Review

Quantitative Proteomics-Based Blood–Brain Barrier Study

Yasuo Uchida

Division of Membrane Transport and Drug Targeting, Graduate School of Pharmaceutical Sciences, Tohoku University; 6–3 Aoba, Aramaki, Aoba-ku, Sendai 980–8578, Japan.

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From the viewpoint of drug discovery, it is an important issue to elucidate the drug permeability at the human central nervous system (CNS) barriers and the molecular mechanisms in the cells forming CNS barriers especially during CNS diseases. I introduced quantitative proteomics techniques into the blood–brain barrier (BBB) study, then quantitatively investigated the transport system at the human BBB and clarified the quantitative differences in protein expression levels and functions of transporters and receptors between animals and humans, or in vitro and in vivo. Based on the difference in the absolute expression level of transporters between in vitro and in vivo, I demonstrated that the drug efflux activity of P-glycoprotein (P-gp) at in vivo BBB can be accurately reconstructed from the in vitro system, not only in mouse models but also monkeys similar to humans and pathological conditions. Furthermore, I discovered Claudin-11 as another tight junction molecule expressed at the CNS barriers, and clarified that it contributes to the disruption of the CNS barriers in multiple sclerosis. Furthermore, it was also elucidated that the P-gp dysfunction causes excessive brain entry of glucocorticoid which causes a nerve damage in cerebral infarct, and it can be suppressed by targeting Abl/Src kinases. These suggest that targeting the tight junctions and transporters, which are important molecules at the CNS barriers, would potentially lead to the treatment of CNS diseases. In this review, I would like to introduce a new CNS barrier study opened by quantitative proteomics research.

Key words pharmacoproteomics (PPx); P-glycoprotein (P-gp); Claudin-11; quantitative targeted absolute proteomics (qTAP); blood–brain barrier; in vitro-to-in vivo reconstruction (IVIVR)

1. WHY IS QUANTITATIVE TARGETED ABSOLUTE PROTEOMICS (QTAP) NEEDED IN DRUG DISCOVERY RESEARCH?

The percentage of compounds finally approved as new drugs is very low among drug candidates that went to clinical trials. Kola and Landis1 found that only 5% for anti-cancer drugs and 8% for central nervous system (CNS) drugs were approved during 1991–2000, and the success rate was no greater between 2000 and 2008. Insufficient efficacy and excessive toxicity in humans have been reported to be the major reasons for the high rate of discontinuation of clinical development.3 Moreover, over 98% of candidate CNS drugs cannot cross the blood–brain barrier (BBB), and therefore cannot give a pharmacologically effective concentration in the brain.3 Thus, the discontinuation of drug development is also caused by unfavorable distribution into target tissues in humans. From these considerations, it is clear that the efficacy, toxicity and distribution of drug candidates in humans are often different from those predicted on the basis of preclinical studies such as in vitro and animal experiments. The activities of many individual functional molecules that interact with the drug, including receptors, channels, transporters and enzymes regulate the efficacy, toxicity and distribution of drugs. Therefore, it is important to quantitatively reveal the differences in the activity of functional molecules between experimental animals and humans, or between in vitro and in vivo, in order to improve the success rate in clinical trials. Furthermore, quantitative evaluation of functional changes of individual molecules in various diseases is also important to develop more effective drugs, so this is one of the essential issues in drug discovery and development.

The activities for a variety of functional proteins including P-glycoprotein (P-gp/multiple drug resistance 1 (MDR1)), Na+/glucose cotransporter 1, CYP2D6 and β-secretase have been shown to correlate well with their protein expression levels.4–9 Hence, we thought that interspecies, in vitro/in vivo differences and disease changes in functional activities may be effectively evaluated by the quantification of protein expression levels, and therefore we established a novel methodology for protein quantification based on LC-linked tandem mass spectrometry (LC-MS/MS) with selected/multiple reaction monitoring (SRM/MRM), called “quantitative Targeted Absolute Proteomics (qTAP).”10,11 Especially, the in vivo activities of target molecules in humans would be able to be reconstructed by integrating the activities measured in in vitro or animal experiments, with in vitro/in vivo or interspecies differences in protein expression levels determined by qTAP. Therefore, qTAP is expected to overcome the major limitations of human studies and to open up the new research field of pharmacoproteomics (PPx).

2. QUANTITATIVE CHARACTERIZATION OF HUMAN BLOOD–BRAIN BARRIER (BBB) BY QTAP TECHNOLOGY

It is an important issue to quantitatively clarify how similar and different the transport molecular mechanisms at the BBB are between human and experimental animals in CNS drug development and human neuroscience studies. Therefore,
we employed the qTAP technique to determine the absolute protein expression level of more than 100 transport carriers at the BBB in humans, monkeys, marmosets, pigs, rats and mice, and quantitatively clarified a large difference in the quantitative protein expression profile between rodents and humans10,12–16) (Fig. 1). These enable us to interpret the causes of the interspecies differences in the concentrations of various substances in brain, such as interspecies difference in brain penetration of P-glycoprotein (P-gp/ABCB1) substrates.

As a multidrug efflux transporter, P-gp has been emphasized in conventional BBB studies centered on rodents, but in humans, breast cancer resistant protein (BCRP/ABCG2) is highly expressed in addition to P-gp15) (Figs. 1b, c), and therefore we advocated the importance of selecting compounds that are not likely to be excreted by BCRP in developing therapeutic agents for CNS diseases.17) The protein expression level of BCRP in the BBB is significantly higher than that of other CNS barriers such as blood–cerebrospinal fluid barrier, inner and outer retinal barriers,16) and therefore this is positioned as a characteristic of the BBB in large animals including humans (Figs. 1d, e).

The second notable finding is that organic anion transporter 3 (oat3) was not detected at all in the human BBB15) (Figs. 1d, e).

Fig. 1. Human-Animal and In Vivo–In Vitro Differences in Absolute Abundances of Transporters, Receptors, Tight Junction Proteins and Other Molecules at the Blood–Brain Barrier

(a) Qualitative profile of drug transporters (reddish color), other transporters (bluish color), receptors and tight junction protein (greenish color) at the blood–brain barrier. (b–f) Quantitative profile opened up by qTAP technology. Whole cell lysate of the brain capillaries isolated from human brain cortices was applied for qTAP experiment, and the absolute abundances of drug transporters (red), other transporters (blue), receptors, tight junction protein, and other molecules (green) were compared with those in the whole cell lysate of mouse (b), rat (c), monkey (d), marmoset (e), hCMEC/D3 cell line (f). U.L.Q, under the limit of quantification. Data were cited from Uchida et al.15) Kamiie et al.10) Hoshi et al.12) Ito et al.13) and Ohtsuki et al.19) (Color figure can be accessed in the online version.)

Biography

Dr. Uchida is currently a lecturer of Tohoku University. In Jan. 2012, he received a Ph.D. degree from the Graduate School of Pharmaceutical Sciences, Tohoku University. He has an achievement in the field of the LC-MS/MS-based central nervous system (CNS) barrier research and opened a new field of “Pharmacoproteomics (PPx).” The transporter study of the CNS barriers is one of his interests, and he is going to comprehensively and rationally clarify (1) what transporter proteins are expressed with the information of absolute abundance, apical/basolateral localization and post-translational modification by the comprehensive absolute quantification method, (2) what role they have physiologically and pharmacokinetically by comprehensive transport assay method, and (3) how they change in the CNS disorders by highly reliable Formalin-Fixed-Paraffin-Embedded (FFPE) tissue proteomics (FFPE-PCT-SWATH). One of his papers, J. Neurochem., 117, 333–345 (2011), has been cited 460 times for 9 years (until Dec. 2020) and he is currently a handling editor for the Journal of Neurochemistry.
l1b, c), although oat3 is known from previous studies of the blood–brain barrier in rodents as a major excretion pathway for harmful brain metabolites such as neurotransmitters, neurosteroids, and uremic substances. Since oat3 was expressed in both rodents and humans at the blood-cerebrospinal fluid barrier, it is regarded as an interspecies difference specific to the BBB.

hCMEC/D3 cells are widely used as in vitro model cells for human brain capillary endothelial cells, and it is an important task to know to what extent they reflect the in vivo transport molecular mechanism. As a result of comparing the protein expression level of the transporter with the in vivo human BBB, the expression levels of glucose transporter type 1 (GLUT1), P-gp and BCRP, which are three important transporters at the BBB, were more than 3 times lower than those in vivo18) (Fig. 1f). Therefore, it is suggested that these transport activities are significantly reduced as compared with in vivo. However, since the expression level of Na+/K+-ATPase (a plasma membrane marker) was also low, the density of the cell membrane in the whole cell may be lower than that in vivo. By comprehensively quantifying all membrane proteins rather than a limited number of transporters, it is possible to determine whether the expression levels of GLUT1, P-gp and BCRP are actually significantly reduced in hCMEC/D3 cells. It will need to be verified.

3. A NEW RESEARCH FIELD OF PPX

It was clarified that there is a large quantitative difference in the transport molecular mechanism between in vitro cultured cells of human brain capillary endothelial cells and in vivo as well as the species difference between humans and experimental animals19) (Fig. 1f). These results mean that conventional animal experiments and in vitro analyzes cannot quantitatively elucidate the substance transport function of the human in vivo BBB. Therefore, we constructed a mathematical model to reconstruct in vivo substance transport via the BBB from in vitro experimental data, which are the expression level of P-gp protein at the BBB and the drug efflux activity per P-gp single molecule measured in in vitro expressing cells20) (Fig. 2a). First, we experimentally demonstrated the mathematical model in normal mouse by reconstructing the P-gp efflux activity of 11 substrates at the in vivo BBB (Fig. 2b). Second, it was demonstrated that, even in cynomolgus monkeys whose expression level of the transport carrier is close to that of humans (Fig. 1d), the intracerebral concentration of the drugs after administration into blood can be reconstructed from the in vitro experimental values by this reconstruction method21) (Fig. 2c). In addition, CNS disorders impair the function of transporting substances at the BBB. Using epilepsy mice and phenytoin-treated mice as models, it was demonstrated that the decreases

Fig. 2. Pharmacoproteomics-Based Reconstruction of in Vivo P-gp Efflux Function at the BBB; Validation in Normal Mice, Cynomolgus Monkey and Disease State

The detailed model and theory of the reconstruction are described in Uchida et al.40) Data were cited from Uchida et al.,20) Uchida et al.,21) and Uchida et al.41) (Color figure can be accessed in the online version.)
in brain penetration associated with pathological conditions and treatment can be reconstructed well\(^{20}\) (Fig. 2d).

It is important in drug development to be able to predict the intracerebral concentration of drugs not only after intravenous administration but also after oral administration. Therefore, we demonstrated a method that can reconstruct the transport activity of P-gp in the small intestine and the absorption rate of P-gp substrates from \textit{in vitro} by the same theory as the BBB above.\(^{22}\) Furthermore, by comprehensively elucidating the protein expression levels of the transport carriers in the small intestinal epithelium of human,\(^{23}\) cynomolgus monkey,\(^{23}\) mouse\(^{22}\) and Caco-2 cultured cells,\(^{24}\) we have constructed a basis for quantitatively estimating the functional differences of transport carriers between laboratory animals or \textit{in vitro} systems and humans \textit{in vivo}.

These were first achieved by “PPx,” a discipline that combines the qTAP method with pharmacokinetics. This PPx is expected to be useful not only for absorption, distribution, metabolism, excretion (ADME) but also for evaluation of drug efficacy and toxicity.

4. CLAUDIN-11 AS ANOTHER MEMBER OF TIGHT JUNCTION PROTEIN AT THE CNS BARRIERS

Elucidation of the molecular mechanism of tight junctions, in addition to transporters, is an important issue in CNS barrier research. The CNS tissue consists of two types of endothelial system barriers, the BBB and the blood–spinal cord barrier (BSCB), which are formed by tight junctions of vascular endothelial cells. Furthermore, there are two types of epithelial system barriers, the blood–cerebrospinal fluid barrier (BCSFB) and the blood–arachnoid barrier (BAB), which are formed by tight junctions of epithelial cells. Tight junction proteins are a group of molecules that hold the key to the formation of these barriers. Although some important molecules such as Claudin-5 and 3 have been identified, the molecular mechanism of tight junctions has not yet been fully explained, suggesting the existence of other tight junction molecules.

We used proteomics analysis to comprehensively search for tight junction proteins in the rat BBB. As a result, in addition to the known Claudin-5, protein expression of Claudin-11, which was shown to be extremely low in the conventional mRNA expression analysis,\(^{25}\) was detected.\(^{26}\) In order to understand how important the Claudin-11 is compared to Claudin-5, the absolute protein expression levels of Claudin-5 and 11 were measured by qTAP analysis. At the BBB, Claudin-11 and Claudin-5 were found to be comparable in rats, but the Claudin-11 expression level was significantly higher (18.4 fmol/µg protein) than that of Claudin-5 (6.56 fmol/µg protein) in humans (Fig. 3a). This suggests that Claudin-11 may play an important role in tight junction formation at the BBB.\(^{26}\) Claudin-11 is originally expressed in oligoden-
drocytes. In order to show that it is certainly expressed in vascular endothelial cells, the expression in human brain capillary endothelial cell lines was quantified, and it was shown that the expression level was significantly higher than that of Claudin-5.\textsuperscript{26} In addition, in immunohistochemical staining analysis using human brain sections, a signal was merged with claudin-5, which is a vascular endothelial cell-specific marker.\textsuperscript{26} Therefore, Claudin-11 was shown to be localized to vascular endothelial cells in addition to oligodendrocytes.

Similarly, at the BSCB, qTAP analysis and immunostaining analysis showed expression of Claudin-11 protein.\textsuperscript{26} Interestingly, Claudin-11 protein expression was significantly lower than that at the BBB.\textsuperscript{26} This may explain previous reports\textsuperscript{27} that the transferred amount of membrane impermeable substances to the spinal cord parenchyma after the administration into the blood is greater than that to the brain parenchyma.

5. CONTRIBUTION OF CLAUDIN-11 TO TIGHT JUNCTION FORMATION AT THE CNS BARRIERS

To clarify the contribution of Claudin-11 to tight junction formation, it was verified whether or not the knockdown with small interfering RNA (siRNA) enhances the paracellular permeability (substance permeability between cells) of the membrane impermeable substance fluorescein isothiocyanate (FITC)-dextran\textsuperscript{26} (Fig. 3b). As a result, the permeability of FITC-dextran was significantly increased in both the monolayer membranes of the endothelium barrier, brain capillary endothelial cells (hCMEC/D3 cells) and the epithelial barrier, choroid plexus epithelial cells (TR-CSFB cells).\textsuperscript{26} Therefore, it was clarified that Claudin-11 contributes to the formation of tight junctions at the CNS barriers, and that its reduced expression reduces the barrier integrity.

To understand how the newly identified claudin-11 contributes to tight junction formation at the human BBB compared to Claudin-5, the relative contributions of Claudin-11 and 5 were estimated by combining the knockdown experiment using \textit{in vitro} hCMEC/D3 cell lines and the qTAP analysis. Consequently, the contribution of Claudin-11 to tight junction formation at the human BBB was almost the same level as that of Claudin-5.\textsuperscript{26} The details for the estimation are previously described.\textsuperscript{26}

6. CNS BARRIER DISRUPTION AND CLAUDIN-11 IN MULTIPLE SCLEROSIS

Multiple sclerosis is one of the neurological autoimmune diseases with many patients in young adults. Autoreactive lymphocytes infiltrate the CNS tissues and cause nerve destruction (demyelination). At present, the current therapeutic agents have not been able to sufficiently suppress the onset and progression of the disease. Analysis using magnetic resonance imaging (MRI) has shown that the CNS barrier has collapsed before lesions and symptoms appear,\textsuperscript{28} so it is believed that the collapse of the CNS barrier occurs early in the disease and triggers the onset of multiple sclerosis. For this reason, the suppression of the collapse of the CNS barrier is a rational therapeutic strategy, but the molecular mechanism of the collapse of the CNS barrier has not been fully elucidated. Multiple sclerosis is more common in young adult women than in men, suggesting that barrier disruption is caused by hormone-dependent molecular mechanisms. Claudin-5 at the BBB and BSCB, and Claudin-3 at the BCSFB are known as important molecules for tight junction formation,\textsuperscript{29,30} but it has not been reported that these functions are regulated by sex hormones.

Interestingly, the expression of Claudin-5 was not decreased in the BBB of multiple sclerosis model mice (Experimental Autoimmune Encephalomyelitis (EAE) mice)\textsuperscript{31} (Fig. 3c).

To clarify whether Claudin-11 is involved in the breakdown of the CNS barrier in multiple sclerosis, an immunohistochemical analysis was done to show whether the protein expression of Claudin-11 is decreased in the blood vessels of the brain and spinal cord of patients with multiple sclerosis and multiple sclerosis model mice (EAE mice). As a result, it was shown that the Claudin-11 signal was significantly weaker in both brain and spinal cord blood vessels than in healthy subjects and normal mice.\textsuperscript{26} When the rate of decrease in expression level was quantified, it was shown that in patients with multiple sclerosis, there was a 39.4% decrease in brain vessels and a 48.4% decrease in spinal blood vessel compared to healthy subjects\textsuperscript{26} (Fig. 3d). Similarly, EAE mice were shown to be 30.8% lower in brain vessels and 41.1% lower in spinal blood vessels than normal mice\textsuperscript{26} (Fig. 3c). By multiplying these decreasing percentages by the absolute protein expression level of Claudin-11 in the cerebral and spinal cord blood vessels of normal mice, the absolute expression level of Claudin-11 in EAE mice was calculated. It was estimated that the expression level at the BSCB was 2.4 times lower than that at the BBB.\textsuperscript{26} Nerve injuries in multiple sclerosis have been reported to be more pronounced in the spinal cord than in the brain.\textsuperscript{26} Therefore, infiltration of autoreactive lymphocytes into the spinal cord parenchyma is more pronounced than in the brain, and the cause would be explained by weaker barrier integrity at the BSCB than BBB, because of the smaller protein expression of Claudin-11 at the BSCB than BBB in multiple sclerosis.

We have shown that Claudin-11 is more highly expressed than the Claudin-3 at the human BCSFB, which is one of the CNS epithelial barriers.\textsuperscript{18} Therefore, it is possible that Claudin-11 also plays an important role in tight junction formation in the epithelial barrier. Therefore, we analyzed whether or not the expression level of Claudin-11 decreased in multiple sclerosis with respect to the BCSFB and the BAB, which are the two CNS epithelial barriers. As a result of analyzing whether the expression level of Claudin-11 was decreased in EAE mice, no change in expression was observed at the BCSFB.\textsuperscript{26} In contrast, regarding the BAB, the Claudin-11 signal on the leptomeninges, which was observed normally in both the brain and spinal cord, disappeared in EAE mice.\textsuperscript{26} Cellular infiltration was observed at the site where expression had disappeared.\textsuperscript{26} Staining with the CD3e antibody showed that the infiltration was lymphocyte infiltration. Therefore, in multiple sclerosis, the expression level of Claudin-11 at the BAB is reduced, and its reduction would affect the infiltration of autoreactive lymphocytes into the CNS tissue.

The incidence of multiple sclerosis is higher in women than in men. It is known that the degree of barrier collapse in multiple sclerosis is inversely proportional to the level of androgen (male hormone) in the body. That is, androgens are thought to maintain the tight junction function of the CNS barrier. In order to clarify whether Claudin-11 is involved in the androgen-induced suppression of barrier disruption, EAE
mouse serum was treated with hCMEC/D3 cells to establish an in vitro multiple sclerosis BBB model, and it was examined whether Claudin-11 expression and the plasma membrane localization were restored by dihydrotestosterone (DHT). As a result, the internalization and loss of Claudin-11 expression observed in the treatment of EAE mouse serum was significantly inhibited by the additional treatment of DHT (Fig. 3e). Therefore, at the BBB, androgens are thought to prevent the disruption of the barrier function by maintaining the expression of Claudin-11 on the cell membrane.

7. P-GP DYSFUNCTION HYPOTHESIS IN CEREBRAL INFARCT

Studies of Claudin-11 in multiple sclerosis have revealed that the CNS barrier plays an important role in the development and progression of CNS diseases. Therefore, the CNS barrier may be an important target in the treatment of CNS diseases, and the molecular mechanisms of the CNS barriers at the CNS diseases should be sufficiently understood for the effective therapies.

P-gp is widely recognized for its role in limiting the transfer of exogenous substances such as drugs and xenobiotics to CNS tissues. However, it physiologically excretes glucocorticoids such as cortisol, and plays a role in regulating the concentrations in the brain. Since an increase in the brain concentration of glucocorticoid causes nerve damage, it is considered that changes in the transport function of P-gp in BBB are closely related to nerve function. Cerebral infarct is one of the diseases related to glucocorticoids, and is one of the diseases in which the number of patients who need long-term care in the subsequent life due to nerve injury at the time of occurrence is extremely large. Advances in antithrombotic therapy have increased the number of cases in which cerebral blood flow can be restored and the proportion of patients who are saved has increased, but the large amount of oxidative stress generated during this reperfusion damages brain function (Fig. 4a). Therefore, it is considered important to prevent this in order to reduce the number of people requiring care after illness.

In cerebral infarct model rats, it has been reported that glucocorticoids damage hippocampal neurons immediately after restoration of blood flow, even though the concentration of glucocorticoids in the blood does not increase. Even in patients with cerebral infarct, the concentration of glucocorticoid in blood is almost the same as that in healthy subjects. The neuronal damage is further accelerated by external pre-administration of glucocorticoid to cerebral infarct model rats, and is significantly suppressed by administration of a glucocorticoid synthesis inhibitor before occurrence of cerebral in-
farct.\textsuperscript{34} It is suggested that glucocorticoid is one of the major causes of nerve injury after cerebral infarct.

Based on these findings, we hypothesized, “Oxidative stress generated by reperfusion therapy after ischemia rapidly reduces the efflux activity of P-gp at the BBB. Therefore, the amount of glucocorticoids that can be transferred from the blood to the brain is increased and then the nerve damage occurs. If this molecular mechanism responsible for the decrease in P-gp activity can be blocked, the increase in brain concentration of glucocorticoids can be prevented and nerve damage can be suppressed (Fig. 4a).”

8. MOLECULAR MECHANISM OF P-GP DYSFUNCTION BY OXIDATIVE STRESS AND THE THERAPY OF CEREBRAL INFARCT BY P-GP ACTIVATION

To prove the above hypothesis, we first examined whether or not the efflux activity of P-gp in BBB was reduced in a short time by oxidative stress stimulation using in situ brain perfusion method. Infusion of H\textsubscript{2}O\textsubscript{2} and the P-gp substrate quinidine and the non-permeable marker raffinose into the common carotid artery of rats for 10 min resulted in a 17-fold increase in quinidine penetration into the brain compared to the H\textsubscript{2}O\textsubscript{2} non-treated group.\textsuperscript{36} By contrast, the penetration of raffinose to the brain did not change, suggesting that the efflux activity of P-gp decreased without the breakdown of tight junctions.

In order to verify whether the same phenomenon occurs in humans, the efflux activity of P-gp substrates after 20 min-treatment with H\textsubscript{2}O\textsubscript{2} was measured using a human brain capillary endothelial cell line (hCMCEC/D3 cells). The efflux transport activity of P-gp was significantly reduced in a concentration-dependent manner of H\textsubscript{2}O\textsubscript{2}.\textsuperscript{36} When blood flow restores after cerebral infarct, the H\textsubscript{2}O\textsubscript{2} concentration locally reaches 0.5 mM or more in the cerebral blood vessels. Even at this 0.5 mM, the efflux activity of P-gp was sufficiently reduced. The expression level of P-gp protein in the plasma membrane fraction decreased in correlation with the decrease in efflux activity, while no change was observed in protein expression level of P-gp in whole cell lysate.\textsuperscript{36} This suggests that the decrease in P-gp efflux activity by oxidative stress was caused by the internalization of P-gp\textsuperscript{36} (Fig. 4c). It was also shown that dynamin is involved in this internalization.

Since the decrease in efflux function by the internalization of P-gp occurs in minutes, it was considered that the intracellular phosphorylation signal could be involved. In order to quantify small amount of phosphorylated protein with high sensitivity and high accuracy, it is important to suppress dephosphorylation during sample pretreatment, purify the phosphorylated peptide with high purity, and quantify it with high sensitivity. We set up a system of quantitative phosphorylation proteomics that combines these, and then analyzed the hCMCEC/D3 cells under oxidative stress stimulation. As a result, it was shown that Caveolin-1 phosphorylation increased about 20 times compared to normal.\textsuperscript{36} Furthermore, as a result of quantifying all the phosphorylation sites of Caveolin-1 (6th, 14th, 25th, and 42nd tyrosine from the N-terminal), the phosphorylation amount of the 14th tyrosine was proportional to the H\textsubscript{2}O\textsubscript{2} treatment concentration.\textsuperscript{36} Its phosphorylation rate was also higher than other tyrosine residues (10–20% of the total amount of Caveolin-1 protein was phosphorylated at the 14th tyrosine).\textsuperscript{36} Therefore, we suspected the involvement of Abl kinase and Src kinase, which are enzymes that phosphorylate the 14th tyrosine of Caveolin-1, and verified using the inhibitors and RNA interference (RNAi) method. As expected, it was clarified that the activation of Abl kinase and Src kinase causes the phosphorylation of Caveolin-1.\textsuperscript{36} As for Caveolin-1, it has been reported that the activity of P-gp decreases and the internalization of caveolae is promoted by Caveolin-1 phosphorylation.\textsuperscript{37,38} Therefore, it was suggested that, in acute oxidative stress stimulation, the molecular mechanism shown in Fig. 4c occurs in the endothelial cells, and consequently the efflux function of P-gp is reduced.

In order to prove that the molecular mechanism identified in the in vitro experiment also contributes in vivo, we used in situ brain perfusion method. As a result, the excess brain entry of P-gp substrate (quinidine) that occurs under oxidative stress conditions was significantly suppressed by inhibition of Abl or Src kinase activity.\textsuperscript{36} Therefore, it was suggested that Abl kinase and Src kinase are responsible molecules even in vivo.

If the excess entry of glucocorticoids into brain can be restricted, nerve damage can be suppressed and the number of patients requiring nursing care can be reduced. As a result of administration of imatinib (an inhibitor of Abl kinase), and PP2 (an inhibitor of Src kinase), we succeeded to significantly suppress cortisol entry into brain (hippocampus, hypothalamus, and other brain regions) under oxidative stress conditions\textsuperscript{36} (Fig. 4b). It has been reported that the administration of imatinib to cerebral infarct model animals significantly reduces the damaged area after cerebral infarct.\textsuperscript{39} The authors speculate that imatinib suppressed the breakdown of tight junctions in the BBB associated with cerebral infarct. Imatinib may suppress the breakdown of tight junctions, as tight junctions weaken over the hours to a day after a stroke occurs. However, tight junctions did not collapse until a few hours after oxidative stress stimulation. We have also confirmed in the in situ brain perfusion experiment that the tight junction of BBB was not disrupted by using the membrane impermeable substance Raffinose.\textsuperscript{36} Therefore, it is considered that the action of imatinib on the initial stage of oxidative stress is not on tight junctions but on the suppression of deterioration of P-gp efflux function (Fig. 4a). Imatinib is a drug that is already on the market for other diseases, and we look forward to the implementation and results of clinical trials aimed at expanding its application to the treatment of cerebral infarct in the future.

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