAFQGN	43232	1012	42.7	1.10E-05
A-kinase anchor protein 2	QVLQSTQSPR	12521	300	41.7	3.20E-05
	SPGALETPSAAGSQGNTASQGK	3921	480	8.17	2.50E-07
Tyrosine-protein phosphatase non-receptor type 11	VYENVGLMQQQK (8M:Oxidation)	10273	304	33.8	2.10E-04
Stromal interaction molecule 1	AEQSLHDLQER	13395	402	33.3	4.20E-04
Alpha-1B adrenergic receptor	NFHEDTLSSTK	1567	54	28.8	0.029
Matrin-3	DTSENADGQSDENK	10374	387	26.8	6.10E-07
Platelet endothelial cell adhesion molecule	DTETVYSEVR	33236	1545	21.5	2.10E-04
	EPLNSDVQYTEVQVSSAESHK	16016	2725	5.88	2.20E-07
HMG box transcription factor BBX	SPTPTVNPR	4043	197	20.6	1.40E-04
Mitochondrial import receptor subunit TOM70	ASPAPGSGHPEGPGAHLDMNSL DR (19M:Oxidation)	16376	806	20.3	0.0026
Mitogen-activated protein kinase 1	VADPDHDHTGFLTEYVATR	31586	1612	19.6	8.90E-04
Caveolin-1	YVDSEGHLYTVPIR	16817	878	19.2	7.20E-05
Zinc finger CCCH domain-containing protein 13	STSPAGQHHSPISSR	3017	170	17.8	0.005
Actin-binding LIM protein 1	STSQGSINSPVYSR	4853	276	17.6	1.50E-08
SHC-transforming protein 1	ELFDDPSYVNVQNLDK	8798	536	16.4	3.10E-07
Serine/arginine repetitive matrix protein 2	SSGHSSSELSPDAVEK	7034	500	14.1	0.0026
	TPPVALNSSR	9105	890	10.2	4.40E-04
	HASSSPESPKPAPAPGSHR	3353	14349	0.230	0.043
	QGSITSPQANEQSVTPQR	251	2348	0.110	2.00E-05
Proteasome subunit alpha type-3	EEDESDDDNM (10M:Oxidation)	3800	273	13.9	0.042
Cytospin-A	TPLSPSPMK (8M:Oxidation)	7151	547	13.1	3.20E-04
Protein disulfide-isomerase	DHENIVIAK	2768	229	12.1	6.50E-04
Disintegrin and metalloproteinase domain-containing protein 9	EVPIYANR	17391	1467	11.9	0.011
Neuroblast differentiation-associated protein AHNAK	AEAPLPSPK	30410	2613	11.6	3.70E-05
	FGTFGGLGSK	21914	2580	8.50	1.50E-05
	GEYDVTMPK (7M:Oxidation)	2880	464	6.20	3.40E-04
	ISMPDIDLNLK (3M:Oxidation)	2983	946	3.15	0.026
	GDLDASVPSMK (10M:Oxidation)	2084	677	3.08	0.039
	GGVTGSPEASISGSK	1415	41325	0.0300	1.20E-05
FK506-binding protein 15	RPSQEQSASASSGQPQAPLNR	2666	232	11.5	4.90E-07
Microtubule-associated protein 1B	GAESPFEEK	7029	641	11.0	0.002
5~-AMP-activated protein kinase subunit beta-1	SHNNFVAILDLPEGEHQYK	6495	604	10.8	0.0019
Tight junction protein ZO-2	HPDIYAVPIK	10941	1048	10.4	0.0019
E3 SUMO-protein ligase RanBP2	SGSSFVHQASFK	6101	604	10.1	0.023
Tensin-3	ASEAASPLPDSPGDK	3499	361	9.69	1.70E-04

Supplementary table 3 (5 mM) List of peptides phosphorylated by 5 mM H_2O_2 incubation for 20 min in hCMEC/D3 cells (continued from page 6 - 13)

Description	Peptide sequence	Average peak intensity (cps)		Peak intensity ratio (H_2O_2/control)	Student's *t*-test p-value
		5 mM H_2O_2	Control		
G patch domain-containing protein 8	GPKPEPPGSGSPAPPR	8370	926	9.04	5.30E-05
DENN domain-containing protein 4C	VPSGIFDVNSR	4721	535	8.83	1.80E-04
Bromodomain adjacent to zinc finger domain protein 1A	ELDQDMVTEDEDDPGSHK (6M:Oxidation)	2855	328	8.70	3.80E-04
Platelet endothelial aggregation receptor 1	GLISEEELGASVASLSSENPYATIR	3147	362	8.69	4.20E-09
Cyclin-dependent kinase 12	SSSPFLSK	4832	559	8.65	2.40E-04
Heterogeneous nuclear ribonucleoprotein U	SSGPTSLFAVTVAPPGAR	9518	1161	8.20	1.20E-04
Ankyrin repeat and KH domain-containing protein 1	LNLTSPK	2991	379	7.89	3.10E-04
Tyrosine-protein phosphatase non-receptor type substrate 1	EITQDTNDITYADLNLPK	5061	642	7.88	3.00E-04
	QPAPKPEPSFSEYASVQVPR	4947	808	6.13	0.027
Tyrosine-protein kinase receptor UFO	IYNGDYYR	5085	671	7.57	6.30E-04
Integrator complex subunit 3	NATQPPNAEEESGSSSASEEEDTKPKPTK	2767	380	7.28	0.0016
Structural maintenance of chromosomes protein 1A	GTMDDISQEEGSSQGEDSVSGSQR (3M:Oxidation)	1863	259	7.19	8.50E-06
Protein disulfide-isomerase A6	DGELPVEDDIDLSDVELDDLGK	2638	369	7.14	3.00E-09
Nuclear mitotic apparatus protein 1	ATSSTQSLAR	15812	2260	7.00	2.50E-05
Condensin complex subunit 2	GHPHSASSPSER	2047	320	6.40	8.70E-04
Ribonucleoside-diphosphate reductase subunit M2	VPLAPITDPQQLQLSPLK	7595	1206	6.30	3.10E-08
Protein LSM14 homolog A	TQLSQGR	6036	970	6.22	0.0019
Heat shock protein beta-1	QLSSGVSEIR	129024	21109	6.11	8.10E-06
Tuftelin-interacting protein 11	GAAEEAELEDSDDEEKPVK	4342	739	5.88	4.50E-06
Vimentin	ISLPLPNFSSLNLR	12223	2183	5.60	5.70E-07
	LLEGEESR	941	2976	0.320	0.0029
Palladin	IASDEEIQGTK	10597	1947	5.44	1.30E-05
Microtubule-associated protein 4	LATNTSAPDLK	14773	2730	5.41	1.30E-06
	DVTPPPETEVVLIK	11210	3091	3.63	0.021
Brefeldin A-inhibited guanine nucleotide-exchange protein 2	GSSLSGTDDGAQEVVK	1989	382	5.21	9.10E-04
Phosphorylated CTD-interacting factor 1	IEIPVTPTGQSVPSSPSIPGTPTLK	2420	499	4.85	4.00E-04
Epsin-1	TALPTSGSSAGELELLAGEVPAR	2041	439	4.65	6.10E-05
Src substrate cortactin	TQTPPVSPAPQPTEER	3425	773	4.43	2.70E-04
Protein enabled homolog	QNSQLPAQVQNGPSQEELEIQR	4683	1075	4.36	4.00E-07
Canalicular multispecific organic anion transporter 2	QLSALSSDGEGQGRPVPR	4678	1074	4.35	1.30E-04
Negative elongation factor E	SISADDDLQESSR	8395	1943	4.32	5.50E-06
Myelin protein zero-like protein 1	SESVVYADIR	15034	3492	4.31	3.00E-05
	SPSDTEGLVK	5510	1471	3.75	3.80E-05
Synaptosomal-associated protein 23	EDEMEENLTQVGSILGNLK (4M:Oxidation)	808	191	4.23	0.002
Protein kinase C delta type	NLIDSMDQSAFAGFSFVNPK (6M:Oxidation)	4895	1178	4.16	1.40E-07
LIM domain only protein 7	GESLDNLDSPR	6246	1545	4.04	4.10E-06
Dystroglycan	LTLEDQATFIK	9896	2487	3.98	5.30E-04
Nucleoprotein TPR	TDGFAEAIHSPQVAGVPR	15244	3867	3.94	5.00E-04
Serine/threonine-protein kinase D1	SVVGTPAYLAPEVLR	4228	1084	3.90	1.10E-07

Supplementary table 3 (5 mM) List of peptides phosphorylated by 5 mM H_2O_2 incubation for 20 min in hCMEC/D3 cells (continued from page 6 - 13)

Description	Peptide sequence	Average peak intensity (cps)		Peak intensity ratio (H_2O_2/control)	Student's *t*-test p-value
		5 mM H_2O_2	Control		
Myosin-9	EQADFAIEALAK	6396	1667	3.84	6.30E-05
Splicing factor 3B subunit 1	TMIISPER (2M:Oxidation)	11066	2888	3.83	0.0073
Protein LAP2	SATLLYDQPLQVFTGSSSSSDLISGTK	5682	1483	3.83	2.80E-11
E1A-binding protein p400	ALSPVTSR	2194	572	3.83	0.0039
MAGUK p55 subfamily member 5	VYESIGQYGGETVK	2732	719	3.80	6.30E-04
Cyclin-L1	GLNPDGTPALSTLGGFSPASKPSSPR	10747	2888	3.72	7.30E-08
Pseudopodium-enriched atypical kinase 1	VPIVINPNAYDNLAIYK	3340	899	3.71	8.50E-05
Actin-binding protein anillin	TQSLPVTEK	4767	1342	3.55	0.017
Bcl-2-associated transcription factor 1	AEGEPQEESPLK	13575	3872	3.51	8.10E-07
	FNDSEGDDTEETEDYR	470	7523	0.0600	3.50E-04
Alpha-actinin-1	AIMTYVSSFYHAFSGAQK	1269	370	3.43	0.002
Membrane-associated progesterone receptor component 2	DFSLEQLR	4082	1209	3.38	0.046
DmX-like protein 1	QEPVITDSYSGSTPSITSLIK	1092	325	3.36	0.0013
DNA repair protein complementing XP-G cells	NAPAAVDEGSISPR	3821	1142	3.35	2.10E-07
Nuclear pore complex protein Nup214	TPSIQPSLLPHAAPFAK	5427	1633	3.32	0.0012
Negative elongation factor B	KPSPAQAAETPALELPLPSVPAPAPL	6432	1941	3.31	3.00E-05
Zinc finger CCCH-type antiviral protein 1	SSLGSLQTPEAVTTR	1366	429	3.18	0.03
Transcription intermediary factor 1-beta	LDLDLTADSQPPVFK	2963	952	3.11	0.0034
Tumor suppressor p53-binding protein 1	FVPAENDSILMNPAQDGEVQLSQNDDK (11M:Oxidation)	5380	1746	3.08	4.40E-06
Histone H4	TVTAMDVVYALK (5M:Oxidation)	14175	4634	3.06	1.30E-07
Epidermal growth factor receptor substrate 15	LNDPFQPFPGNDSPK	4320	1410	3.06	0.0012
Proto-oncogene tyrosine-protein kinase Src	LIEDNEYTAR	5172	15848	3.06	7.60E-06
DNA topoisomerase 2-alpha	FTMDLDSDEDFSDFDEK (3M:Oxidation)	6532	2149	3.04	1.10E-09
H(+)/Cl(-) exchange transporter 7	VGHMSSVELDDELLDPDMDPPHPFPK (4M:Oxidation|18M:Oxidation)	5239	1746	3.00	6.60E-06
Transcription activator BRG1	AENAEGQTPAIGPDGEPLDETSQMSDLPVK (24M:Oxidation)	1040	3116	0.330	3.30E-10
Cutaneous T-cell lymphoma-associated antigen 5	AFLSPPTLLEGPLR	390	1186	0.330	7.60E-06
E3 UFM1-protein ligase 1	DDDSDDESQSSHTGK	19079	63806	0.299	2.40E-06
Multidrug resistance-associated protein 1	HHNSTAELQK	3565	12381	0.290	0.021
AT-rich interactive domain-containing protein 1A	GPSPSPVGSPASVAQSR	1481	5105	0.290	3.50E-04
Transcription factor AP-1	LASPELER	1127	3882	0.290	4.60E-04
Actin, cytoplasmic 1	DLTDYLMK	1008	3812	0.260	0.014
	EITALAPSTMK	517	2638	0.200	0.0028
Forkhead box protein K1	SAPASPTHPGLMSPR (12M:Oxidation)	2866	11411	0.250	7.90E-05
Coiled-coil domain-containing protein 6	LDQPVSAPPSPR	506	2461	0.210	6.10E-05
Ankycorbin	SITSTPLSGK	2113	11591	0.180	0.0021
Myb-binding protein 1A	EIPSATQSPISK	1679	9537	0.180	0.034
A-kinase anchor protein 12	SATLSSTESTASEMQEEMK (14M:Oxidation|18M:Oxidation)	613	3377	0.180	1.90E-04

Supplementary table 3 (5 mM) List of peptides phosphorylated by 5 mM H_2O_2 incubation for 20 min in hCMEC/D3 cells (continued from page 6 - 13)

Description	Peptide sequence	Average peak intensity (cps)		Peak intensity ratio (H_2O_2/control)	Student's *t*-test p-value
		5 mM H_2O_2	Control		
Zinc finger CCCH domain-containing protein 18	ASDLEDEESAAR	599	3740	0.160	2.50E-04
A-kinase anchor protein 5	STQDLSEGISR	355	2195	0.160	8.60E-06
U5 small nuclear ribonucleoprotein 200 kDa helicase	EEASDDDMEGDEAVVR (8M:Oxidation)	2006	13658	0.150	4.20E-12
Double-strand break repair protein MRE11A	NYSEVIEVDESDVEEDIFPTTSK	280	1904	0.150	1.60E-09
Paralemmin-1	TEVVMNSQQTPVGTPK (5M:Oxidation)	873	6209	0.140	1.30E-04
RNA-binding protein 12B	SPTEDFR	628	4472	0.140	0.0019
Apoptosis-stimulating of p53 protein 2	NQSSEDILR	500	3539	0.140	0.02
Zinc finger protein 638	AVIVSSPK	388	2977	0.130	0.0043
Nuclear factor 1 C-type	SPFNSPSPQDSPR	735	6381	0.120	8.80E-05
Rho GTPase-activating protein 23	SYSPSFQR	452	3660	0.120	0.044
Catenin delta-1	SGDLGDMEPLK (7M:Oxidation)	386	3445	0.110	1.50E-05
Arf-GAP domain and FG repeat-containing protein 1	GTPSQSPVVGR	2430	22045	0.110	1.90E-05
Caveolin-2	ADVQLFMDDDSYSHHSGLEYA DPEK (7M:Oxidation)	497	4692	0.110	0.0094
Zinc finger protein 185	GGQGDPAVPAQQPADPSTPER	275	2558	0.110	6.40E-04
Alpha-actinin-4	HEAFESDLAAHQDR	176	1785	0.100	2.30E-05
	HRPELIEYDK	192	6503	0.0300	0.0022
Ribosomal RNA processing protein 1 homolog B	VAEPGAEATSSTGEESGSEHPPA VPMHNK (26M:Oxidation)	250	2986	0.0800	0.027
Centromere protein I	GPSPEPLK	237	2815	0.0800	0.034
Nucleolar and coiled-body phosphoprotein 1	VADNSFDAK	181	2387	0.0800	8.20E-05
Antigen KI-67	SPPPELTDTATSTK	281	4231	0.0700	5.60E-05
Pre-mRNA-splicing regulator WTAP	EGNTTEDDFPSSPGNGNK	185	2607	0.0700	0.011
Disintegrin and metalloproteinase domain-containing protein 12	KPPDSYPPK	994	15329	0.0600	0.011
SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily A member 5	TPTSPLK	511	8842	0.0600	2.20E-04
Putative pre-mRNA-splicing factor ATP-dependent RNA helicase DHX16	LLEDSEESSEETVSR	327	5171	0.0600	3.80E-04
Treacle protein	QEDSESSEEESDSEEAAASPAQV K	194	3335	0.0600	5.10E-05
Msx2-interacting protein	DLEPGEVPSDSDEDGEHK	201	3267	0.0600	9.00E-04
Thyroid hormone receptor-associated protein 3	SPEIHR	1070	23218	0.0500	0.0097
BCL2/adenovirus E1B 19 kDa protein-interacting protein 3-like	NGGLEHVPSSSSIHNGDMEK (18M:Oxidation)	258	4966	0.0500	0.029
Tight junction protein ZO-1	HSPQQPSNGSLR	227	5011	0.0500	0.033
Methyl-CpG-binding protein 2	AETSEGSGSAPAVPEASASPK	132	2714	0.0500	5.60E-04
Tyrosine-protein kinase BAZ1B	LAEDEGDSEPEAVGQSR	623	14117	0.0400	9.50E-10
Sister chromatid cohesion protein PDS5 homolog B	AESPESSAIESTQSTPQK	586	13727	0.0400	2.60E-05
Splicing factor, arginine/serine-rich 19	SPFLKPDER	208	5344	0.0400	0.017
Segment polarity protein dishevelled homolog DVL-2	GSTGGAPNLR	521	15554	0.0300	0.043
Serine/arginine-rich splicing factor 11	DYDEEEQGYDSEK	251	9316	0.0300	2.00E-06
Zinc finger and BTB domain-containing protein 7A	GGAPDPSPGATATPGAPAQPSSP DAR	112	4319	0.0300	4.90E-06
Protein PML	VVVISSSEDSDAENSSSR	108	3326	0.0300	0.0025
Histone H3.1t	EIAQDFK	187	8444	0.0200	0.015

Supplementary table 3 (5 mM) List of peptides phosphorylated by 5 mM H_2O_2 incubation for 20 min in hCMEC/D3 cells (continued from page 6 - 13)

Description	Peptide sequence	Average peak intensity (cps)		Peak intensity ratio (H_2O_2/control)	Student's *t*-test p-value
		5 mM H_2O_2	Control		
Coagulation factor VIII	TSNNSATNR	491	24347	0.0200	0.045
Drebrin	LSSPVLHR	209	10013	0.0200	4.50E-05
Isthmin-2	EEEEAPLLPR	175	7375	0.0200	0.023
Pericentriolar material 1 protein	VTNDISPESSPGVGR	62	3897	0.0200	6.60E-06
PHD and RING finger domain-containing protein 1	TISINSPK	1095	74057	0.0100	0.0088
Zinc finger Ran-binding domain-containing protein 2	YNLDASEEEDSNK	240	16876	0.0100	3.30E-08
Nuclear ubiquitous casein and cyclin-dependent kinases substrate	NSQEDSEDSEDK	105	8621	0.0100	2.10E-04
Lysosome-associated membrane glycoprotein 1	DNTTVTR	106	24667	0.0043	0.018
Golgi phosphoprotein 3-like	WVNDPQR	10	5316	0.0018	0.023
Pejvakin	INFDHSLIR	0	15385	0.0000	0.028
Chromatin assembly factor 1 subunit B	GSSPGPRPVEGTPASR	0	10271	0.0000	0.0011

hCMEC/D3 cells were treated with 5 mM H_2O_2 or vehicle (Control) for 20 min, and then whole-cell lysate (1800 μg protein) was digested with lysyl endopeptidase and trypsin. The phosphorylated peptides in the digests were enriched by the HAMMOC method. To increase the sensitivity of LC-MS/MS detection, the phosphorylated peptides were dephosphorylated by incubation with hydrofluoric acid. Finally, dephosphorylated peptides were measured by nanoLC-MS/MS in the shotgun mode (information-dependent acquisition mode) in triplicate experiments. The peak intensities of the precursor ions of the dephosphorylated peptides were comprehensively compared between 5 mM H_2O_2- and vehicle-treated conditions using 2D-ICAL software. Among the identified proteins, we focused only on peptides including either S, T or Y in this experiment, because serine (S), threonine (T) and tyrosine (Y) are generally considered potentially phosphorylatable. The average values of peak intensities from the three different measurements were calculated and used to calculate the ratio in the H_2O_2 and control groups. The identified proteins whose peptide(s) exhibited more than 3-fold differences with statistical significance ($p < 0.01$, Student's *t*-test) between two groups were finally selected and are listed here (165 peptides; 147 proteins). In this experiment, it is assumed that the difference in the peak intensities of dephosphorylated peptides detected is consistent with the difference in the phosphorylation levels in the peptides between the two conditions before the dephosphorylation step by hydrofluoric acid (possible phosphorylation sites are indicated with underlines).

Supplementary table 4 Protein expression and phosphorylation amounts of Cav1 in whole cell lysate of H_2O_2 treated-hCMEC/D3 cells

	Protein expression or phosphorylation amounts (fmol/µg protein)					
H_2O_2 concentration	0 mM	0.25 mM	0.5 mM	1 mM	2.5 mM	5 mM
Total Cav1	30.7 ± 4.4	31.9 ± 5.3	31.6 ± 6.0	29.9 ± 4.1	29.0 ± 4.1	28.9 ± 3.6
Tyr6 phosphorylated Cav1	U.L.Q. (< 0.00654)	0.0942 ± 0.0432	0.176 ± 0.107	0.171 ± 0.047	0.164 ± 0.070	0.156 ± 0.026
Phosphorylation ratio (%)	*< 0.0213*	*0.296 ± 0.135*	*0.559 ± 0.340*	*0.573 ± 0.156*	*0.565 ± 0.242*	*0.541 ± 0.091*
Tyr14 phosphorylated Cav1	U.L.Q. (< 0.000500)	1.41 ± 0.31	2.87 ± 0.30	3.36 ± 0.57	4.34 ± 0.74	4.79 ± 0.60
Phosphorylation ratio (%)	*< 0.00163*	*4.44 ± 0.96*	*9.08 ± 0.94*	*11.2 ± 1.9*	*15.0 ± 2.5*	*16.6 ± 2.1*
Tyr6 and Tyr14 phosphorylated Cav1	U.L.Q. (< 0.00275)	0.0145 ± 0.0052	0.0437 ± 0.0113	0.0473 ± 0.0171	0.0482 ± 0.0136	0.0434 ± 0.0245
Phosphorylation ratio (%)	*< 0.00896*	*0.0456 ± 0.0165*	*0.138 ± 0.036*	*0.158 ± 0.057*	*0.166 ± 0.047*	*0.150 ± 0.084*

Protein expression amount and phosphorylation amounts of Cav1 in whole-cell lysate of hCMEC/D3 cells were determined by LC-MS/MS analysis after enrichment of phosphorylated peptides by the HAMMOC method. hCMEC/D3 cells were incubated with H_2O_2 in ECF buffer for 20 min. The whole-cell lysate of hCMEC/D3 cells was digested with lysyl endopeptidase and trypsin. The digest (1800 µg protein) was loaded on to a HAMMOC column to enrich phosphorylated peptides. The phosphorylated peptides-enriched fraction was subjected to LC-MS/MS. The phosphorylation levels represents the mean±SD of 9-12 SRM/MRM transitions in three independent analyses. The expression amount of phosphorylated Cav1 was determined by quantification of the peptide YVDSEGHLYTVPIR derived from Cav1. YVDSEGHLYTVPIR peptide contains two phosphorylation sites, so Tyr6-phosphorylated, Tyr14-phosphorylated and both Tyr6/Tyr14-phosphorylated peptides were separately quantified in this study. The phosphorylation ratio (%) were calculated by dividing the expression amount of phosphorylated Cav1 by that of total Cav1. U.L.Q., under the limit of quantification

Supplementary table 5 Probe peptides and SRM/MRM transitions for LC-MS/MS quantification

Synonym	Probe Sequence	Number of AA	Position of stable isotope-labeled AA from the N terminal	Stable isotope-labeled AA	Standard peptide Q1-Q3 (m/z)	Internal standard peptide Q1-Q3 (m/z)
Caveolin-1 (total)	HLNDDVVK	9	9	K	470.2-251.2	474.3-251.2
					470.2-595.2	474.3-595.2
					470.2-345.3	474.3-353.3
					470.2-246.2	474.3-254.2
Caveolin-1 (Tyr6 phosphorylated)	pYVDSEGHLYTVPIR	14	14	R	576.9-693.9	580.3-698.9
					576.9-343.1	580.3-343.1
					576.9-748.4	580.3-758.4
					576.9-636.3	580.3-641.3
Caveolin-1 (Tyr14 phosphorylated)	YVDSEGHLpYTVPIR	14	14	R	576.9-733.9	580.3-738.9
					576.9-385.3	580.3-395.3
					576.9-676.3	580.3-681.3
					576.9-828.4	580.3-838.4
Caveolin-1 (Tyr6 and Tyr14 phosphorylated)	pYVDSEGHLpYTVPIR	14	14	R	603.6-733.9	606.9-738.9
					904.9-385.3	909.9-395.3
					603.6-385.3	606.9-395.3
					603.6-676.3	606.9-681.3
Caveolin-1 (Tyr25 phosphorylated)	EQGNIpYK	7	7	K	466.2-390.2	470.2-398.2
					466.2-674.3	470.2-682.3
					466.2-503.3	470.2-511.3
					466.2-258.1	470.2-258.1
Caveolin-1 (Tyr42 phosphorylated)	QVpYDAHTK	8	8	K	347.8-228.6	350.5-232.6
					347.8-407.7	350.5-411.7
					521.2-248.2	525.2-256.2
					521.2-814.3	525.2-822.3
Src kinase (Tyr419 phosphorylated)	LIEDNEpYTAR	10	10	R	652.3-1077.4	657.3-1087.4
					652.3-948.4	657.3-958.4
					652.3-590.3	657.3-600.3
					652.3-833.4	657.3-843.4

Unlabeled (standard, St) and stable isotope (^{13}C and ^{15}N)-labeled peptides (internal standard, IS) were designed by employing the *in silico* peptide selection criteria reported previously (Uchida *et al., Fluids Barriers CNS.* **10 (1),** 21, 2013), and synthesized by Thermo Fisher Scientific (Sedanstrasse, Germany) or Scrum Inc. (Tokyo, Japan) with > 95% peptide purity. The concentrations of peptide solutions were determined by quantitative amino acid analysis using a HPLC-UV system with post-column ninhydrin derivatization (LaChrom Elite, Hitachi, Tokyo, Japan). The SRM/MRM transitions were determined from MS/MS spectra obtained by direct infusion of 1 μM peptide solution at a flow rate of 5 μL/min with a syringe pump (Harvard) into the mass spectrometer. Doubly charged precursor ions were selected (Q1). Four transitions per peptide (Q3-1, -2, -3 and -4), corresponding to high-intensity fragment ions, were

selected. The declustering potentials and collision energies were optimized to maximize signal strength. For the stable isotope-labeled peptides, precursor ions and transitions corresponding to those of the unlabeled peptides were selected, with the same declustering potentials and collision energies as for the unlabeled peptides. pY indicates phosphorylated tyrosine. AA: amino acid.

Reference

Uchida Y, Tachikawa M, Obuchi W, et al. A study protocol for quantitative targeted absolute proteomics (QTAP) by LC-MS/MS: application for inter-strain differences in protein expression levels of transporters, receptors, claudin-5, and marker proteins at the blood-brain barrier in ddY, FVB, and C57BL/6J mice. *Fluids Barriers CNS* 2013; 10: 21.

Supplementary method 1 Uptake assay to estimate P-gp- or MRP1-mediated [^{3}H]vinblastine efflux activity

hCMEC/D3 cells were seeded onto Cultrex® Rat Collagen (R&D Systems)-coated 24-well plates at a density of 5 × 10^4 cells/well. Uptake assay was conducted at 6 days after seeding, except for siRNA-transfected cells. The cell surface was washed twice with 500 μL of extracellular fluid (ECF) buffer (122 mM NaCl, 25 mM NaHCO3, 3 mM KCl, 0.4 mM K2HPO4, 10 mM d-glucose, 1.4 mM CaCl2, 1.2 mM MgSO4·7H2O, 10 mM HEPES, pH 7.4, 300 ± 10 mOsm) and pre-incubated with 200 μL ECF buffer for 30 min at 37°C. To initiate uptake, the pre-incubation buffer was removed and 200 μL ECF buffer containing 50 nM [^{3}H]vinblastine was added. After incubation for 20 min at 37°C, the cell surface was quickly washed four times with 500 μL of ice-cold ECF buffer. When cells were treated with PSC833, MK571, H_2O_2 or inhibitors (dynasore, imatinib, PP2, SKF-96365, bis-ANS, KRIBB3), we used ECF buffer containing these compounds during both the pre-incubation and uptake procedures. Stock solutions were prepared as follows: PSC833, MK571, dynasore, imatinib, PP2 and KRIBB3 were dissolved in dimethyl sulfoxide, SKF-96365 was dissolved in water, and bis-ANS was dissolved in methanol. During the uptake assay, the final concentration of organic solvent from the stock solution was less than 0.1%. Vehicle controls included the same concentration of the corresponding solvent. NaOH solution (5 mol/L, 200 μL) was added to the wells and the plates were incubated at room temperature overnight to lyse the cells. HCl solution (5 mol/L. 200 μL) was added for neutralization and the radioactivity in each well was measured by liquid scintillation counting. Protein amount in each well was measured by the Lowry method using DC protein assay reagent (Bio-Rad, Hercules, CA, USA). Cell-to-medium ratio (C/M ratio) (μL/mg protein) was calculated from the vinblastine concentration in the cell (mol/mg protein) divided by that in the incubation buffer (mol/μL). Efflux activity of P-gp was estimated according to Hoshi et al (2017).

Reference

Hoshi Y, Uchida Y, Tachikawa M, et al. Actin filament-associated protein 1 (AFAP-1) is a key mediator in inflammatory signaling-induced rapid attenuation of intrinsic P-gp function in human brain capillary endothelial cells. *J Neurochem* 2017; 141: 247-262.

Supplementary method 2 siRNA transfection of hCMEC/D3 cells

Abl kinase siRNA (sense: 5'-GAAGGGAGGGUGUACCAUUtt-3′) was synthesized by Nihon Bio-Service (Saitama, Japan). Stealth RNAi™ siRNA Negative Control Med GC Duplex (Invitrogen, Carlsbad, CA) were used as a negative control for the experiments. siRNA was transfected using Lipofectamine RNAiMAX (Invitrogen). The siRNA treatment of hCMEC/D3 cells was performed according to the manufacturer's instructions. hCMEC/D3 cells were seeded on collagen-coated 24-well plates (for uptake assay) or 10 cm dishes (for LC-MS/MS analysis) at a density of 2 x 10^4 cells/cm^2. Cells were cultured in the medium without antibiotics for 12 to 18 hours, and then treated with 33 nM siRNA against Abl kinase or 33 nM negative control siRNA using Lipofectamine RNAiMAX and Opti-MEM® I Reduced Serum Medium (Invitrogen). The cells were cultured for 72 hours, and used for experiments.

Supplementary method 3 Quantitative targeted absolute proteomics (QTAP) in hCMEC/D3 cells, human and rat brain capillaries

Samples (50 μg protein) were suspended in solubilizer (500 mM Tris-HCl (pH 8.5), 7 M guanidium hydrochloride and 10 mM EDTA), and the proteins were S-carbomoylmethylated. The alkylated proteins were precipitated with a mixture of methanol and chloroform. The precipitates were dissolved in 6 M urea in 100 mM Tris–HCl (pH 8.5), and diluted 5-fold with 100 mM Tris–HCl (pH 8.5). The dissolved samples were mixed with ProteaseMAX surfactant (Promega, Madison, WI, USA) and treated with lysyl endopeptidase (LysC; Wako Pure Chemical Industries) at 0.05% final concentration and an enzyme/substrate ratio of 1:100. The mixture was incubated at 30°C for 3 hours, and then treated with TPCK-treated trypsin (Promega) at an enzyme/substrate ratio of 1:100 at 37°C for 16 hours. The tryptic digests were mixed with isotope-labeled peptides as internal standards. The peptide samples were cleaned up for LC-MS/MS analysis by using SDB and GC tip (GL Science, Tokyo, Japan) according to the manufacturer's instructions. Peptide solution acidified with formic acid was centrifuged at 4°C and 17,360 g for 5 min, and the supernatants were subjected to LC-MS/MS analysis.

Supplementary method 4 LC-MS/MS conditions for QTAP analysis

QTAP analysis was performed with an electrospray ionization (ESI) triple quadrupole mass spectrometer (QTRAP5500; Sciex, Framingham, MA) equipped with a Turbo V ion source (Sciex) and coupled with an

Eksigent Expert™ microLC 200 system (Eksigent Technologies, Dublin, CA, USA). Samples equivalent to 10 μg protein were injected onto a HALO C18 column (2.7 μm, 0.5 × 50 mm, Eksigent Technologies) at a flow rate of 10 μL/min, together with isotope-labeled peptides. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The peptides were separated and eluted from the column using the following gradient sequences 1% B (0-2 min), 1-30% B (2-32 min), increased to 30-100% B (32-35 min), maintained at 100% B (35-37 min), reduced to 100-1% B (37-39 min) and then maintained at 1% B (39-55 min). The eluted peptides were simultaneously and selectively detected by means of electro-spray ionization in a multiplexed selected/multiple reaction monitoring (SRM/MRM) mode. The source/gas parameters were as follows: ion source gas 1 (20 psi), ion source gas 2 (40 psi), curtain gas (40 psi), collision gas (12 psi), ion spray voltage floating (5500 V), interface heater temperature (500°C). The dwell time was 10 msec per SRM/MRM transition. Each molecule was monitored with four sets of SRM/MRM transitions (Q1/Q3-1, Q1/Q3-2, Q1/Q3-3, Q1/Q3-4) derived from one set of unlabeled (standard) and isotope-labeled (internal standard) peptides. Non-phosphorylated peptides of caveolin-1 and phosphorylated peptides of caveolin-1 and Src kinase (pTyr6, pTyr14, both pTyr6 and pTyr14, pTyr25 and pTyr42 caveolin-1 and pTyr419 Src kinase) were monitored using the peptides and SRM/MRM transitions listed in Supplementary Table 1, and P-gp and MRP1 were monitored using those reported by Uchida et al. (2011). Chromatogram ion counts were determined by using an auto analysis system established in our laboratory [Uchida et al., 2013]. Signal peaks with a peak area count of over 5000 detected at the same retention time as an isotope-labeled peptide were defined as positive. When positive peaks were observed in three or four sets of SRM/MRM transitions, the molecules were considered to be expressed, and the protein expression amounts were determined as the average of the three or four quantitative values. The limit of quantification was calculated as described previously [Uchida et al. 2011].

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Uchida Y, Ohtsuki S, Katsukura Y, et al. Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. *J Neurochem* 2011; 117: 333-345.

Uchida Y, Tachikawa M, Obuchi W, et al. A study protocol for quantitative targeted absolute proteomics (QTAP) by LC-MS/MS: application for inter-strain differences in protein expression levels of transporters, receptors, claudin-5, and marker proteins at the blood-brain barrier in ddY, FVB, and C57BL/6J mice. *Fluids Barriers CNS* 2013; 10: 21.

Supplementary method 5 LC-MS/MS conditions for phosphoproteomic analysis

Phosphoproteomic analysis was performed with an electrospray ionization (ESI) triple TOF 5600 mass spectrometer (Sciex) equipped with a NanoSpray III ion source (Sciex) and coupled with a nanoLC ultra 2D plus (Eksigent Technologies). The NanoLC is connected to a cHiPLC® nanoflex (Eksigent Technologies) with a column-switching system that can handle two sets of gradient pumps and analytical columns. The peptide solution (1 μg protein) was loaded onto one analytical column (75 μm x 15 cm, ReproSil-Pur 3 μm, C18-AQ 120Aº) and eluted at a flow rate of 300 nL/min. During analysis using one column, the next peptide solution was preliminarily loaded onto the other analytical column at a flow rate 300 nL/min. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The LC-gradient design was as follows: for sample analysis: 0-50% B (0-140 min), increased to 50-100% B (140-141 min), maintained at 100% B (141-150 min), reduced to 0% B (150-150.1 min), and then maintained at 0% B (150.1-160 min), for sample loading: 0% B (0-160 min). The column temperature was not set (room temperature). The eluted peptides were sprayed through a PicoTip™ nanospray emitter (New Objective, Woburn, MA). The source/gas parameters were as follows: ion source gas 1 (20 psi), ion source gas 2 (0 psi), curtain gas (20 psi), ion spray voltage floating (2300 V), interface heater temperature (150°C). The scan parameters were as follows: TOF MS scan (350-1250 Da), TOF MS accumulation time (0.25 sec), MS/MS scan (100-1600 Da), MS/MS accumulation time (0.1 sec), declustering potential (80 V), collision energy (35 V), collision energy spread (15 V). After TOF MS survey scan, a maximum of 20 intense precursor ions per cycle were selected and fragmented within the 1.3 sec cycle time. Data analysis for peak alignment between different measurements and the calculation of peak intensities were conducted using the two-dimensional image-converted analysis of liquid chromatography-mass spectrometry (2D-ICAL) software package (Mitsui Knowledge Industry Co. Ltd, Tokyo, Japan) with the analytical settings described by Nurdin et al. (2016). Candidate phosphoproteins were determined as described previously [Hoshi et al., 2017]. Identified peptides that exhibited more than 3-fold difference

with statistical significance ($p < 0.01$, Student's t-test) between the two groups were finally selected and are listed in Supplementary table 2 and 3.

References

Nurdin A, Hoshi Y, Yoneyama T, et al. Global and Targeted Proteomics of Prostate Cancer Cell Secretome: Combination of 2-dimensional image-converted analysis of liquid chromatography and mass spectrometry and in silico selection selected reaction monitoring analysis. *J Pharm Sci* 2016; 105: 3440-3452.

Hoshi Y, Uchida Y, Tachikawa M, et al. Actin filament-associated protein 1 (AFAP-1) is a key mediator in inflammatory signaling-induced rapid attenuation of intrinsic P-gp function in human brain capillary endothelial cells. *J Neurochem* 2017; 141: 247-262.

Supplementary method 6 *In situ* brain perfusion

Rats were anaesthetized with a mixture of ketamine hydrochloride (125 mg/kg body weight) and xylazine hydrochloride (1.22 mg/kg body weight), and kept warm on a hot plate. The external carotid arteries were exposed on the left side by midline incision at the neck, and cannulated using polyethylene tubing (SP-10; Natume, Tokyo, Japan) to allow perfusion of modified Ringer's solution (128 mM NaCl, 4.2 mM KCl, 2.4 mM KH_2PO_4, 24 mM $NaHCO_3$, 0.9 mM $MgCl_2 \cdot 6H_2O$, 1.5 mM $CaCl_2$, 9 mM D-glucose, 3.9% w/v 100 kDa dextran, 0.1% w/v BSA, pH 7.4) as described by Hosoya et al. (2001). Rats received 30 min constant infusion of DMSO (vehicle), 328 μM imatinib, or 328 μM PP2 dissolved in modified Ringer's solution at 50 μL/min for 30 min (Harvard pump 11; Harvard Apparatus Inc., MA). All vehicle controls were infused with the same concentrations of DMSO as used in the test conditions. During the infusion, the final concentrations of imatinib and PP2 at the vessel lumen of the BBB were close to 5 μM, because the blood flow rate at the internal carotid artery was estimated to be 840 μL/min [Pardridge et al., 1985]. After pre-infusion, a solution of 1 μM quinidine or 1 μM cortisol with 500 μM raffinose and 5 mM hydrogen peroxide dissolved in modified Ringer's solution (37 °C) was perfused at 2 ml/min for 10 min. For imatinib- or PP2-treated rats, 275 nM imatinib or 275 nM PP2 was simultaneously perfused. This concentration was estimated to be the same as the free concentration of 5 μM imatinib in plasma, because the free fraction of imatinib in rat plasma is reported to be 5.5% [Kretz et al., 2004]. At the end of the perfusion, rats were sacrificed, and cerebrum, hippocampus, and hypothalamus of the left hemisphere were collected and weighed. Tissues were homogenized in 10 mM ammonium acetate (4 mL : 1 g tissue) and then 300 μL (whole cerebrum), 120 μL (hippocampus) or 60 μL (hypothalamus) of homogenate and 1200 μL of acetonitrile containing 1% formic acid and internal standard (1 μM cinchonine for quinidine; 500 μM sucrose for raffinose; 1 μM dexamethasone for cortisol) were mixed and shaken for 20 min. The homogenates were centrifuged at 4°C and 17,360 g for 5 min. The supernatant was collected and evaporated by centrifugation under vacuum, and the residue was reconstituted in 100 μL of 0.1% aqueous formic acid. This solution was centrifuged at 4°C and 17,360 g for 5 min and the supernatant was subjected to LC-MS/MS analysis. Perfusate solutions were diluted 10-fold with 0.1% formic acid in water and mixed with internal standards. Aliquots were used for LC-MS/MS analysis.

The LC-MS/MS analyses for the quantification of low-molecular-weight molecules were performed with a QTRAP5500 coupled with an UPLC system (Waters, Milford, MA). For quinidine and cortisol, AQCUITY UPLC BEH C18 (2.1 × 50 mm, 1.7 μm particles; Waters) at a flow rate of 500 μL/min was used. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The gradient sequences were 1% B (0-1 min), increased to 1-100% B (1-3 min), maintained at 100% B (3-4 min), reduced to 100-1% B (4-4.1 min) and then maintained at 1% B (4.1-6 min). The source/gas parameters were as follows: ion source gas 1 (50 psi for quinidine; 60 psi for cortisol), ion source gas 2 (50 psi for quinidine; 40 psi for cortisol), curtain gas (15 psi for quinidine; 20 psi for cortisol), collision gas (6 psi for quinidine; 12 psi for cortisol), ion spray voltage floating (5500 V for quinidine and cortisol), interface heater temperature (600°C for quinidine and cortisol). Quinidine, cinchonine, cortisol and dexamethasone were positively ionized and monitored at 325.3/160.3, 295.3/130.1, 363.3/121.0 and 393.1/373.3, respectively. For raffinose, ZIC®-pHILIC (2.1 x 100 mm, 5 μm particles; Millipore) at a flow rate of 250 μL/min was connected to the UPLC system. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. The gradient sequences were 98% B (0 min), reduced to 98-30% B (0-6 min), maintained at 30% B (6-7.6 min), increased to 30-98% B (7.6-7.7 min) and then maintained at 98% B (7.7-10 min). The source/gas parameters were as follows: ion source gas 1 (60 psi), ion source gas 2 (60 psi), curtain gas (30 psi), collision gas (10 psi), ion spray voltage floating (-4500 V), interface heater temperature (550°C). Raffinose and sucrose were negatively ionized

and monitored with 503.2/179.1 and 341.1/88.8, respectively. The brain-to-perfusate concentration ratio (R_{br}, μL/g tissue) was calculated individually for each rat by dividing the tissue concentration (mol/g tissue) by the perfusate concentration (mol/μL).

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