Full Paper

Altered Brain Penetration of Diclofenac and Mefenamic Acid, but Not Acetaminophen, in Shiga-Like Toxin II-Treated Mice

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Abstract. It is well accepted that bacterial and virus infections elevate the levels of cytokines in serum and cerebrospinal fluids. Such high levels of cytokines might alter the integrity of the blood-brain barrier (BBB) and/or blood-cerebrospinal fluid barrier (BCSFB), subsequently affecting brain penetration of drugs. However, few reports have addressed this issue. Thus, we investigated brain penetration of cyclooxygenase (COX) inhibitors, commonly used as antipyretics, in mice treated with Shiga-like toxin II (SLT-II) derived from *E. coli* O157:H7, which significantly elevates cytokine levels. As antipyretics, we used diclofenac, mefenamic acid, and acetaminophen. We found that SLT-II significantly increased the brain-to-plasma concentration ratio (K_p) of diclofenac and mefenamic acid, but not of acetaminophen. Moreover, the K_p of diclofenac and mefenamic acid was increased by probenecid, an anionic compound. These results suggest that efflux anion transporters might be involved in the transport of diclofenac and mefenamic acid. Western blot analysis revealed that SLT-II decreased the expression of organic anion transporter-3, an efflux transporter located on the BBB and/or BCSFB. Taken together, these results suggest that SLT-II and/or SLT-II-stimulated cytokines might change brain penetration of drugs and could possibly increase the risk of their side-effects by altering the expression of transporters.

Keywords: blood-brain barrier, Shiga-like toxin II, organic anion transporter, cyclooxygenase inhibitor

Introduction

Cyclooxygenase (COX) inhibitors are widely used as antipyretic drugs in patients with virus or bacteria infection with high fevers. The treatment with COX inhibitors is generally effective. However, when COX inhibitors are used in children, there may be central nervous system (CNS) complications such as Reye's syndrome (1). Moreover, the influenza-associated encephalopathy in the Asian population is reported to be worsened by COX inhibitors (2, 3), associated with higher levels of cytokines in serum (4, 5) and in cerebral

spinal fluids (6).

It has been reported that cytokines might regulate the functions of transporters at the blood-brain barrier (BBB) and blood-brain cerebrospinal barrier (BCSFB) (7 – 9). Thus, it is likely that the influenza and influenza-like symptoms might stimulate the release of cytokines (4 – 6) that impair BBB function. Accordingly, BBB and/or BCSFB dysfunction might alter the penetration of COX inhibitors into the CNS, subsequently causing CNS damage. However, because of a lack of animal models, no reports have addressed this issue.

Recently, it has been demonstrated that Shiga-like toxin type II (SLT-II), a bacterial toxin from *E. coli* O157, stimulates significant release of cytokines in

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rodents (10–12) and that SLT-II altered drug penetration into the brain (11). For example, the brain-to-plasma concentration ratio (K_p) of fluorescein isothiocyanate labeled dextran (FD-4), a marker for the penetration through the tight junction of brain capillary endothelial cells at the BBB and BCSFB, was significantly increased (11). Moreover, it has been reported that the K_p of doxorubicin was also increased in SLT-II-treated animals (11). One of the possible explanations for this alteration of the function of the BBB and/or BCSFB could be the significant release of inflammatory cytokines including tumor necrosis factor (TNF)- α , interleukin (IL)-6, IL-10, and nitric oxide (NO) by SLT-II (10–12), although the precise mechanism is not yet known.

Recently, various transporters have been found on the BBB and BCSFB that act as influx/efflux transporters (13, 14). It has been reported that the ability of several transporters to transport their substrates is competitively blocked by COX inhibitors (15, 16), suggesting that several COX inhibitors are transported at the BBB and/or BCSFB. For example, among organic anion transporters (OATs), OAT2 and OAT3 have been reported to be localized on the BBB and BCSFB (13, 15, 17–19). The OAT1- and OAT2-mediated transports in the transfected LLC-PK1 cells were blocked by various COX inhibitors (diclofenac, ketoprofen, indometacin, and others) (16, 20, 21). Moreover, organic anion transporting polypeptide (Oatp) 14 was localized on the BBB and BCSFB (22). The transport of prothyroid hormone in Oatp14-expressed HEK 293 cells was blocked by ketoprofen. Furthermore, two Oatps have been localized in the rat choroid plexus epithelium; Oatp2 is expressed at both the basolateral and apical membranes, while Oatp3 was identified at the apical membrane (13). The Oatp2-mediated transports were also blocked by ketoprofen and indometacin (23). Moreover, it has been reported that monocarboxylic acid transporters (MCTs) can transport several COX inhibitors that have a carboxyl residue (24). These results suggest the importance of transporters on the penetration of COX inhibitors into brain. However, the transporters responsible for COX inhibitors transport at the BBB and their functional and biochemical changes in response to infection including influenza are not well understood.

In the present study, we investigated brain penetration of COX inhibitors in a mouse model of infection associated with high levels of cytokines. We selected SLT-II-treated mice as the BBB failure model during the infection. Among COX inhibitors, diclofenac, mefenamic acid, and acetaminophen were studied.

Materials and Methods

Materials

Diclofenac, mefenamic acid, and probenecid were purchased from Sigma (St. Louis, MO, USA). Acetaminophen was purchased from Wako (Osaka). All other reagents were commercially available and were of analytical grade. All chemicals were dissolved in physiological saline before use.

Preparation of SLT-II

A clinically isolated *E. coli* O157:H7 strain NGY12 was used for the production of SLT-II as reported previously (12). This strain does not produce SLT-I. The absence of SLT-I gene was confirmed by PCR with specific primers. The strain was grown in 500 ml Luria broth (LB) by constant shaking for 12 h at 37°C. The culture supernatant was obtained by centrifugation and the protein fraction was precipitated with 60% saturated ammonium sulfate at 4°C. The precipitate was collected by centrifugation, dissolved in 2 ml of phosphate-buffered saline (PBS) (pH 7.2) and dialyzed overnight against PBS at 4°C. After the dialysis, the dialysate (approximately 2.5 ml) was used as the crude SLT-II preparation. The concentration of SLT-II in the crude preparation was determined to be 20 μ g/ml by using a reverse passive latex agglutination kit (Vtec-Rpla; Denka Seiken, Tokyo).

Animals

Male ddY mice (Japan SLC, Hamamatsu), weighing 25–30 g, were used in this study. The mice were housed under controlled environmental conditions (about 25°C) with commercial food and water freely available. All animal experiments were carried out in accordance with the guidelines of Nagoya University School of Medicine for the care and use of laboratory animals.

Establishment of SLT-II-treated mice

SLT-II-treated mice were established by injecting SLT-II (0.3 μ g/animal) into the tail vein of mice 24 h before starting the experiments (11). Control mice were injected with saline instead of SLT-II.

Protein binding experiments

To measure the protein binding of each drug in the control and SLT-II-treated mice, the protein binding experiment was done by using seamless cellulose tubing (Viskase Sales Corp., Willowbrook, IL, USA). Plasma samples were obtained from control mice and mice treated 24 h earlier with SLT-II (0.3 μ g/animal). Before doing the experiment, the seamless cellulose tubing was boiled for 1 h. Plasma sample was put into one side of a

dialysis cell divided by the seamless cellulose tubing, and PBS containing each drug (10 $\mu\text{g}/\text{ml}$) was put into the other side. Then, they were incubated for 6 h at 37°C. The total (plasma solution) and unbound (PBS solution) concentrations of each drug were measured by high-performance liquid chromatography (HPLC).

In vivo experiments

Mice were anesthetized with pentobarbital (25 mg/kg) and cannulated with polyethylene tubes (PE 28; Natsume, Tokyo) in the right jugular vein for drug administration and blood collection. After surgical preparation, mice received a loading dose (LD) and a maintenance dose (MD) of COX inhibitors at a rate of 0.3 ml/h until the end of the study. The LD and the MD of each drug were as follows: diclofenac: LD = 1.70 mg/kg, MD = 1.88 mg $\cdot$ h⁻¹ $\cdot$ kg⁻¹; mefenamic acid: LD = 15.35 mg/kg, MD = 12.32 mg $\cdot$ h⁻¹ $\cdot$ kg⁻¹; acetaminophen: LD = 46.27 mg/kg, MD = 12.10 mg $\cdot$ h⁻¹ $\cdot$ kg⁻¹. Based on the preliminary experiments to obtain plasma concentrations-time profiles of COX inhibitors after the bolus intravenous injection of diclofenac (10 mg/kg), mefenamic acid (10 mg/kg), and acetaminophen (100 mg/kg), the corresponding pharmacokinetic parameters were calculated and LD and MD were decided upon. Expected plasma concentrations of COX inhibitors were 15 $\mu\text{g}/\text{ml}$ of diclofenac, 15 $\mu\text{g}/\text{ml}$ of mefenamic acid, and 10 $\mu\text{g}/\text{ml}$ of acetaminophen. After 2-h infusion, the mice were killed by exsanguinations, and blood and brain samples were collected.

To evaluate the function of anion transporter in the brain, a typical anion transporter inhibitor, probenecid, was administered to the mice. Probenecid was administered (100 mg/kg, i.p.) to control and SLT-II-treated mice 30 min before they were killed. The dose of probenecid was selected from the literature and is known to induce efflux transport of anionic compounds from the brain (25, 26). The control and SLT-II group without probenecid were administered saline (0.1 ml/10 g, i.p.) instead of probenecid. The mice were killed by exsanguinations, and blood and brain samples were collected.

Blood samples were immediately centrifuged at 6,000 rpm for 10 min at 4°C to yield plasma. Brain samples were homogenized with a 4-fold volume of saline by an ice-cold glass homogenizer. All plasma and brain samples were stored at -80°C until analysis.

The K_p value of each drug is presented as the brain-to-plasma concentration ratio:

$$K_{p_{\text{total}}} = \text{brain concentration} / C_{\text{ss}}$$

$$K_{p_{\text{unbound}}} = \text{brain concentration} / C_{\text{ss}} \times \text{unbound fraction}$$

Extraction of COX inhibitors from plasma and brain tissue

For quantification of mefenamic acid and diclofenac, 50 μl of plasma samples or 200 μl of brain samples were vortexed for 10 min with 10 μl of internal standard (I.S.), 10 μl of HCl, and 1000 μl of hexane / isopropanol (99 : 1). Mefenamic acid (50 $\mu\text{g}/\text{ml}$) was used as I.S. for diclofenac measurement and diclofenac (50 $\mu\text{g}/\text{ml}$) for mefenamic acid measurement. After the centrifugation at 12,000 rpm for 5 min at 4°C, 900 μl of upper organic solvent layer was collected into test tubes and evaporated to dryness under a stream of nitrogen gas at 30°C. The dried residues were reconstituted with 120 μl of the mobile phase and injected into the HPLC instrument.

To extract acetaminophen from plasma and brain tissue, 50 μl of plasma samples or 200 μl of brain samples were vortexed for 10 min with 500 μl of methanol containing antipyrine (5 $\mu\text{g}/\text{ml}$) as an I.S. After the centrifugation at 12,000 rpm for 5 min at 4°C, 450 μl of the upper organic solvent layer was collected into test tubes and evaporated to dryness under a stream of nitrogen gas at 40°C. The residue was reconstituted with 120 μl of the mobile phase and injected into the HPLC instrument.

Analysis of COX inhibitors in plasma and brain samples by HPLC

After the extraction, plasma and brain concentrations of each drug were determined by HPLC.

The HPLC apparatus was an LC-10A_{VP} system (Shimadzu, Kyoto) consisting of an LC-10A_{VP} liquid pump, an SPD-10A_{VP} ultraviolet detector, and an SIL-10A_{VP} autoinjector. A Cosmosil 5C18-MS-II column (4.6 $\times$ 150 mm; Nacalai Tesque, Kyoto) was used with a column oven (OTC-10A_{VP}, Shimadzu) heated to 40°C. A voltage stabilizer (CV-1000; Denken Co., Ltd., Aomori) was also used.

In order to analyze diclofenac and mefenamic acid, 0.1 M phosphate buffer (pH 5.0) : acetonitrile = 60 : 40 (vol : vol) was used as the mobile phase at 1.2 ml/min flow rate, and the column eluate was monitored at a UV wavelength of 279 nm (27, 28).

To analyze for acetaminophen, 0.1 M phosphate buffer (pH 5.0) : methanol = 85 : 15 (vol : vol) was used as the mobile phase at 1.0 ml/min flow rate, and the column eluate was monitored at a UV wavelength of 245 nm (29).

Standard curves for measuring COX inhibitors in the plasma and brain tissue were linear for concentrations ranging from 0.15 to 20 $\mu\text{g}/\text{ml}$, with a correlation coefficient of 0.999. The intra- and inter-assay coefficients of variation for the HPLC assay were less than 6%

at concentrations of 0.15 to 20 $\mu\text{g/ml}$. The detection limit of COX inhibitors was 0.05 $\mu\text{g/ml}$.

Western blot analysis

Brain obtained from control mice (saline) and mice treated 24 h after SLT-II (0.3 $\mu\text{g/animal}$, i.v.) was kept at -80°C until analysis. Each brain was homogenized with a 5-fold volume of homogenate buffer (0.1 M Tris-HCl buffer (pH 8.0) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), aprotinin, leupeptin, pepstatin) in a glass homogenizer at 4°C . Then the homogenate was re-homogenized with a loose dounce homogenizer. After obtaining the homogenate supernatant by centrifugation at 8,500 rpm for 15 min at 4°C , the supernatant was transferred to an ultracentrifuge tube and centrifuged at 40,000 rpm for 30 min at 4°C . Subsequently, the supernatant was removed and the pellet was dissolved in homogenate buffer containing 1% of NP-40.

The protein concentration in the solution was measured using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA, USA) with bovine serum albumin (Sigma) as a standard. The protein (25 μg per lane) was subjected to electrophoresis on a 10% sodium dodecylsulfate (SDS)-polyacrylamide gel. The separated proteins were electrotransferred onto a nitrocellulose membrane, and the membrane was blocked with PBS containing 0.1% Tween 20 (PBS-T) and 5% nonfat dry milk for 1 h at room temperature. After washing three times with PBS-T for 5 min, the membrane was incubated with anti-OAT3 antibody (Transgenic, Kumamoto) for 1 h at room temperature. After washing three times, the membrane was allowed to bind a horseradish peroxidase-labeled anti-rabbit IgG antibody (Amersham Biosciences UK Ltd., Little Chalfont, UK) diluted 1:1000 in PBS-T for 1 h at room temperature. After washing three times, the bands were detected using ECL plus (Amersham Biosciences UK Ltd.). To quantify the relative levels of OAT3 in membranes, intensity of the stained bands was measured by the NIH image program (National Institutes of Health, Bethesda, MD, USA).

The statistical analysis

Results are expressed as the mean $\pm$ S.E.M. Data were analyzed by one-way analysis of variance (ANOVA) and post-hoc comparison was carried out with the Scheffe's post-hoc test. All statistics reported in these experiments were generated using StatView (version 4.5; Abacus Concepts, Berkeley, CA, USA) and *P*-values less than 0.05 were considered statistically significant.

Results

Plasma, brain concentrations and K_p of each COX inhibitor in control mice

COX inhibitors were continually infused until plasma steady-state concentrations were reached, at which point plasma and brain concentrations of COX inhibitors were measured. The ratio of brain concentration to total plasma concentration of COX inhibitors ($K_{p_{\text{total}}}$) varied between COX inhibitors. The rank of order of $K_{p_{\text{total}}}$ was observed to be acetaminophen (0.773 ± 0.074) > diclofenac (0.048 ± 0.003) > mefenamic acid (0.039 ± 0.006) when calculated using total plasma concentrations (Table 1). Since drug binding to plasma protein could affect drug distribution and elimination kinetics, the protein binding of the three COX inhibitors was also measured. The unbound fraction of diclofenac, mefenamic acid, and acetaminophen was $0.068 \pm 0.008\%$, $19.58 \pm 1.48\%$, and $89.55 \pm 2.28\%$, respectively (Table 1). Based on these results, the ratio of brain concentration to unbound plasma concentration of COX inhibitors ($K_{p_{\text{unbound}}}$) was calculated. The rank of order of $K_{p_{\text{unbound}}}$ was diclofenac (71.057 ± 3.786) $\gg$ acetaminophen (0.863 ± 0.083) > mefenamic acid (0.198 ± 0.029) (Table 1).

Effects of SLT-II on protein binding and K_p of COX inhibitors

To investigate the effect of SLT-II on the penetration of COX inhibitors into brain, K_p of each drug was studied in mice pretreated with SLT-II. In SLT-II-treated mice, for each COX inhibitor tested, protein binding was not altered. The unbound fraction of diclofenac, mefenamic acid, and acetaminophen was $0.065 \pm 0.004\%$, $16.71 \pm 0.08\%$, and $85.55 \pm 1.09\%$, respectively (Table 2). $K_{p_{\text{total}}}$ of acetaminophen was not significantly different between control and SLT-II-treated mice (Fig. 1, Table 2). However, $K_{p_{\text{total}}}$ of diclofenac and mefenamic acid was significantly increased in SLT-II-treated mice (Fig. 1, Table 2). No significant differences in the plasma concentrations of each drug were observed between the control and SLT-II-treated mice (Table 2).

Effects of probenecid on K_p in control and SLT-II-treated mice

In order to investigate the involvement of the anion transport system in brain penetration of COX inhibitors, we coupled administration of COX inhibitors with the typical anionic compound probenecid. In control mice, probenecid significantly increased the $K_{p_{\text{total}}}$ of both diclofenac and mefenamic acid (Fig. 2). However, in SLT-II-treated mice, probenecid did not affect the $K_{p_{\text{total}}}$

Table 1. Plasma, brain concentration, and protein binding (unbound fraction: fu) of each COX inhibitor in control mice

Drugs	Plasma ($\mu\text{g/ml}$)	Brain ($\mu\text{g/ml}$)	fu (%)	K _p (total)	K _p (unbound)
Diclofenac	15.11 $\pm$ 1.72	0.72 $\pm$ 0.06	0.068 $\pm$ 0.008	0.048 $\pm$ 0.003	71.06 $\pm$ 3.79
Mefenamic acid	18.05 $\pm$ 2.96	0.59 $\pm$ 0.06	19.58 $\pm$ 1.48	0.039 $\pm$ 0.006	0.198 $\pm$ 0.029
Acetaminophen	7.83 $\pm$ 1.23	5.79 $\pm$ 0.44	89.55 $\pm$ 2.28	0.773 $\pm$ 0.074	0.863 $\pm$ 0.083

Data represent the mean $\pm$ S.E.M. of 4 to 5 animals.

Table 2. Plasma, brain concentration, and protein binding (unbound fraction: fu) of each COX inhibitor in SLT-II-treated mice

Drugs	Plasma ($\mu\text{g/ml}$)	Brain ($\mu\text{g/ml}$)	fu (%)	K _p (total)	K _p (unbound)
Diclofenac	12.08 $\pm$ 2.14	0.84 $\pm$ 0.13	0.065 $\pm$ 0.004	0.072 $\pm$ 0.007 ^a	110.38 $\pm$ 10.02 ^a
Mefenamic acid	16.34 $\pm$ 2.00	1.78 $\pm$ 0.27 ^b	16.71 $\pm$ 0.08	0.115 $\pm$ 0.015 ^b	0.690 $\pm$ 0.091 ^b
Acetaminophen	7.64 $\pm$ 1.13	5.58 $\pm$ 0.95	85.55 $\pm$ 1.09	0.725 $\pm$ 0.035	0.848 $\pm$ 0.041

Mice were treated with SLT-II (0.3 $\mu\text{g/animal}$) into the tail vein 24 h before drug administration. Data represent the mean $\pm$ S.E.M. of 4 to 5 animals. ^a P <0.05, ^b P <0.01 vs control group (Scheffe's post-hoc test).

Fig. 1. K_p of Dic, MA, and Ace in SLT-II-treated mice. SLT-II was injected (0.3 $\mu\text{g/animal}$, i.v.) into the tail vein of mice 24 h before starting the experiments. Dic, MA, or Ace was continuously infused in SLT-II-treated mice. Two hours after the infusion of COX inhibitors, mice were killed to obtain K_p. Each column represents the ratio of the control mice and is presented as the mean $\pm$ S.E.M. (n = 4 – 5). ^a P <0.05 vs control. ^b P <0.01 vs control.

of both diclofenac and mefenamic acid (Fig. 2).

Expression of OAT3 in SLT-II-treated mice

Western blotting analysis revealed that the expression of organic anion transporter 3 (OAT3) in the brain was significantly decreased to 57 $\pm$ 5% of the control after 24 h of SLT-II treatment (Fig. 3).

Discussion

It is generally accepted that influenza virus infections stimulate the significant release of cytokines (4, 5), subsequently causing an infection-related syndrome including fever, diarrhea, and other symptoms. There are many reports indicating that cytokines affect the properties of the BBB and BCSFB by altering the function of the tight junction and drug/nutrition transporters (8, 10 – 12). However, the impact of the infection on brain penetration of antipyretic COX inhibitors remains unclear. Thus, we investigated brain penetration profiles of three COX inhibitors, diclofenac, mefenamic acid, and acetaminophen, that are popularly used as antipyretics, in the presence of SLT-II, a bacterial toxin, that stimulates cytokine release and alters the function of transporters as well as tight junctions in the brain (10 – 12).

Initially, we compared the brain penetration of each COX inhibitor using the ratio of brain concentration to unbound plasma concentration (K_p_{unbound}). Our results indicated that K_p_{unbound} differed between COX inhibitors, with the rank of order of K_p_{unbound} being diclofenac $\gg$ acetaminophen > mefenamic acid. These data suggest that diclofenac might potentially penetrate into brain more so than other COX inhibitors used in this study. Secondly, the effect of SLT-II on brain penetration of COX inhibitors was investigated. We found that K_p values of diclofenac and mefenamic acid were significantly increased, but not that of acetaminophen. In addition, SLT-II did not affect protein binding of all COX inhibitors. Taken together, these data show that SLT-II changed brain penetration of diclofenac and mefenamic acid, but not acetaminophen. Moreover, it

Fig. 2. Effect of probenecid on *Kp* of Dic and MA in control and SLT-II-treated mice. SLT-II was injected ($0.3 \mu\text{g}/\text{animal}$, i.v.) into the tail vein of mice 24 h before starting the experiments. Dic or MA was continuously infused in control mice and SLT-II-treated mice. Two hours after the infusion of COX inhibitors, mice were killed to obtain *Kp*. Probenecid was administered ($100 \text{ mg}/\text{kg}$, i.p.) 30 min before they were killed. Each column represents the mean $\pm$ S.E.M. ^a $P < 0.05$ vs control. ^b $P < 0.01$ vs control. ^c $P > 0.05$ vs SLT-II.

Fig. 3. Effect of SLT-II on the protein level of OAT3 in brain (A) and ratio of relative staining intensity for OAT3 in control and SLT-II-treated mice (B). Brain samples were obtained from control mice (saline) and mice treated 24 h after SLT-II ($0.3 \mu\text{g}/\text{animal}$, i.v.). The band was detected at approximately 50 kDa. Each column represents the mean $\pm$ S.E.M. ^a $P < 0.01$ vs control.

has been suggested that among the COX inhibitors investigated, it would be easier to control acetaminophen dosage because less consideration for potential CNS side-effects is needed.

In a previous study, we demonstrated that the same dosing regimen of SLT-II increased the brain to plasma ratio of fluorescein isothiocyanate labeled dextran with average molecular weight 4.4 kDa (FD-4), to approximately 150% of the control (11). In the present experiment, brain penetration of diclofenac and mefenamic acid increased to $148 \pm 13\%$ and $297 \pm 39\%$ of the control. Thus, SLT-II might alter an active transport system for COX inhibitors, in addition to changing brain capillary integrity. We also previously demonstrated that SLT-II increased the brain to plasma ratio of doxorubicin (11). Considering the fact that doxorubicin is a typical substrate for one of the efflux transporters, P-glycoprotein, it would be expected that SLT-II decreased P-glycoprotein expression, subsequently decreasing the efflux of doxorubicin into the blood circulation. However, unexpectedly, SLT-II did not decrease P-glycoprotein expression, but rather increased it (11). Thus, these results suggest that SLT-II might modify the expression of other transporters sensitive to doxorubicin (11).

In the present study, SLT-II increased brain penetration of diclofenac and mefenamic acid, but not acetaminophen. Strictly speaking, diclofenac and mefenamic acid, but not acetaminophen, having a carboxyl group is negatively charged under the physiological condition. Thus, it is likely that SLT-II might alter the expression of anion transporters rather than P-

glycoprotein. Indeed, there are numerous reports demonstrating the existence of anion transporters including OATs and Oatps (8, 30), although the direction of transport and specific location are not fully identified yet. Thus, we investigated the effect of anionic compounds on the brain penetration of COX inhibitors. So far, selective compounds for each anion transporter are not available. Thus, we selected probenecid due to the following two reasons: 1) it is a strong anion (31, 32), and 2) it can passively diffuse into tissues due to its lipophilicity. Thus, it would be expected that applying probenecid with COX inhibitors could estimate the function of both influx and efflux anion transporters at the BBB and/or BCSFB. Indeed, Galinsky et al. have demonstrated that probenecid can be used to evaluate the function of efflux anion transporters at the BBB in an in vivo animal model (33). Our data indicate that probenecid significantly increased K_p of both diclofenac and mefenamic acid. These results suggest that anion transporters might be involved in the transport of diclofenac and mefenamic acid. Moreover, considering the fact that the K_p of diclofenac and mefenamic acid was increased, rather than decreased, we suggest that probenecid may inhibit the efflux transporters at the BBB and/or BCSFB.

Thus, considering the finding that SLT-II increased brain penetration of these two COX inhibitors, it has been expected that SLT-II decreases the expressions/functions of efflux transporter for anionic compounds at the BBB and/or BCSFB. Recently, various efflux transporters are found on the BBB and BCSFB. Among them, Oatp2 and OAT3 have been reported as anion transporters (34–37). However, immunohistochemical studies have demonstrated that Oatp2 is located on the apical sides of the BBB, probably acting as an influx transporter (30). Thus, considering the results using probenecid, we investigated the expression of OAT3 in SLT-II-treated mice. Our results indicated that SLT-II significantly decreased the expression of OAT3. This suggests that the decreased expression of OAT3 might be one candidate efflux transporter to change brain penetration of COX inhibitors by SLT-II. In this study, we did not evaluate the impact of other anion transporters on the brain penetration of COX inhibitors. Thus, further work is required to fully understand this issue. Moreover, we must also determine which factors are important for regulating the BBB function after SLT-II treatment among the cytokines released by SLT-II or SLT-II itself since cytokines might regulate the function of transporters (7–9) and SLT-II increases the levels of cytokines including TNF- α and NOx in the plasma of rodents (11, 12, 38). However, the altered brain penetration of drugs was varied between the bacterial toxins

since another bacterial toxin, endotoxin, did not alter K_p of either FD-4 or doxorubicin, even though endotoxin stimulated the release of cytokines (39).

In conclusion, SLT-II differentially affected the brain penetration of COX inhibitors. Among COX inhibitors investigated, SLT-II might preferentially alter brain penetration of diclofenac and mefenamic acid, rather than acetaminophen. Thus, considerable attention would be necessary when using COX inhibitors in patients with *E. coli* O157 infection. Moreover, one of the candidate transporters that may contribute to the change in brain penetration of COX inhibitors, OAT3, was evaluated. Together, these results caution us that bacterial and virus infections such as *E. coli* O157 (10, 11) and influenza virus (4, 5) might increase the risk of CNS side-effects of drugs transported by OAT3 and other efflux transporters.

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