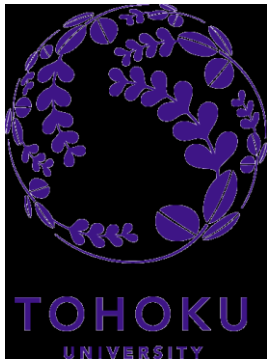

"10th Meet the Experts: The Transporter Conference – 2019" at JW Marriott Hotel, Seoul, South Korea

12:40 – 13:20 (30 min + 10 min), Nov., 14, 2019

Keynote:

Regulation Mechanism of P-gp in the Blood-Brain Barrier

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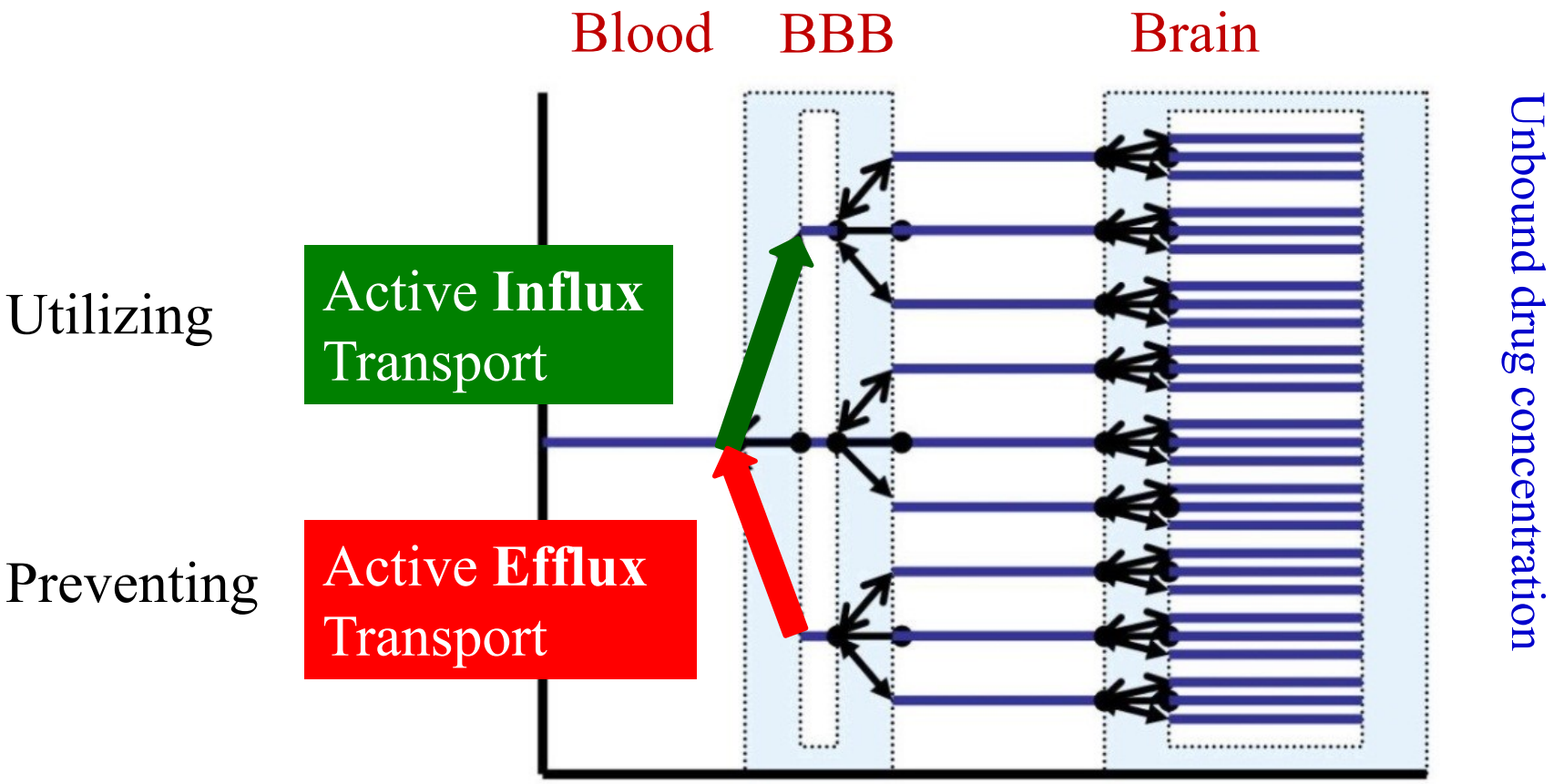
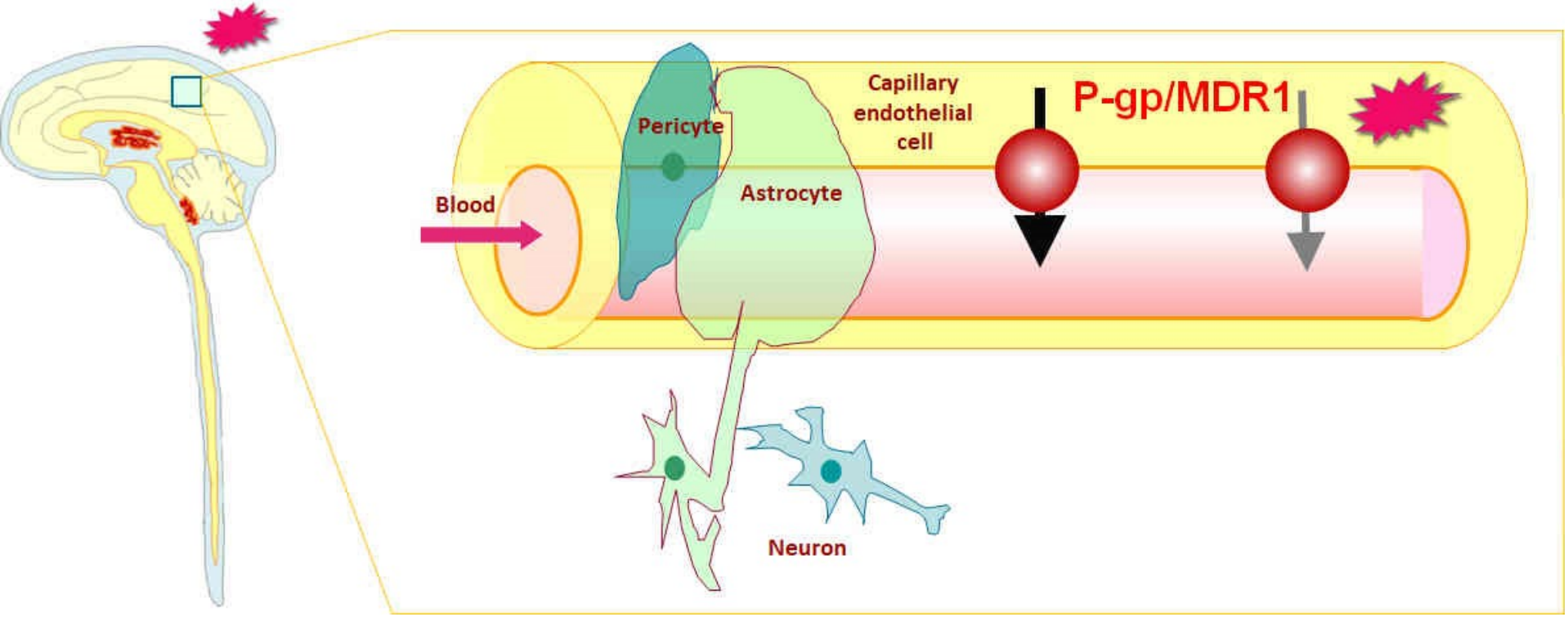
BBB transport activity generate the unbound drug concentration gradient between brain and blood (Kp, uu)



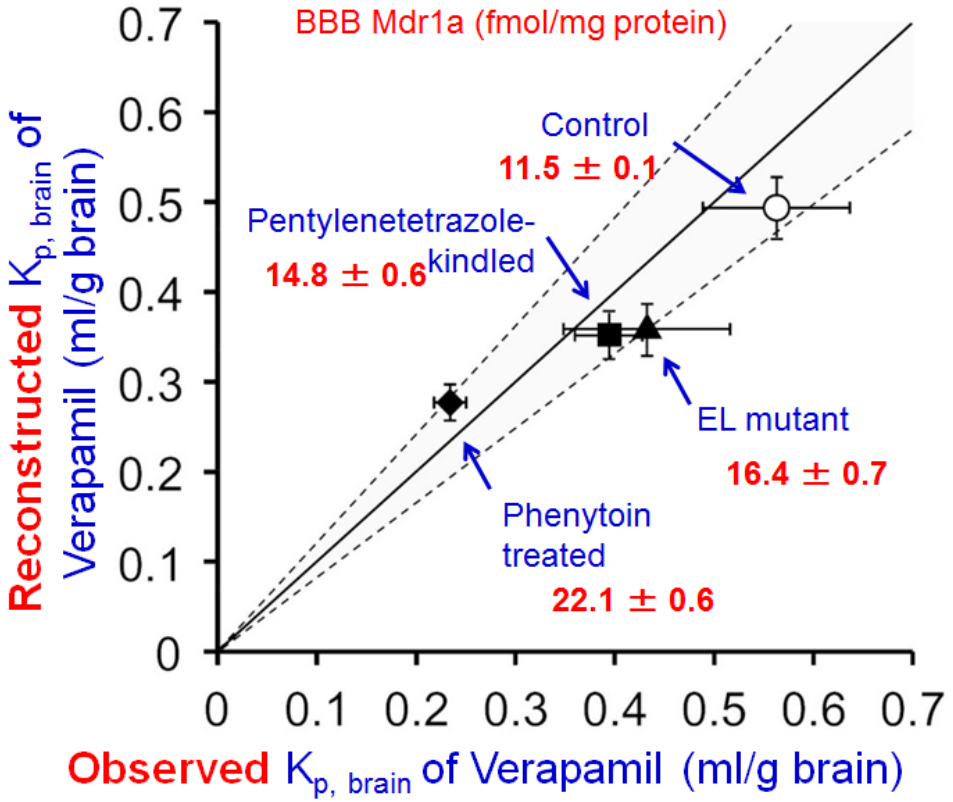
Now, we could reconstruct/predict the gradient (Kp, uu) generated by P-gp efflux function in the animal model.

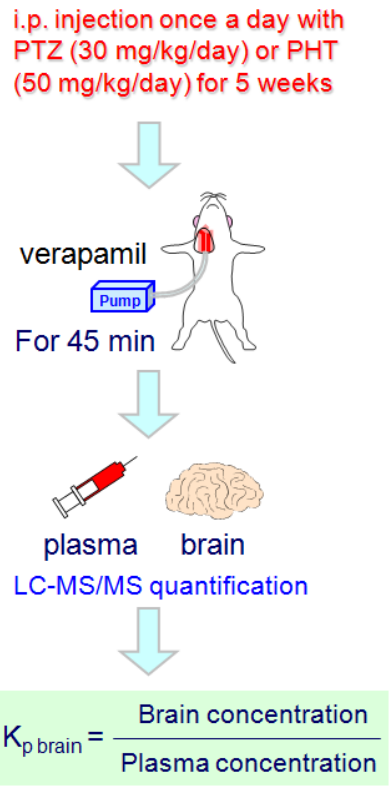
Understanding changes in transporter function and regulatory mechanisms at the blood–brain barrier (BBB) is important to predict and control drug uptake into the brain in patients with central nervous system (CNS) diseases.



P-glycoprotein (P-gp) is the most important drug efflux transporter at the BBB. However, P-gp transport activity is altered in several CNS diseases.

Assuming the intrinsic P-gp activity is the same to that of normal mice, a significant coincidence was demonstrated for the observed Kp,brain and the reconstructed Kp brain values of verapamil in PTZ-kindled, EL, PHT-treated, and control mice





Chronic phase

5 weeks treatment

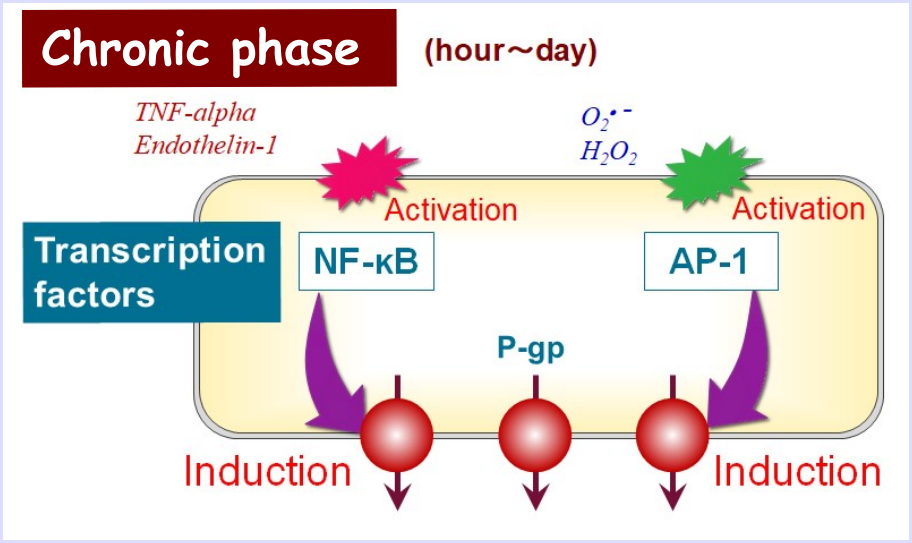
Cited from our paper published in Drug Metab Dispos. 42: 1719-1726 (2014).

Induction of P-gp protein is determinant for in vivo P-gp efflux activity in the epileptic model and Phenytoin treated mouse.

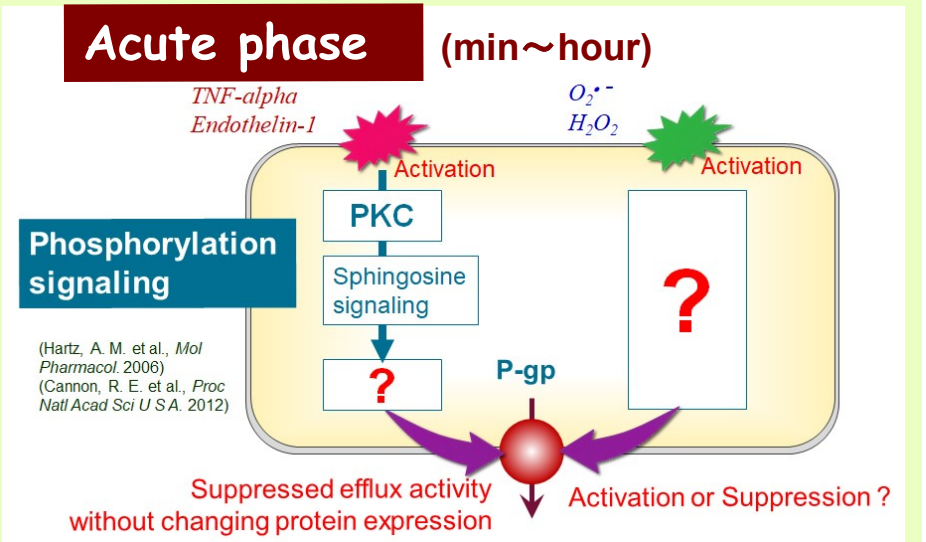
Molecular mechanism of induction will be

Glutamate initiated; NfκB Drug initiated; Nuclear receptor

Inflammation and oxidative stress are associated with a variety of CNS disease.



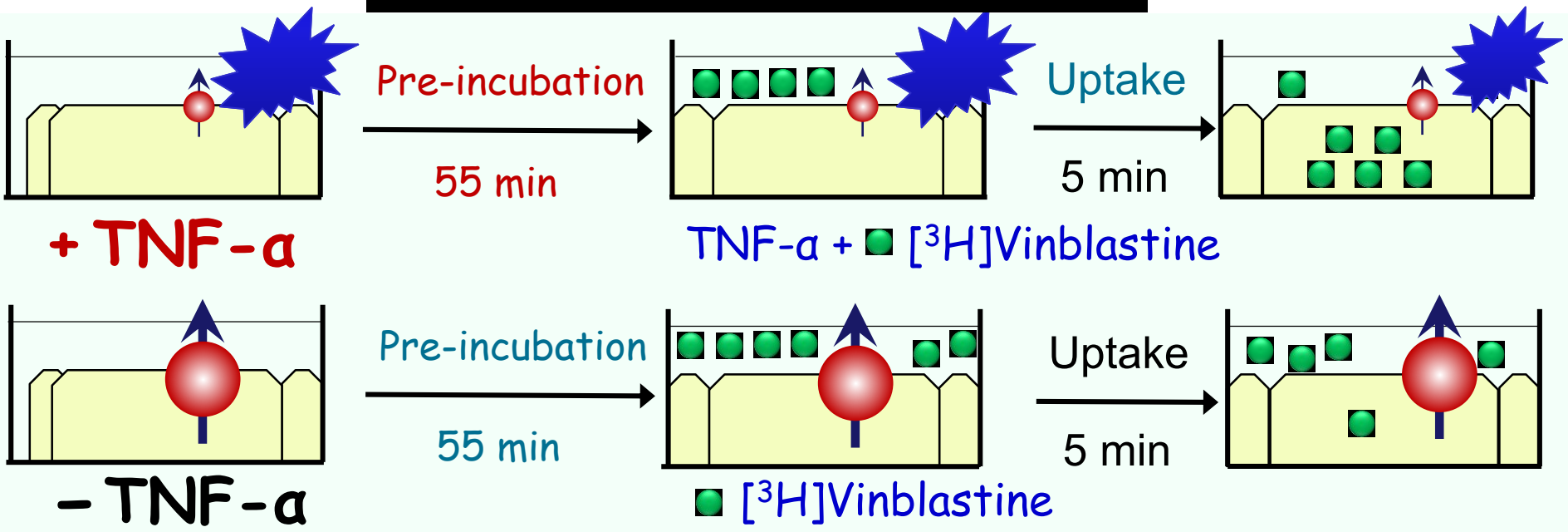
NF-κB and AP-1 are known to the key molecule of P-gp induction in the chronic disorder



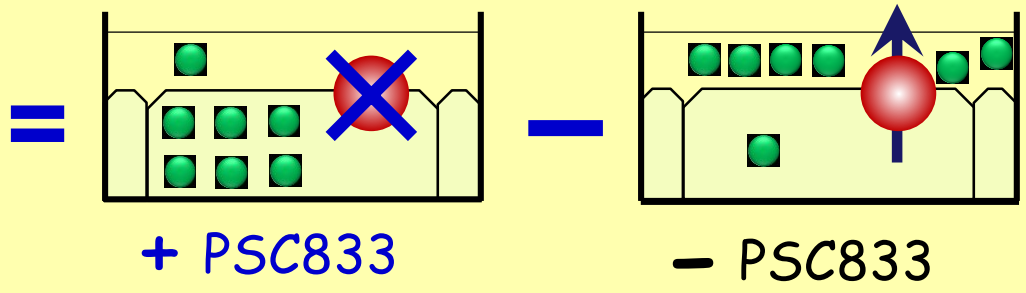
Only limited information is available for the regulatory mechanism of P-gp function in the acute phase of CNS disorder

- ✓ The purpose of the present work was to clarify regulatory mechanism in inflammatory or oxidative stress-induced decrease in apparent P-gp/MDR1 efflux function in human brain capillary endothelial cells.
- ✓ TNF-alpha treatment and hydrogen peroxide treatment were used for inflammation model and oxidative stress model, respectively.

TNF-α effect was evaluated



P-gp efflux activity was estimated from the difference of substrate accumulation in the presence and absence of P-gp inhibitor



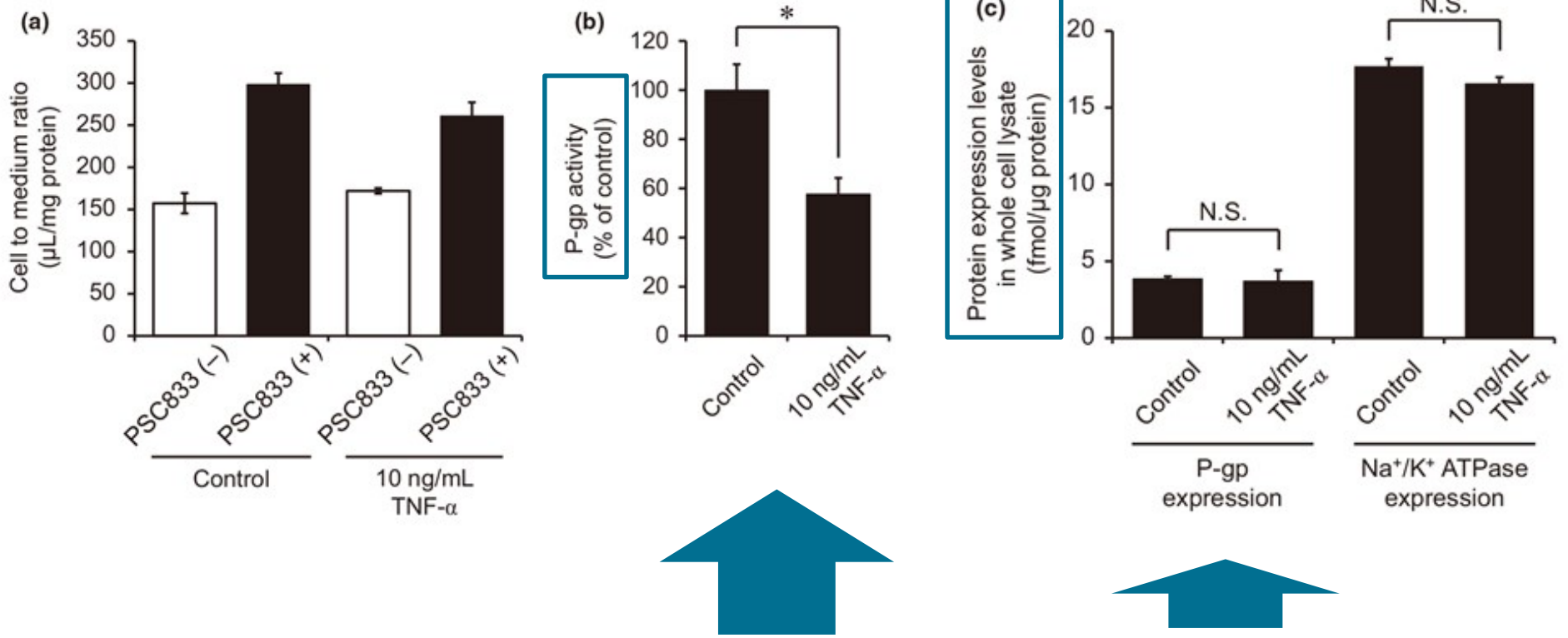
Inflammation model: TNF-α (10 ng/mL x 1 hour) treatment

In vitro human BBB model: hCMEC/D3 cell line

P-gp substrate : [3H]Vinblastine

P-gp inhibitor : PSC833

Effect of inflammatory mediator tumor necrosis factor-a (TNF-a) on P-gp efflux activity and protein expression level in hCMEC/D3 cells



TNF-a treatment reduced P-gp efflux activity significantly

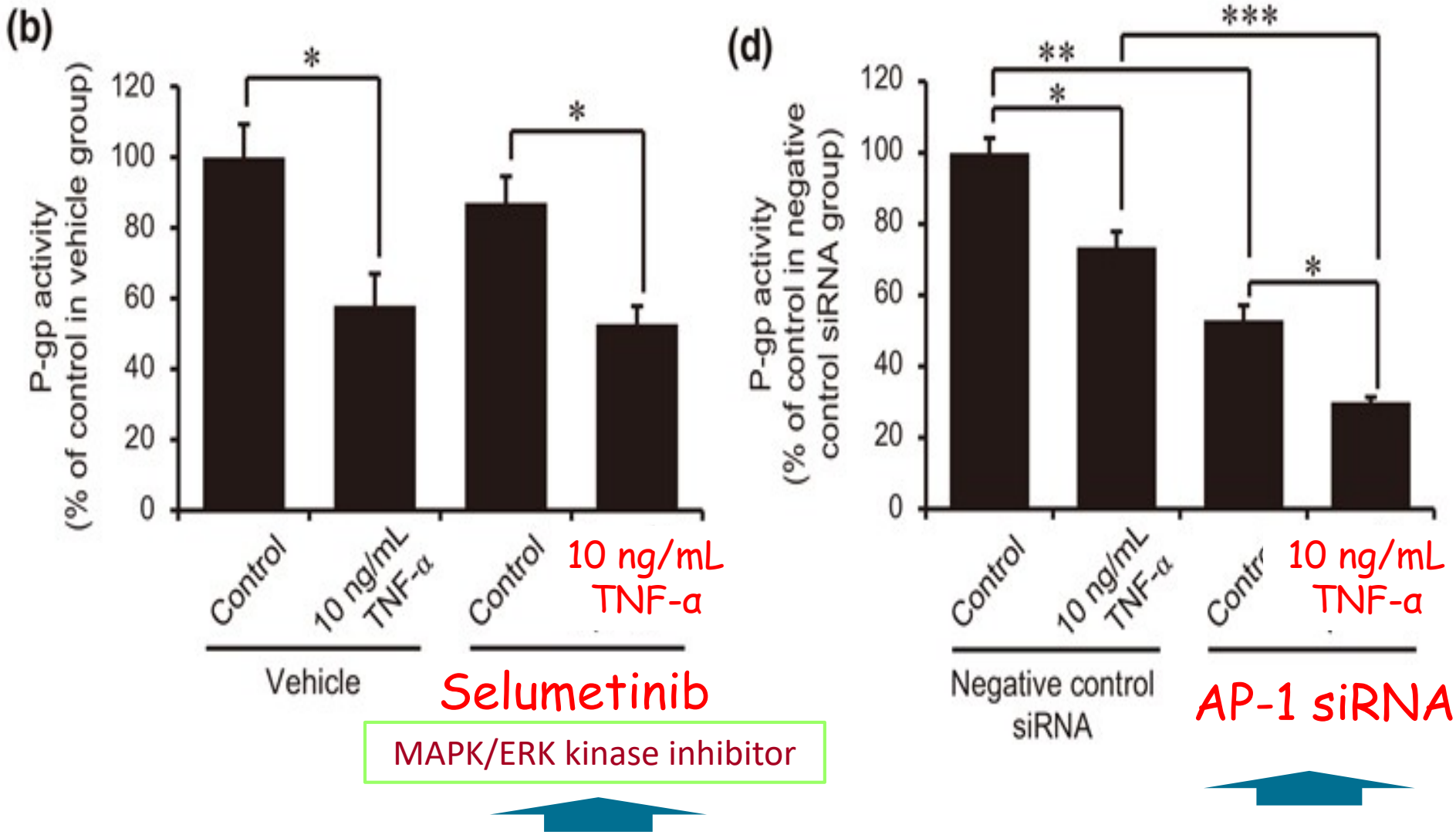
P-gp protein expression was not changed

To clarify the non-transcriptional mechanism that causes the decrease of intrinsic efflux activity of P-gp in acute inflammation, we applied comprehensive quantitative phosphoproteomics to compare hCMEC/D3 cells treated with TNF-α and vehicle (control).

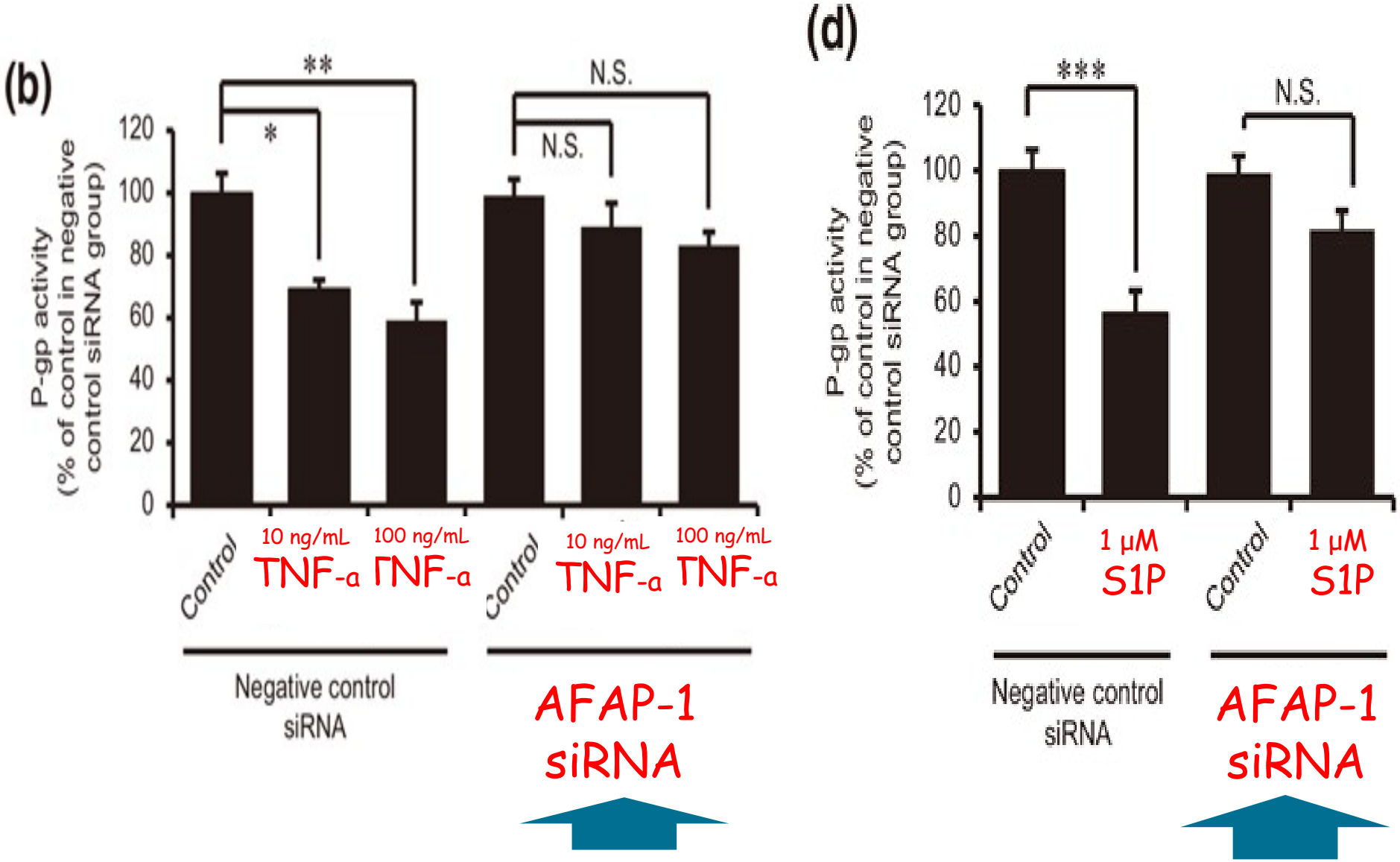
Table 3 Three candidate proteins selected based on the results of phosphoproteomic analysis

Protein name		Peak intensity ratio (10 ng/mL TNF-α/control)	
		1 h treatment	6 h treatment
Actin filament-associated protein 1 (AFAP-1)	AFAP-1	x 3.96 increase	Not detected[a]
Mitogen-activated protein kinase 1 (MAPK)	MAPK	x 3.03 increase	Not detected[a]
Transcription factor AP-1 (AP-1)	AP-1	x 5.08 increase	2.74
		x 4.09 increase	5.28

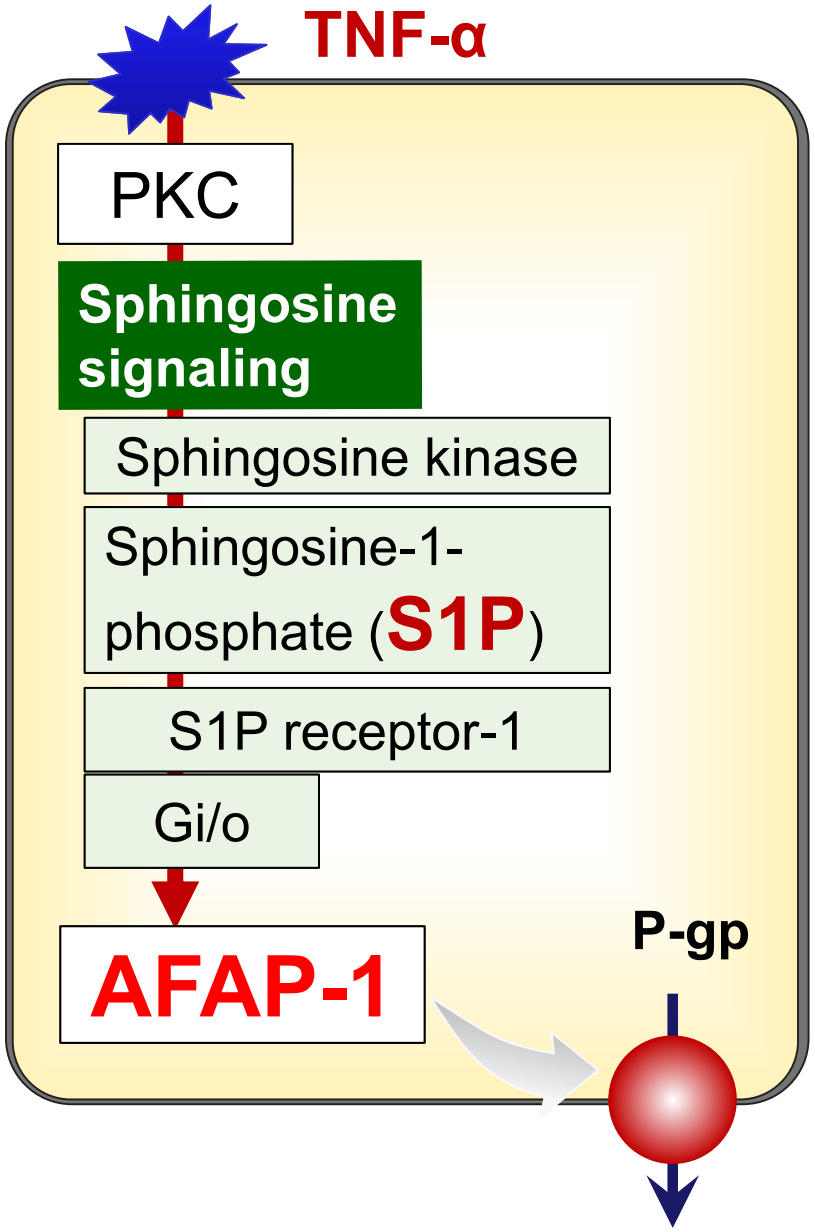
Actin filament-associated protein-1 (AFAP-1), MAPK1, and transcription factor AP-1 (AP-1) were **significantly phosphorylated** in TNF-α-treated cells, and were selected as candidate proteins.



Attenuation of P-gp efflux activity by TNF-α was ***NOT*** *blocked* by MAPK inhibitor or AP-1 depletion.



Attenuation of P-gp efflux activity by TNF-a or sphingosine-1-phosphate (S1P) was ***blocked*** by **knockdown of AFAP-1**



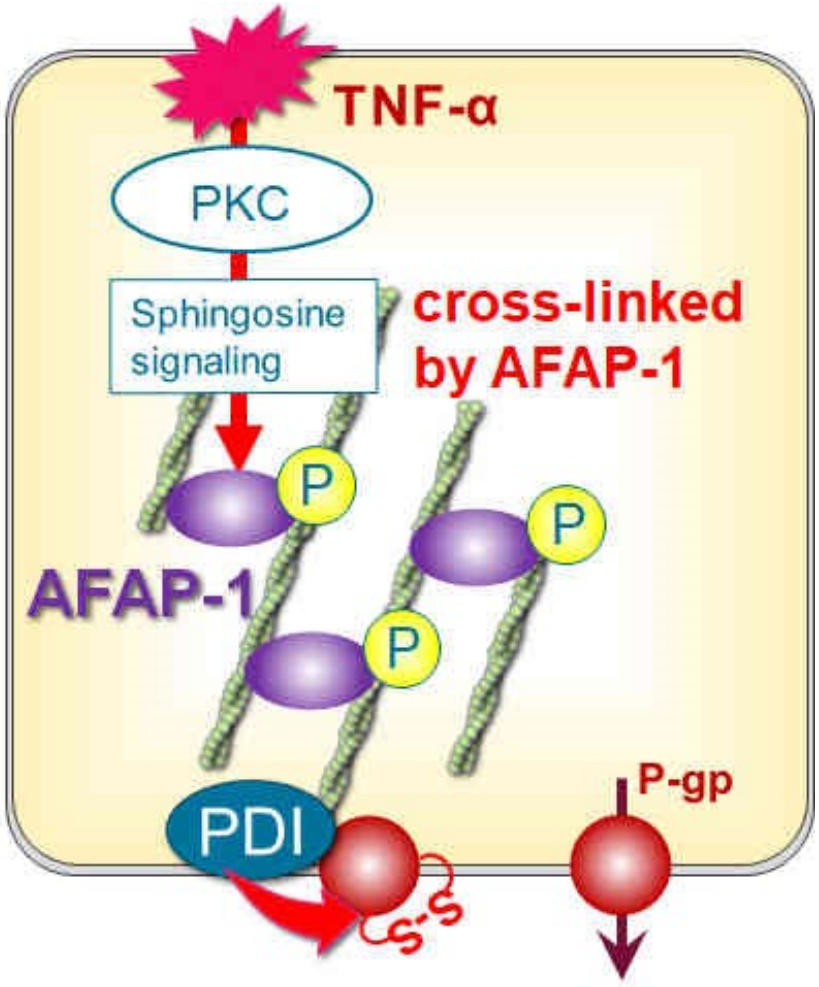
(Hartz, A. M. et al., *Mol Pharmacol.* 2006)
(Cannon, R. E. et al., *Proc Natl Acad Sci U S A.* 2012)

Among the molecules identified in the inflammatory signaling pathway regulating the P-gp efflux activity at the BBB, S1P, and its receptor are located downstream of PKC activation (Cannon et al. 2012).

The AFAP-1 siRNA treatment attenuated the reduction in P-gp efflux activity by S1P, supporting the idea that **AFAP-1 is located downstream of S1P.**

- ✓ AFAP-1 protein was detected only in the cytosol fraction, whereas P-gp was localized mostly in the plasma membrane fraction.
- ✓ AFAP-1 may not directly regulate P-gp function at the plasma membrane.

		Protein expression levels (fmol/μg protein)			
		Negative control siRNA		AFAP-1 siRNA	
Protein name	Subcellular fraction	Vehicle	10 ng/mL TNF-α	Vehicle	10 ng/mL TNF-α
AFAP-1	Cytosol fraction	1.58 ± 0.22	1.50 ± 0.22$^{N.S.}$	ULQ (< 0.495)	ULQ (< 0.493)
	Plasma membrane fraction	ULQ (< 0.552)	ULQ (< 0.556)	ULQ (< 0.552)	ULQ (< 0.562)
	Whole-cell lysate	ULQ (< 0.592)	ULQ (< 0.566)	ULQ (< 0.590)	ULQ (< 0.606)
P-gp	Cytosol fraction	ULQ (< 0.659)	ULQ (< 0.679)	ULQ (< 0.668)	ULQ (< 0.704)
	Plasma membrane fraction	8.47 ± 0.22	9.67 ± 0.93$^{N.S.}$	11.4 ± 1.0	10.7 ± 0.4$^{N.S.}$
	Whole-cell lysate	2.01 ± 0.29	2.11 ± 0.16$^{N.S.}$	2.62 ± 0.25	2.27 ± 0.02$^{N.S.}$
Na^+/K^+ ATPase	Cytosol fraction	ULQ (< 0.659)	ULQ (< 0.718)	ULQ (< 0.667)	ULQ (< 0.757)
	Plasma membrane fraction	51.4 ± 2.8	53.0 ± 1.9$^{N.S.}$	54.0 ± 2.3	49.5 ± 3.5$^{N.S.}$
	Whole-cell lysate	12.6 ± 0.9	11.9 ± 0.2$^{N.S.}$	13.5 ± 0.2	14.6 ± 1.0 $^{N.S.}$

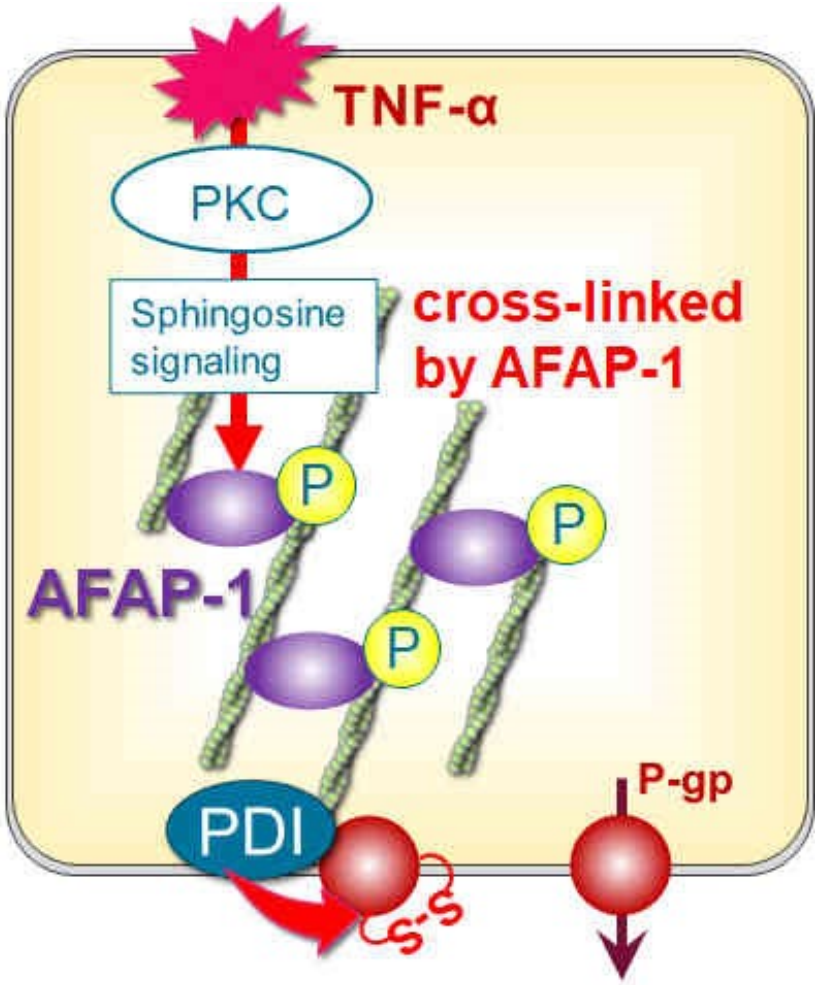


Reduce P-gp activity

- ✓ Recently, protein disulfide isomerase (PDI) was identified as P-gp binding protein in rat brain microvessels (Tome et al. 2015). PDI was reported to interact with β-actin (Sobierajska et al.2014).
- ✓ Cell surface expression of PDI was inhibited by actin filament disruption (Wan et al. 2012) and because AFAP-1 is necessary to form the actin stress fibers (Dorfleutner et al.2007).

- ✓ These findings raise the possibility that **AFAP-1 is associated with the interaction between P-gp and PDI through the actin filaments.**

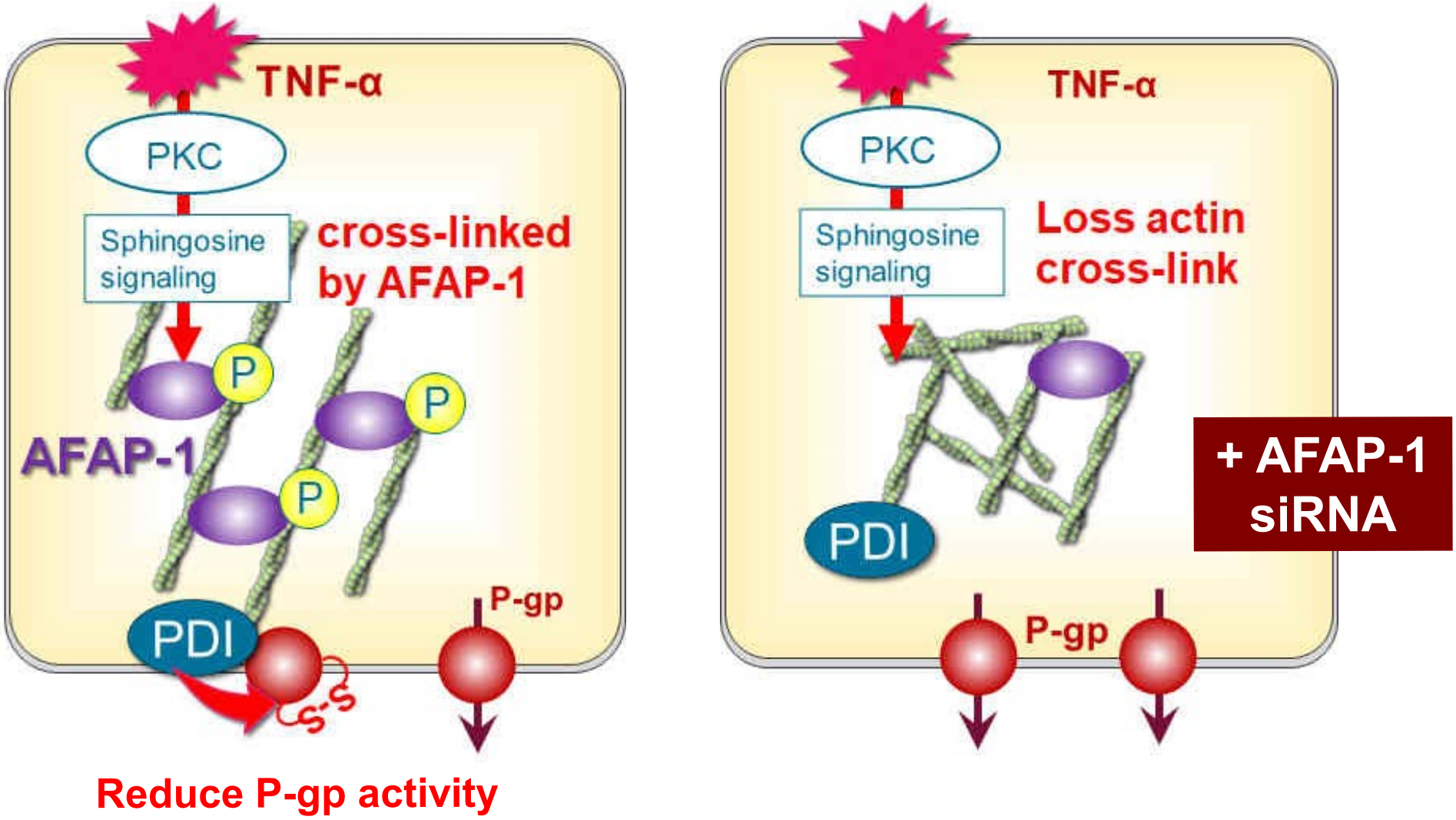
(Tome, M. E et al., *J Neurochem.* 2015) (Sobierajska, K. et al., *J Biol Chem.* 2014) (Dorfleutner A. et al., *J Cell Physiol.* 2007) (Wan SW. et al., *J Cell Biochem.* 2012) (Wilkinson, B. and Gilbert, H. F. et al., *Biochim Biophys Acta.* 2004) (Urbatsch, I. L. et al., *J Biol Chem.* 2001)



Reduce P-gp activity

- ✓ Interestingly, the ATPase activity of purified wild-type P-gp was activated by dithiothreitol, which can reduce disulfide bonds (Urbatsch et al. 2001).
- ✓ The primary role of PDI is introducing disulfide bonds into proteins (Wilkinson and Gilbert 2004).
- ✓ This may indicate that **the formation of disulfide bonds in human wildtype P-gp keeps the ATPase activity low.**

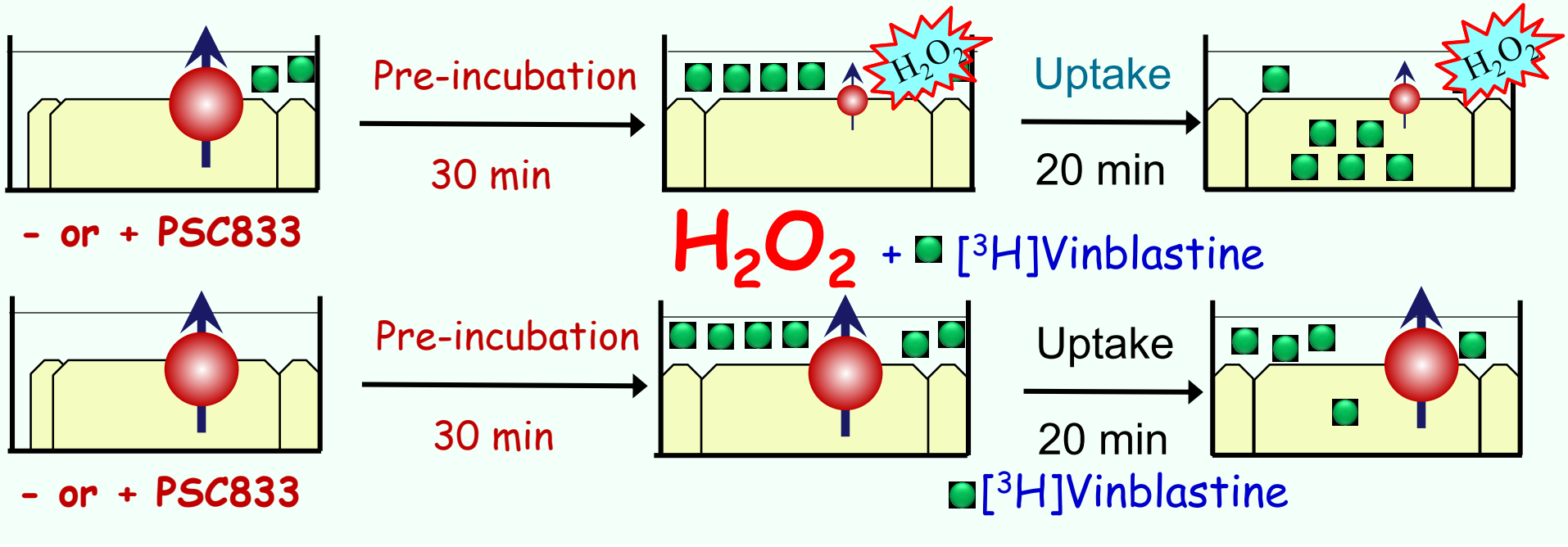
(Tome, M. E et al., *J Neurochem.* 2015) (Sobierajska, K. et al., *J Biol Chem.* 2014) (Dorfleutner A. et al., *J Cell Physiol.* 2007) (Wan SW. et al., *J Cell Biochem.* 2012) (Wilkinson, B. and Gilbert, H. F. et al., *Biochim Biophys Acta.* 2004) (Urbatsch, I. L. et al., *J Biol Chem.* 2001)



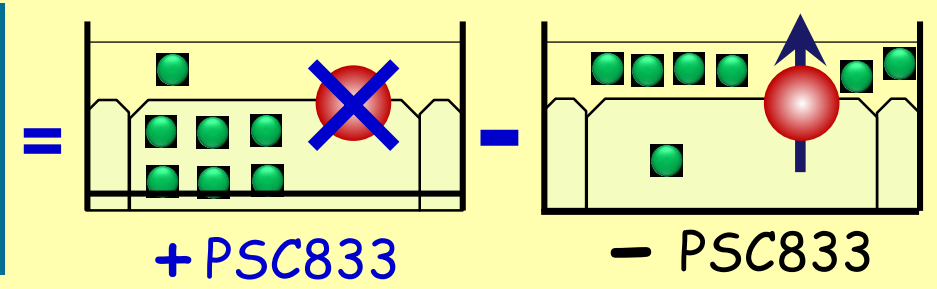
Reduce P-gp activity

In conclusion, AFAP-1 is a key molecule in the TNF-a-mediated inflammatory signaling pathway that leads to a rapid decrease in P-gp intrinsic transport activity without its translocation in human brain capillary endothelial cells.

H_2O_2 effect was evaluated

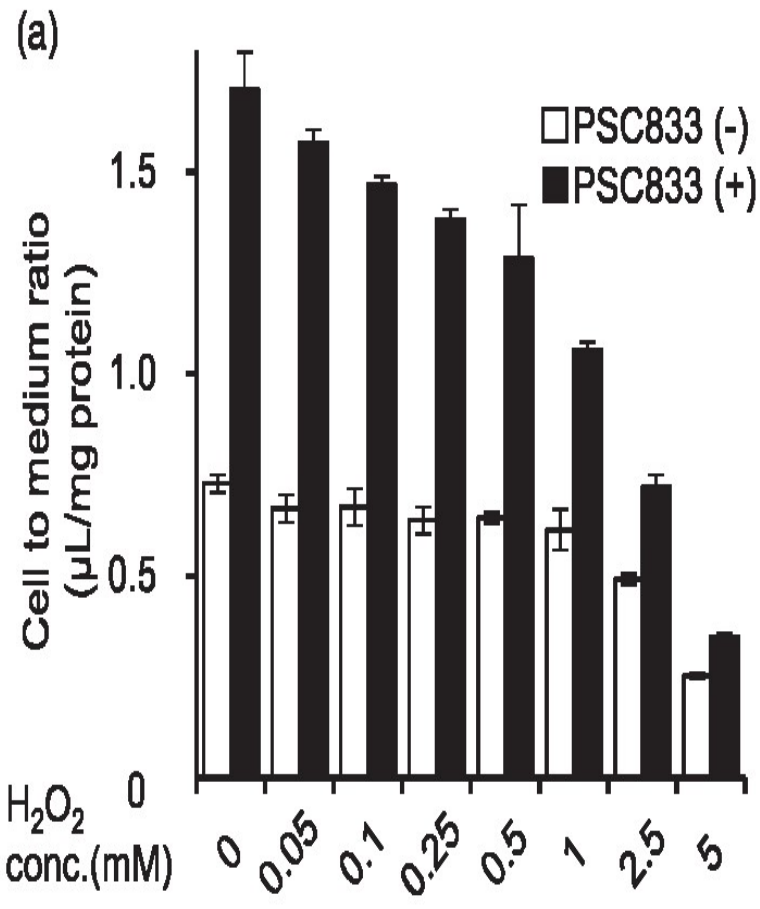


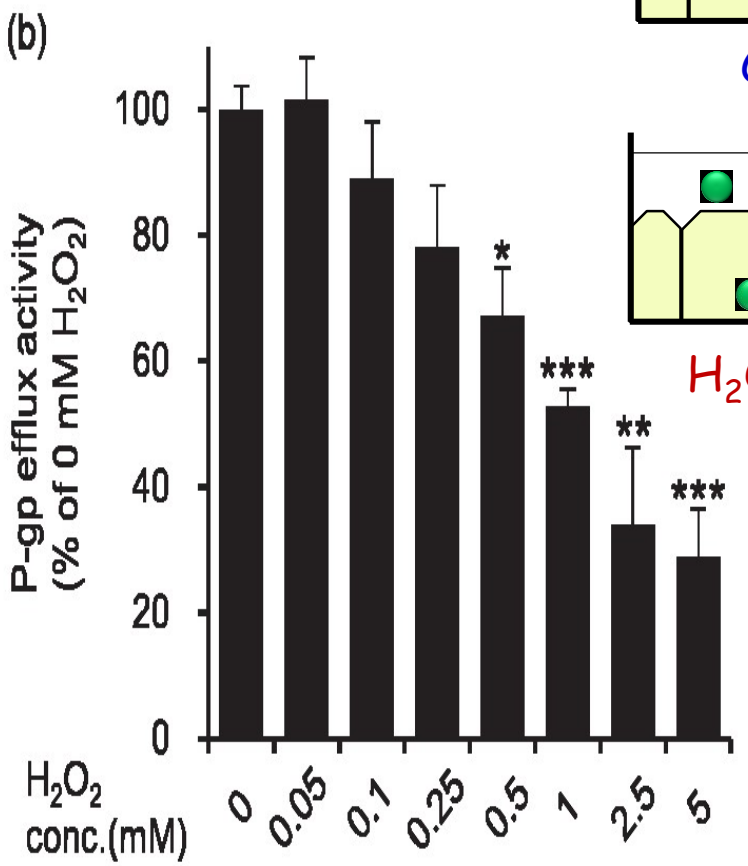
P-gp efflux activity was estimated from the difference of Vinblastine accumulation in the presence and absence of P-gp inhibitor

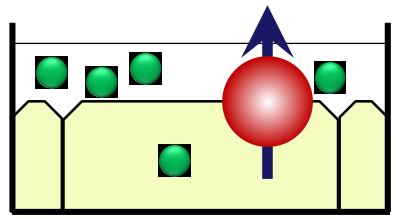


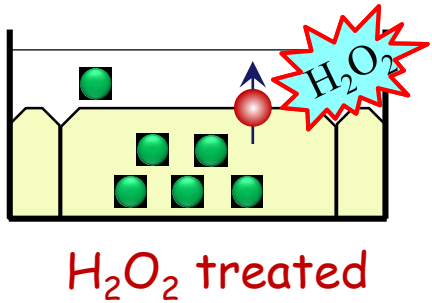
Oxidative stress model: H_2O_2 (0.05 ~ 5 mM x 20 min) treatment
In vitro human BBB model: hCMEC/D3 cell line
P-gp substrate : [^{3}H]Vinblastine
P-gp inhibitor : PSC833

Oxidative stress to the P-gp function in the BBB

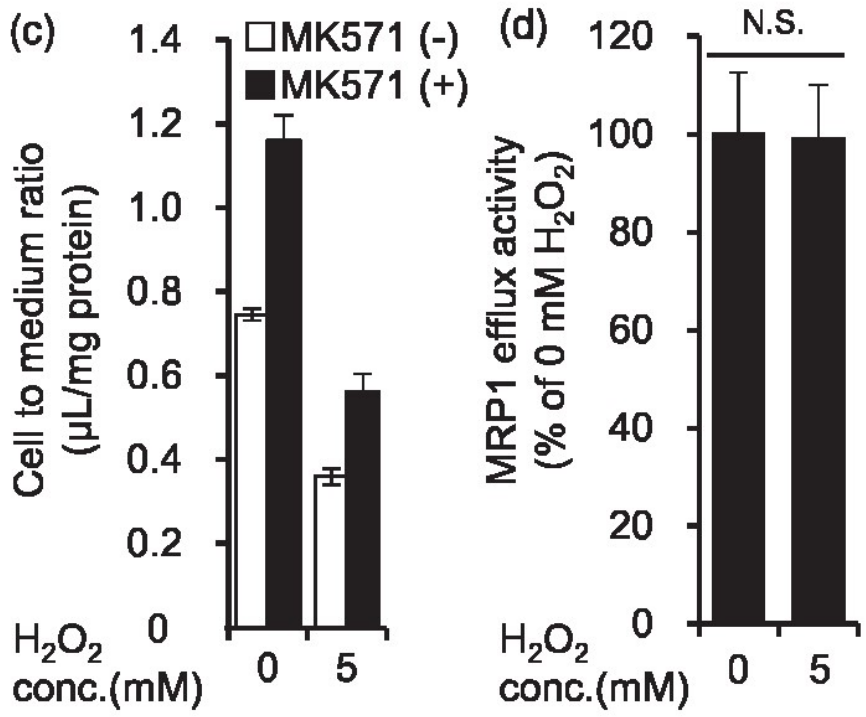








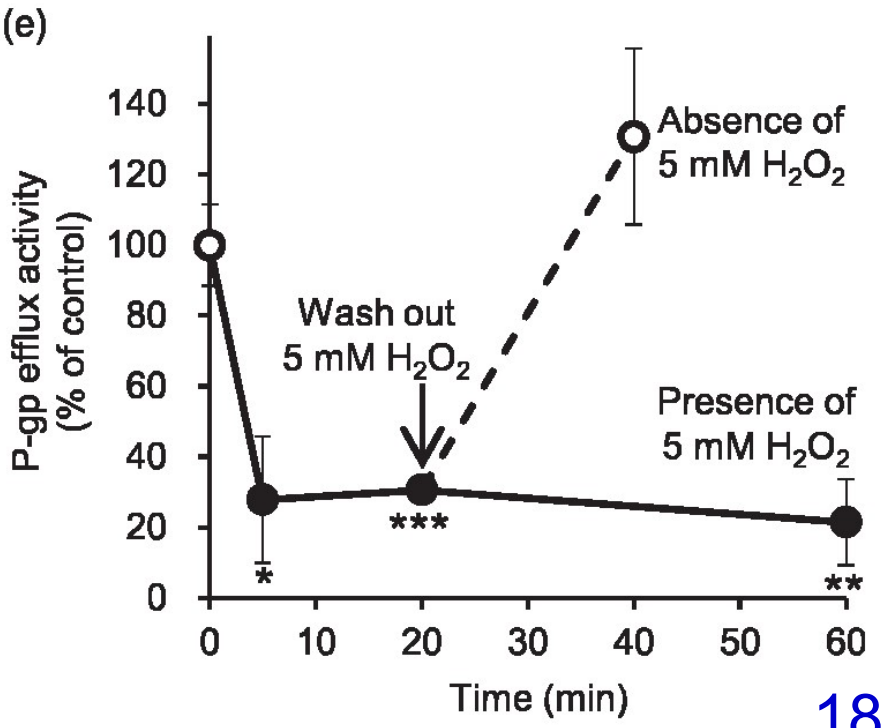
P-gp efflux activity (PSC833 sensitive **vinblastine** efflux) of the human CMEC/D3 was reduced by H_2O_2 treatment in a concentration dependent manner



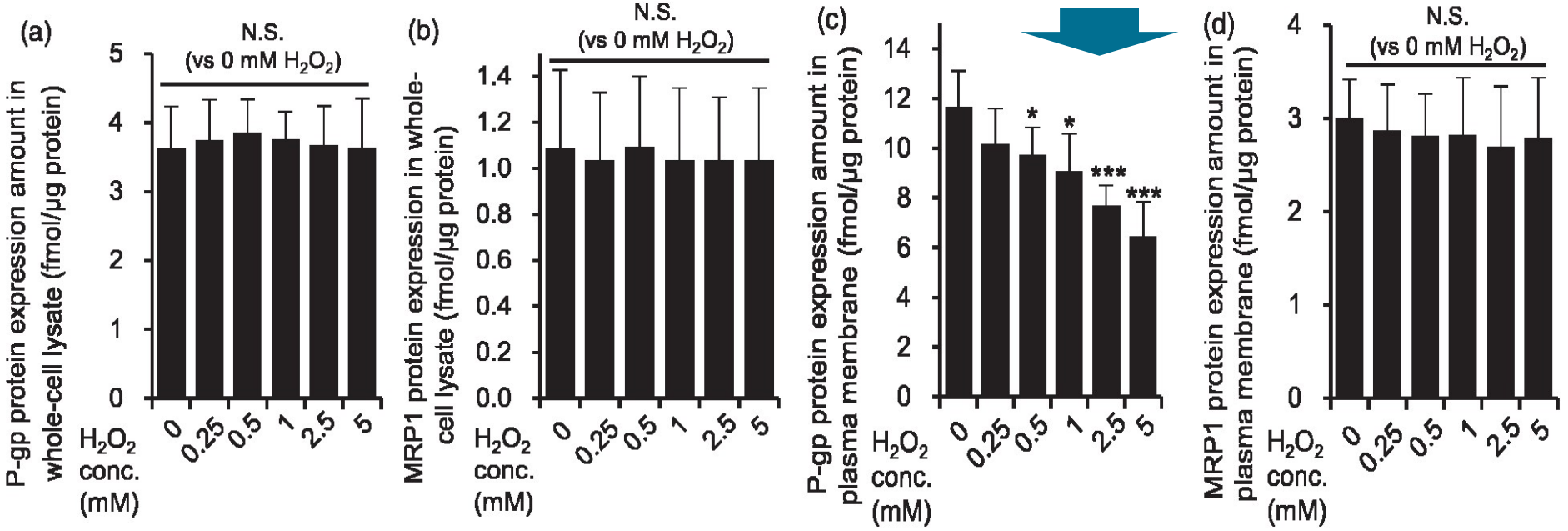
We also examined the effect of MK571, an inhibitor of multidrug resistance-associated protein (MRP) transporters. The MK571-sensitive vinblastine efflux transport activity was not affected by treatment with 5mM H_2O_2 for 20 min.

The reduction of vinblastine efflux transport activity by H_2O_2 **does not involve MRP transporters**

The decrease of P-gp efflux transport activity by H_2O_2 is both **rapid and reversible.**



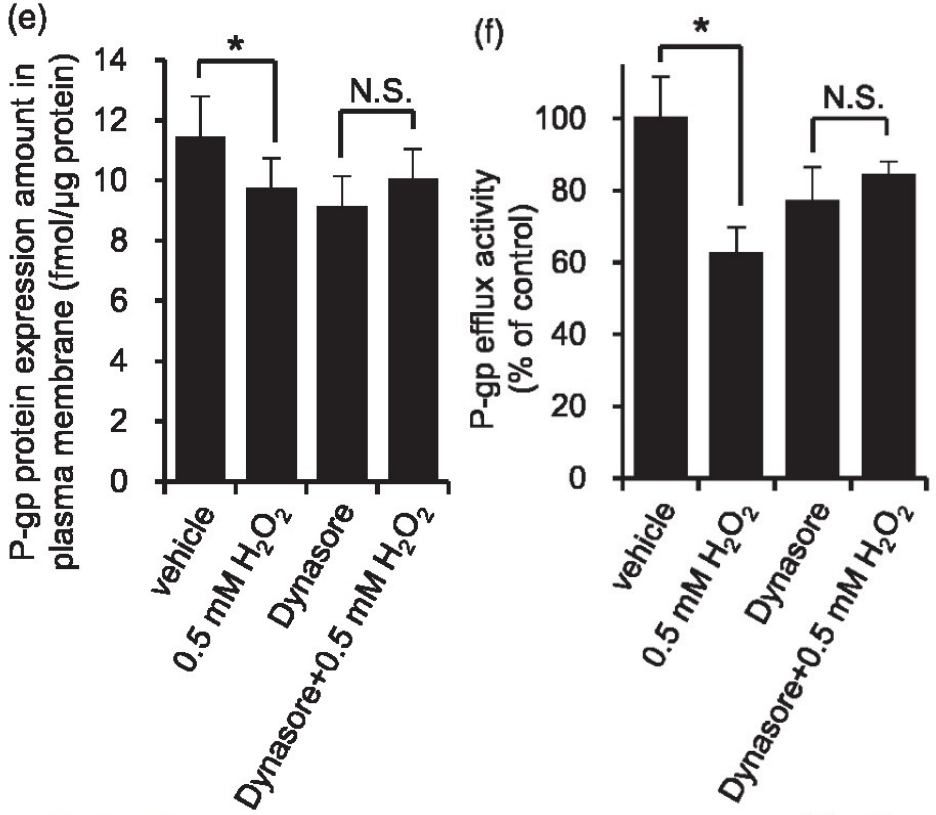
P-gp MRP1 P-gp MRP1



Protein amount in **whole cell** Protein amount in **plasma membrane**

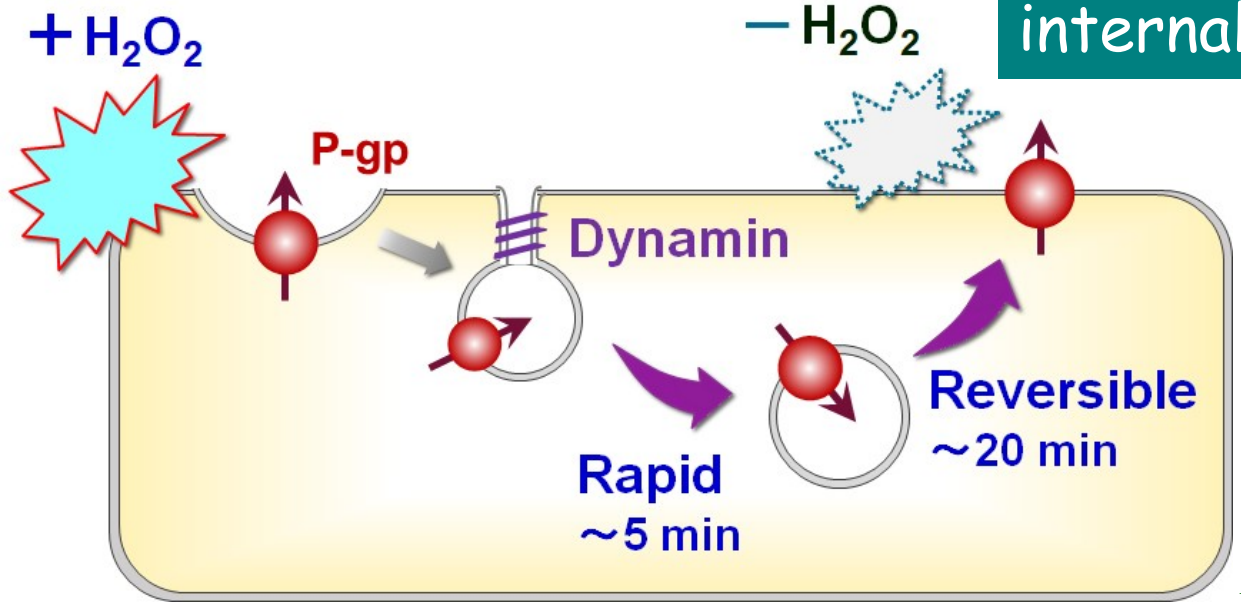
The P-gp protein expression amount in whole-cell lysate did not change during treatment with H_2O_2 . In contrast, P-gp in the plasma membrane fraction was decreased in a H_2O_2 concentration-dependent manner. There was no change in MRP1 expression either in whole-cell lysate or in plasma membrane fraction.

H_2O_2 induces a change of P-gp localization from the cell membrane to an intracellular site.



Dynasore (dynamin GTPase inhibitor) significantly suppressed the decrease of P-gp efflux transport activity and P-gp protein expression in plasma membrane by 0.5mM H_2O_2.

The reduction of P-gp efflux transport activity by H_2O_2 is due to dynamin-dependent internalization of P-gp.



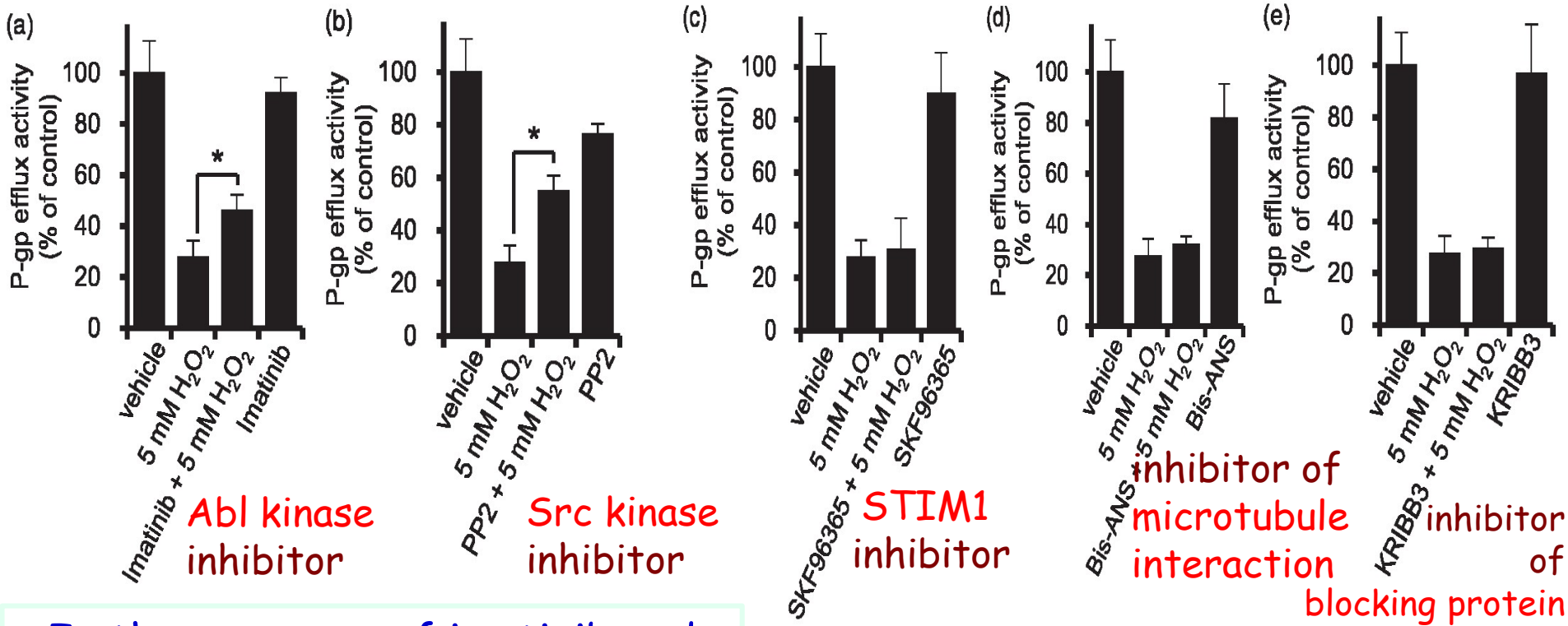
- ✓ Since phosphorylation is a rapid and transient regulatory mechanism, we hypothesized that it could play a role in the change of P-gp efflux function in response to oxidative stress.

- ✓ To find candidate substrates, we conducted comprehensive comparative phosphoproteomics.

- ✓ We focused on the nine phosphorylated proteins that commonly changed their phosphorylation levels in cells treated with 0.5mM and with 5mM H_2O_2.

✓ Among them, we further examined four for which phosphorylation inhibitors are available: **Cav1**, stromal interaction molecule 1 (**STIM1**), microtubule-associated protein 4 (**MAP4**), and heat shock factor binding protein 1 (**HSBP1**).

Description		H_2O_2 Conc.	Average peak intensity (cps)		Peak intensity ratio	Students *t*-test
			H_2O_2	Control	(H_2O_2/control)	p-value
Caveolin-1 (Cav1)		0.5 mM	6221	319	19.5	6.28E-06
		5 mM	16817	878	19.2	2.43E-05
Stromal interaction molecule 1 (STIM1)		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 (MAP4)		0.5 mM	14773	2730	5.41	1.36E-03
		5 mM	12494	3054	4.09	1.92E-04
Platelet endothelial cell adhesion molecule	DIETVYSEVR	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 factor binding protein 1 (HSBP1)		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



Abl kinase inhibitor

Src kinase inhibitor

STIM1 inhibitor

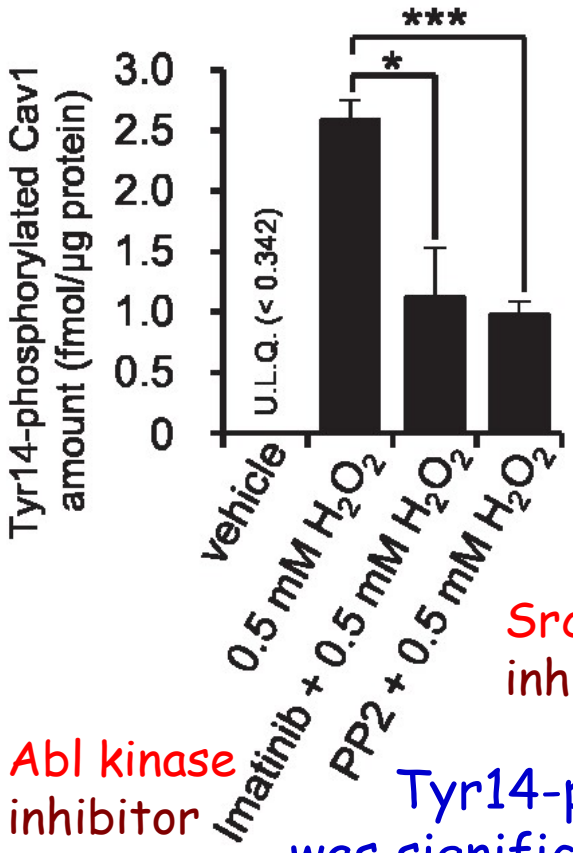
inhibitor of microtubule interaction

inhibitor of blocking protein kinase C dependent phosphorylation of HSPB1

In the presence of imatinib and PP2, the decrease of P-gp efflux transport activity by 5mM H_2O_2 was significantly attenuated.

SKF96365, Bis-ANS and KRIBB3 had no effect.

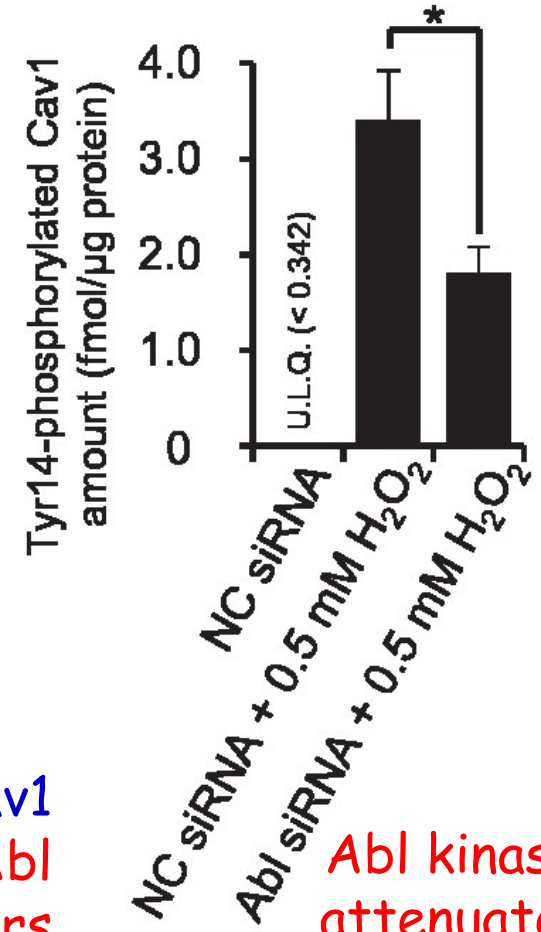
Phosphorylation of Cav1 by Abl kinase and Src kinase is involved in the decrease of P-gp function at the BBB under acute oxidative stress.



Src kinase inhibitor

Abl kinase inhibitor

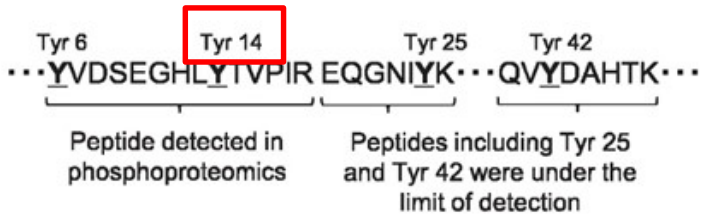
Tyr14-phosphorylated Cav1 was significantly increased. Abl and Src kinase inhibitors suppressed the increase of Tyr14-phosphorylated Cav1



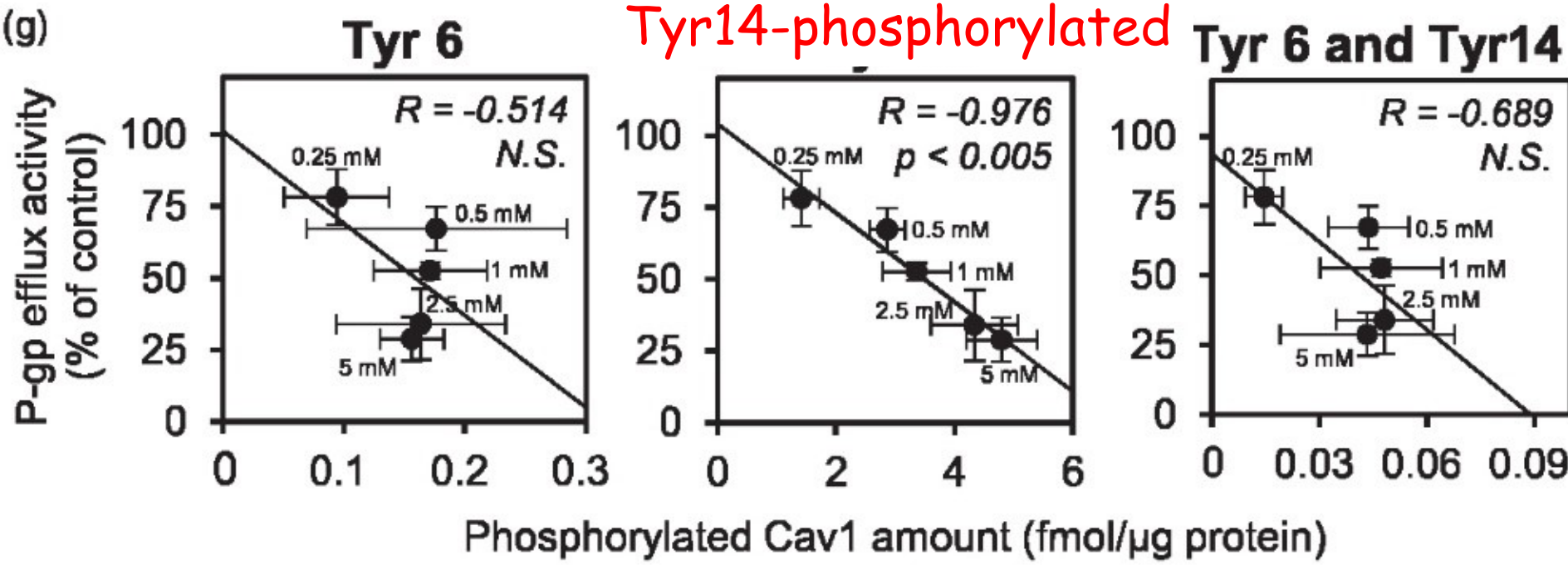
Abl kinase knockdown attenuated the H_2O_2-induced increase of Tyr14-phosphorylated Cav1

The expression amount of Tyr14-phosphorylated Cav1 is regulated through both Abl kinase and Src kinase at the BBB in the presence of oxidative stress.

Cav1 sequence

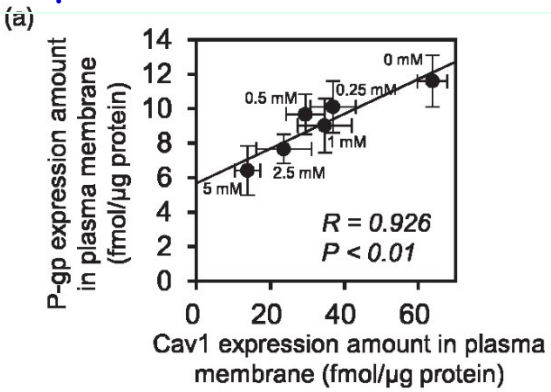


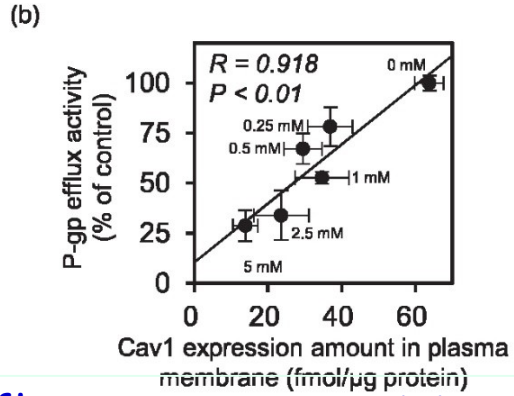
Quantity of Tyr14-phosphorylated peptide was significantly correlated with the decrease in efflux transport activity of P-gp, while Tyr6- or both Tyr6 and Tyr14-phosphorylated peptides were not.



Tyr14 of Cav1 is a major phosphorylation site involved in the decrease of efflux transport activity of P-gp.

A statistically significant correlation between the amounts of P-gp and Cav1 in the plasma membrane fraction.

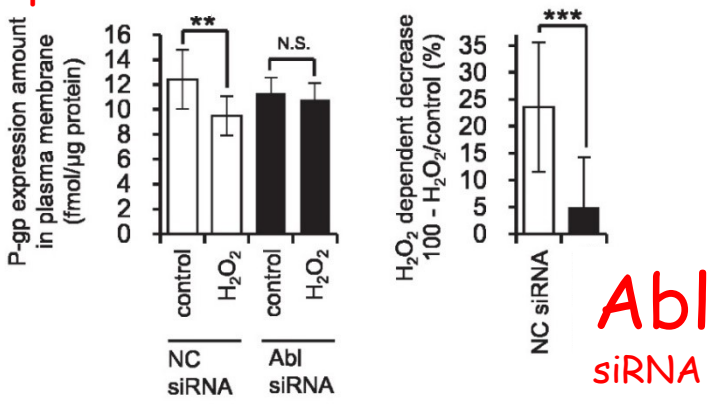




The efflux transport activity of P-gp was also well correlated with the expression level of Cav1 in the plasma membrane fraction.

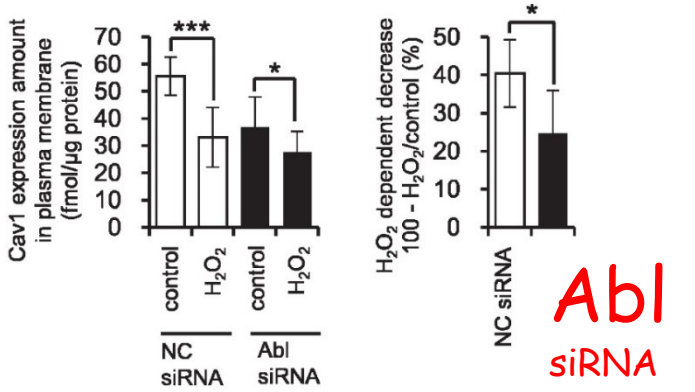
P-gp is internalized with Cav1 at the BBB under conditions of oxidative stress.

P-gp protein



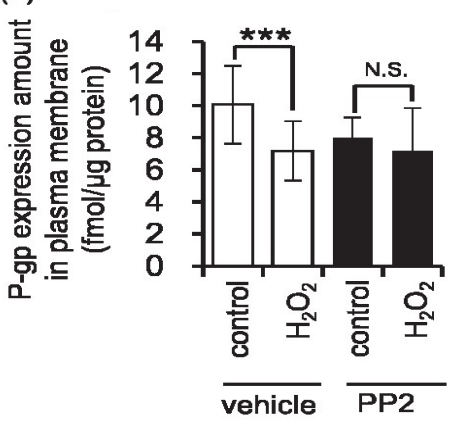
Abl kinase depletion significantly attenuated the H_2O_2-induced decrease in the plasma membrane expression of P-gp.

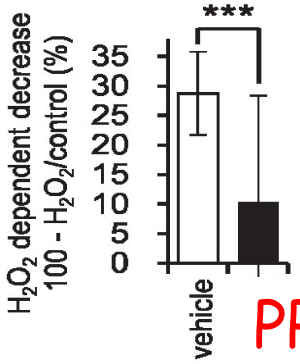
Cav1 protein



The amount of internalized Cav1 was also suppressed.

(d) P-gp protein

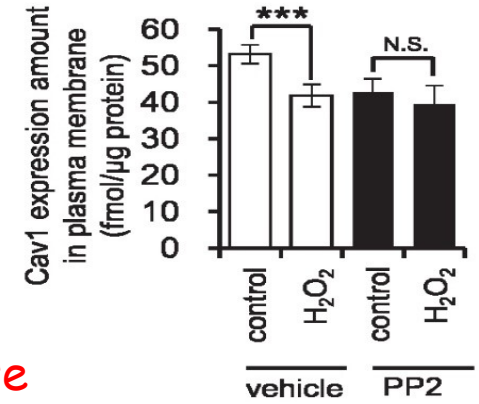


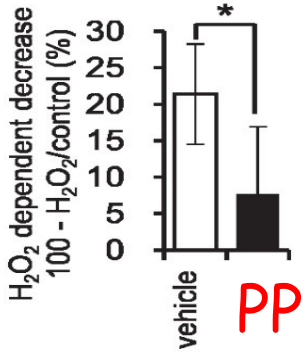


PP2

Src kinase inhibitor

(f) Cav1 protein



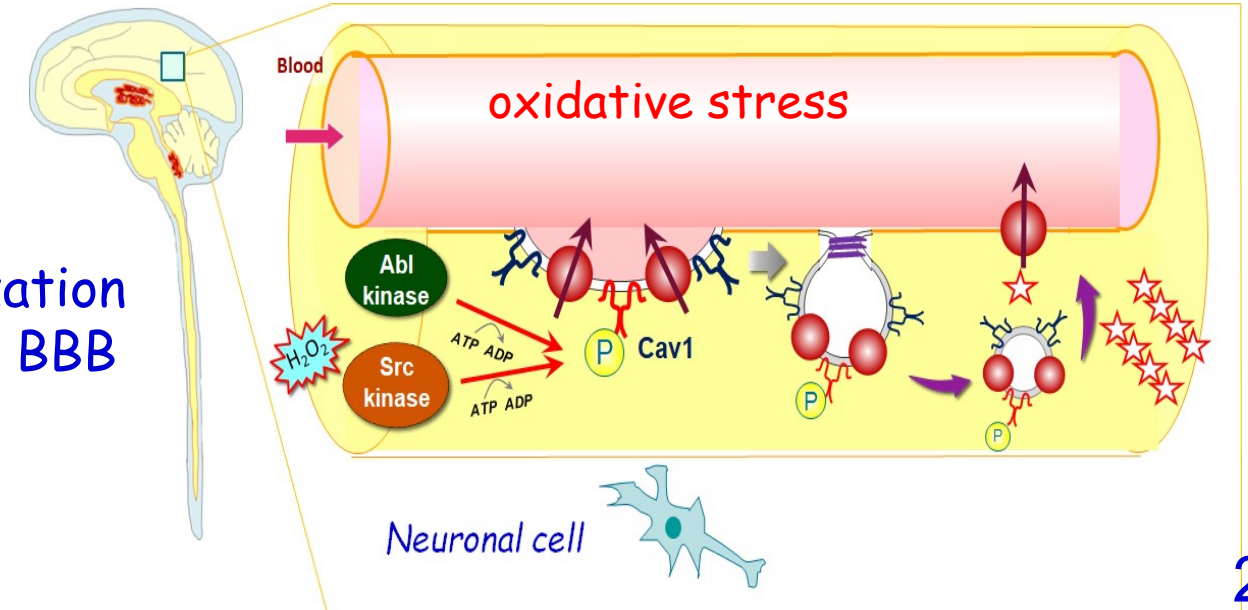


PP2

Src kinase inhibitor

Src kinase inhibitor also significantly attenuated the H_2O_2-induced decrease of P-gp and Cav1.

Possible model of rapid and reversible reduction of P-gp efflux activity in the BBB



Activation of both Abl kinase and Src kinase mediates the internalization of P-gp and Cav1 at the BBB under conditions of oxidative stress.

To see whether the same mechanism operates **in vivo**, we conducted a brain perfusion study to measure P-gp efflux function at the rat BBB using a typical P-gp substrate, quinidine.

Pre-treatment

Constant infusion (30 min)

Rat, 8wk, male

Imatinib Abl inhibitor

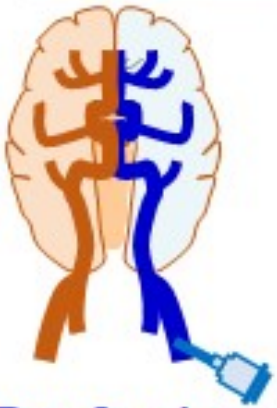
[5 uM in total blood]

PP2 Src inhibitor

[5 uM in total blood]

Extra carotid artery infusion

in situ brain perfusion



Perfusion (10 min)

H_2O_2 [5 mM in perfusate]

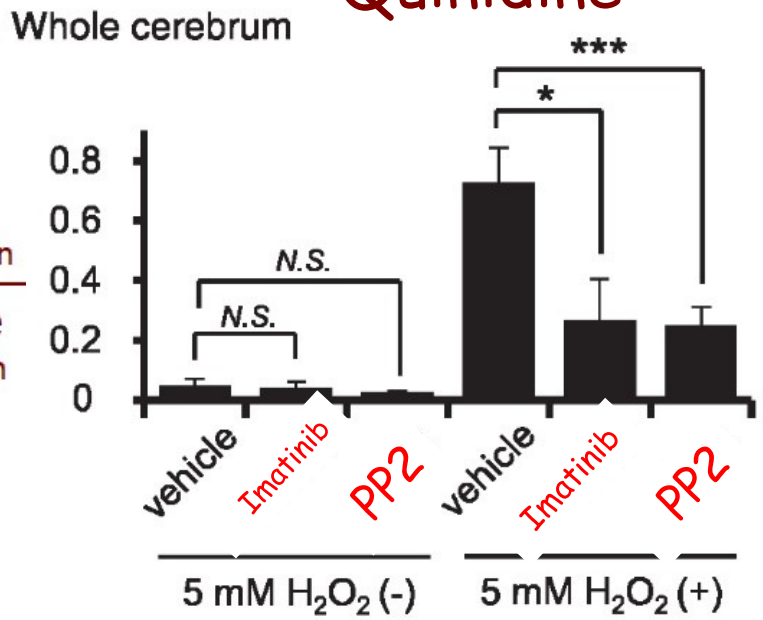
Quinidine (P-gp substrate)

Raffinose Plasma membrane non-permeable and non-degradative sugar in the cell (Vascular space marker)

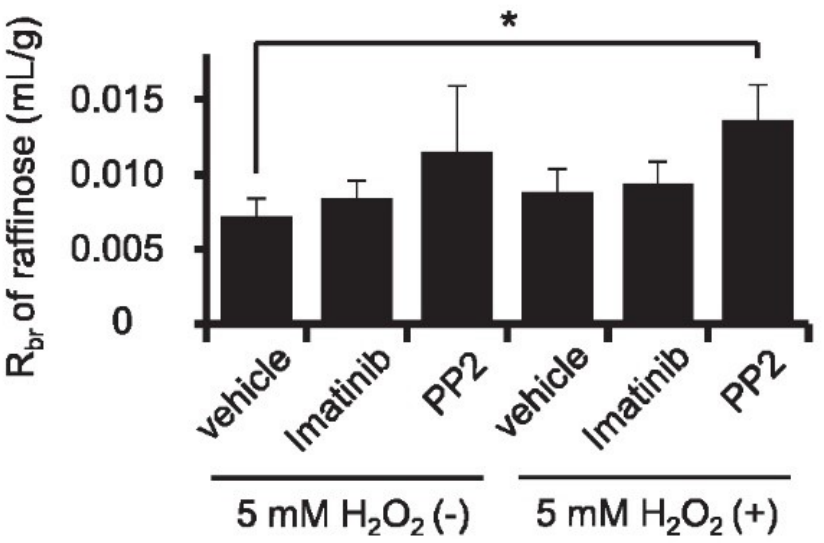
- ✓ The ratio of brain concentration per perfusate concentration of quinidine, Rbr, was increased significantly treated with 5mM H_2O_2. This increase was significantly suppressed by imatinib or PP2.
- ✓ No marked change of raffinose was observed, indicating that tight-junction integrity was well maintained.

Quinidine

Rbr = Brain Concentration / Perfusate Concentration (mL/ g brain)



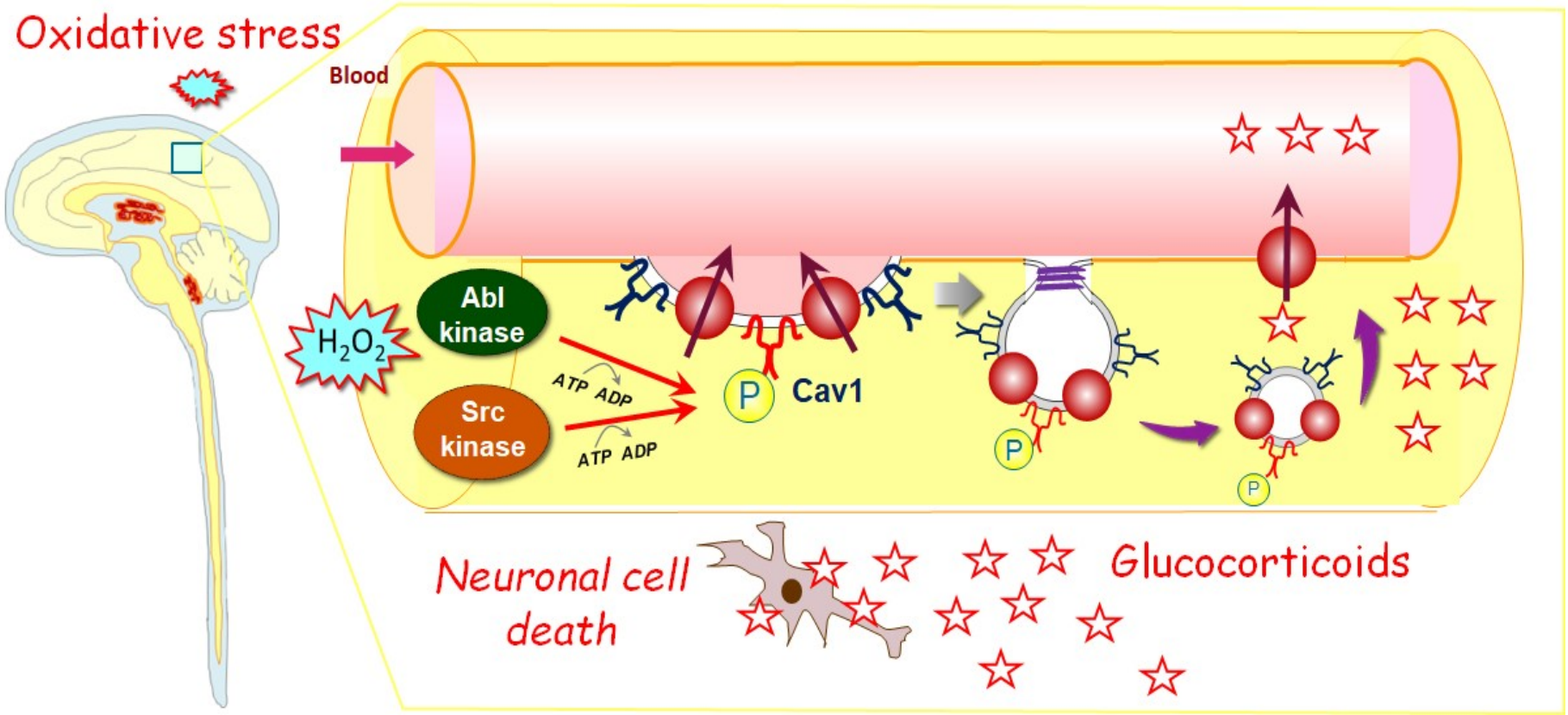
Raffinose



H_2O_2 decreases the efflux transport activity of P-gp through the activation of Abl kinase and Src kinase in vivo.

Exposure to high levels of glucocorticoids in the brain induces hippocampal neuronal cell death

(Anacker C, et al., Neuropsychopharmacology 2013; Krugers HJ, et al., Stroke 2000)



① Stroke (Ischemia, hypo-xia, reperfusion) ⇨ ② Oxidative stress ⇨ ③ Internalization of P-gp mediated by Abl and Src kinase in the BBB ⇨

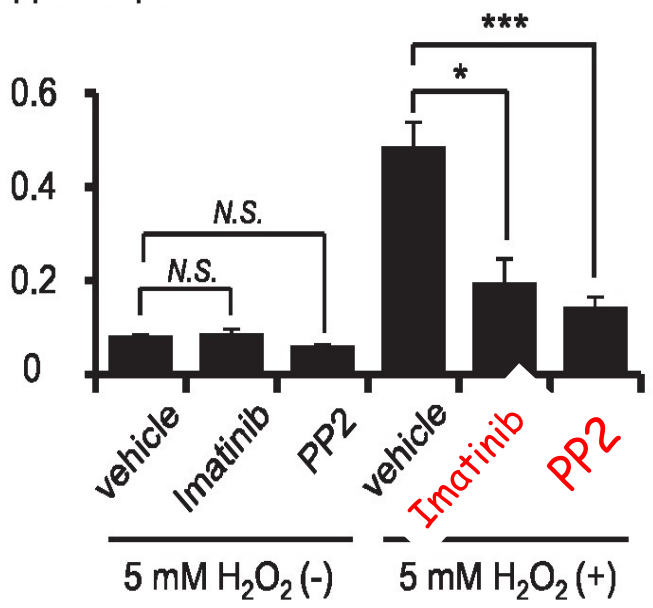
④ Accumulation of glucocorticoids in the brain ⇨ ⑤ Hippocampal neuronal cell death

The Rbr values of cortisol in hippocampus, hypothalamus and other cerebrum regions were significantly elevated after the administration of 5mM H_2O_2. These elevations were significantly attenuated by imatinib (Abl kinase inhibitor) or PP2 (Src kinase inhibitor), while there was no significant alteration in the Rbr of raffinose (not shown).

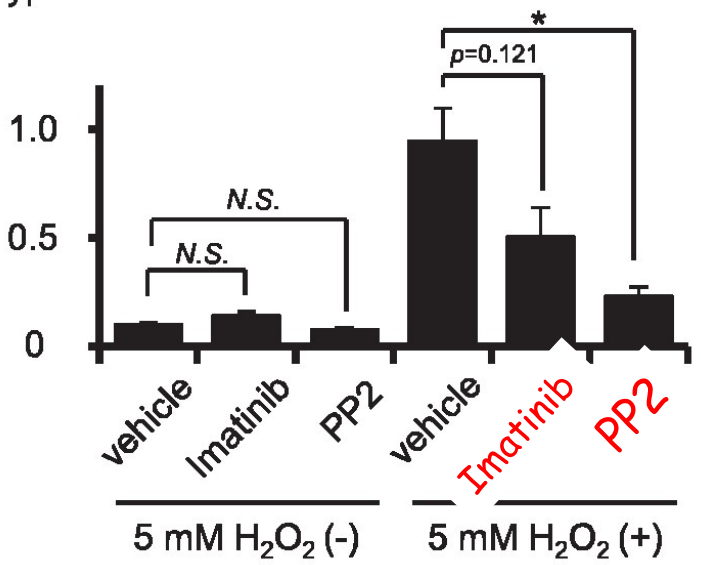
Cortisol

Rbr = Brain Concentration / Perfusate Concentration (mL/ g brain)

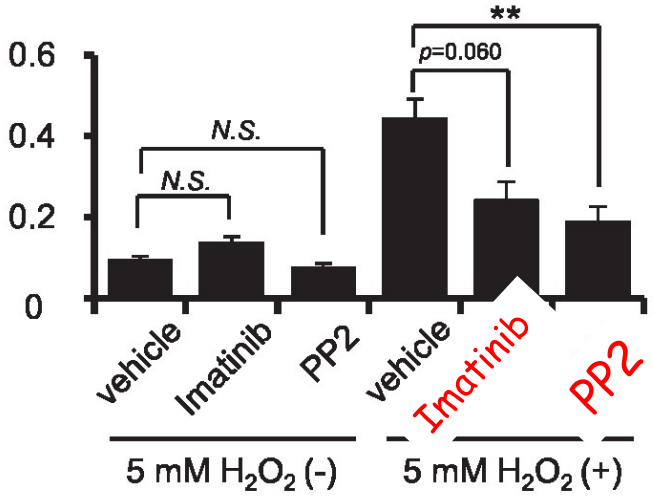
(b) Hippocampus



(c) Hypothalamus



(d) Other cerebrum region

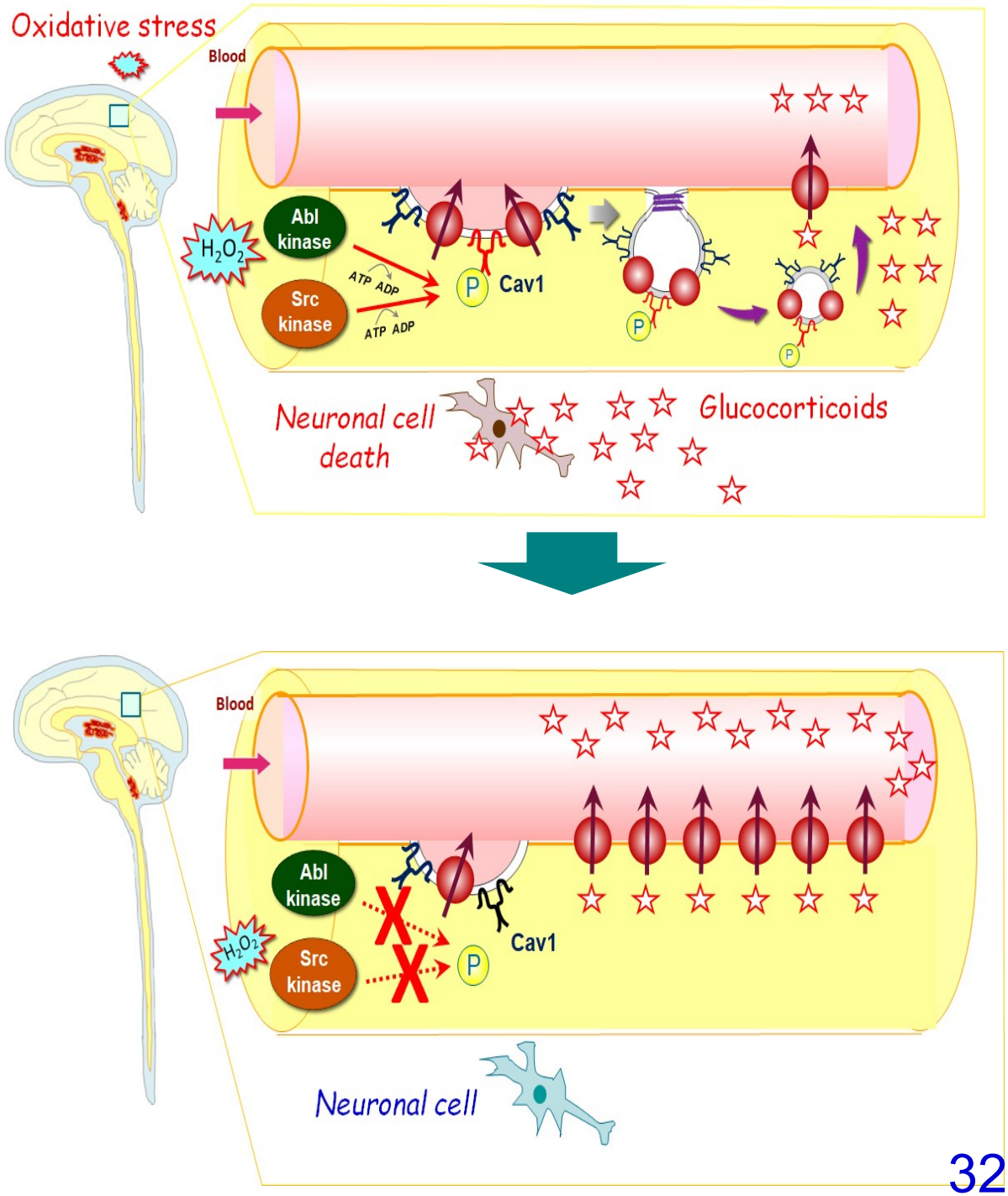


An oxidative stress-induced, Abl kinase and Src kinase-mediated decrease of P-gp efflux transport activity contributes to the elevation of cortisol concentration in the brain.

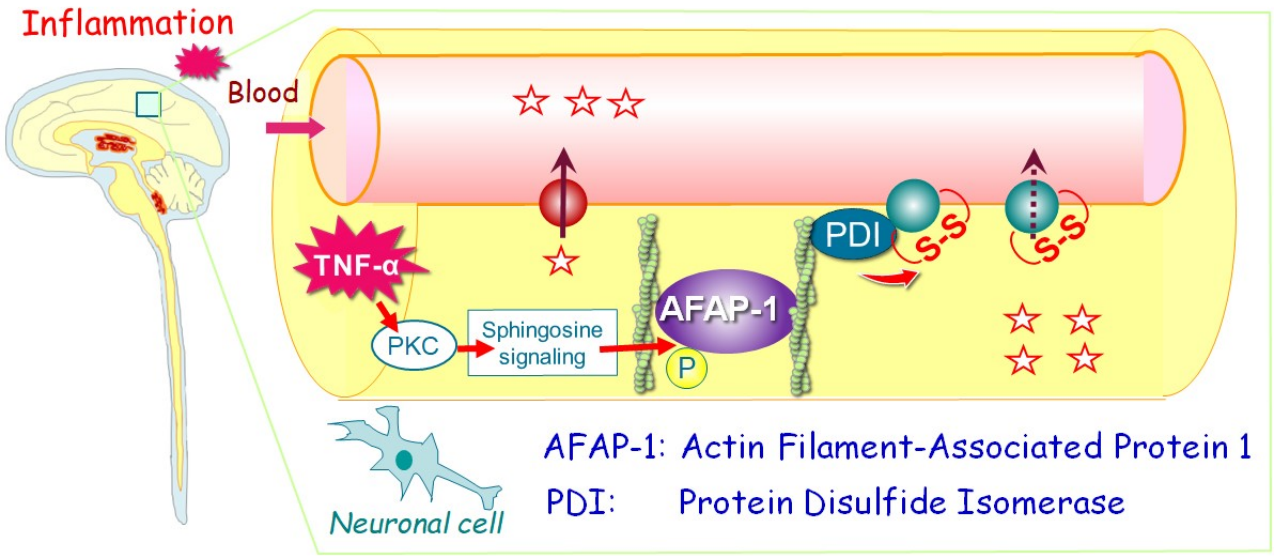
The suppression of elevated cortisol concentration in the brain

may contribute to

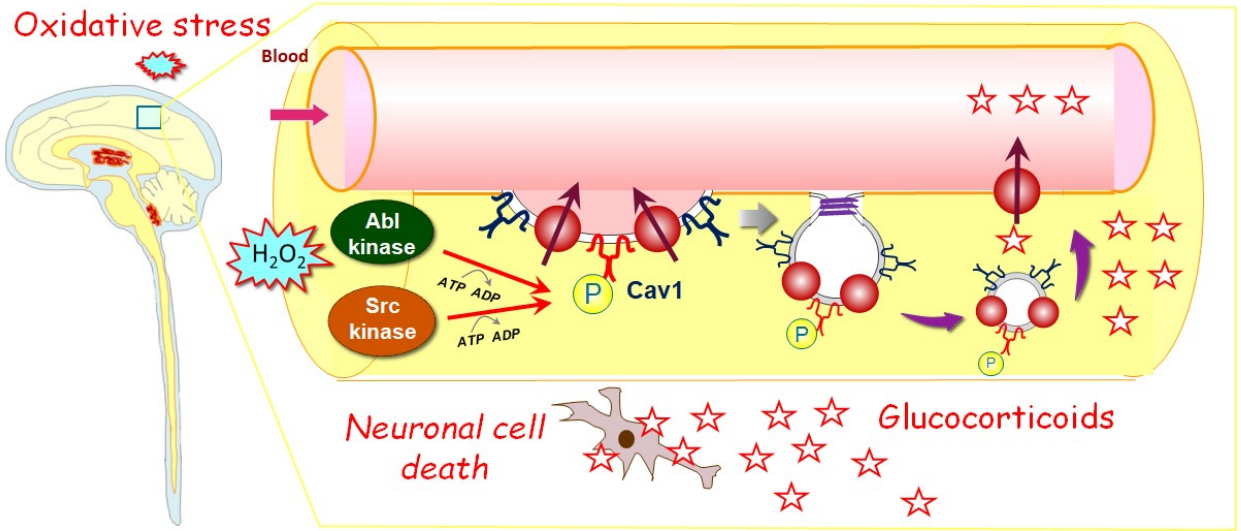
the **improvement of cognitive function** by **Abl kinase and Src kinase inhibitors**



Conclusion



The efflux activity of P-gp per protein was reduced by the treatment of TNFa in the BBB. Actin Filament-Associated Protein 1 (AFAP-1) will be a key mediator for the signaling-induced rapid attenuation of P-gp efflux activity.



The apparent P-gp efflux activity in the BBB was reduced by oxidative stress. Internalization of P-gp protein was facilitated via the signaling pathway of Abl and Src kinases.

Key References

1. Hoshi Y, Uchida Y, Tachikawa M, Ohtsuki S and Terasaki T, 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.***, 141(2):247-262 (2017). doi: 10.1111/jnc.13960.

2. Hoshi Y, Uchida Y, Tachikawa M, Ohtsuki S, Couraud PO, Suzuki T and Terasaki T, 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, ***J. Cereb. Blood Flow Metab.***, 2019 Jan 9:271678X18822801. doi: 10.1177/0271678X18822801.