Regular Article

Inflammation-Induced Attenuation of Prostaglandin D₂ Elimination across Rat Blood–Brain Barrier: Involvement of the Downregulation of Organic Anion Transporter 3 and Multidrug Resistance-Associated Protein 4

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Prostaglandin (PG) D₂ is a lipid mediator, and in the brain, overproduction of PGD₂ is reportedly involved in the progression and exacerbation of neuroinflammation. The objective of this study was to elucidate PGD₂ efflux transport, under normal and inflammatory conditions, across the blood–brain barrier (BBB), which is formed by brain capillaries. Elimination of [³H]PGD₂ across the BBB of normal and lipopolysaccharide (LPS)-induced inflammatory rats was examined by the intracerebral microinjection technique. After intracerebral injection, the percentage of [³H]PGD₂ remaining in the ipsilateral cerebrum decreased with time, with a half-life of 13 min. This [³H]PGD₂ elimination across the BBB was significantly inhibited by the co-administration of unlabeled PGD₂, which suggests carrier-mediated PGD₂ efflux transport at the BBB. In isolated rat brain capillaries, mRNA expression of organic anion transporter (Oat) 3, organic anion-transporting polypeptide (Oatp) 1a4, and multidrug resistance-associated protein (Mrp) 4 was observed. In addition, co-administration of substrates/inhibitors for Oat3, Oatp1a4, and/or Mrp4, such as benzylpenicillin and cefmetazole, reduced [³H]PGD₂ elimination across the BBB. Data suggest that Oat3 and Mrp4, but not Oatp1a4 are involved in PGD₂ elimination across the BBB, as Oatp1a4-expressing Xenopus (X.) oocytes did not show the significant [³H]PGD₂ uptake compared with water-injected X. oocytes. In LPS-treated rats, [³H]PGD₂ elimination across the BBB and mRNA expression levels of Oat3 and Mrp4 were significantly decreased. Our data suggest that Oat3- and Mrp4-mediated PGD₂ elimination across the BBB is attenuated under inflammatory conditions.

Key words blood–brain barrier; lipopolysaccharide; prostaglandin D₂; organic anion transporter 3; multidrug resistance-associated protein 4

INTRODUCTION

Prostaglandin (PG) D₂ is a lipid mediator produced by a series of enzymatic reactions via cytosolic phospholipase A₂, cyclooxygenase, and prostaglandin D synthase (PGDS) from the cellular membrane.¹ In the brain, PGD₂ is reportedly the most abundant PG,² and has known neural roles including regulation of body temperature and sleep-wake cycles.³,⁴ Trimarco et al. reported that myelination is controlled by PGD₂, which is produced via lipocalin-type PGDS.⁵ On the other hand, cerebral PGD₂ which is overproduced via hematopoietic PGDS in microglia, is reportedly involved in the progression and exacerbation of neuroinflammation.⁶ Iwamoto et al. have reported that the amount of PGD₂ is increased in patients of Alzheimer-type dementia.⁷ In this neurodegenerative disease, it has been reviewed that neural inflammation is one of the pathologies.⁸ Taken together, it is considered that the PGD₂ level in the brain is strictly regulated to maintain homeostasis of neural functions in addition to avoidance of neuroinflammation and development of neurodegenerative diseases.

The metabolic inactivation and elimination of PGD₂ are considered to play major roles in cerebral PGD₂ clearance. The activity of nicotinamide adenine dinucleotide-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH), an enzyme mediated by the first step of PGD₂ inactivation, is reportedly undetectable in the brain parenchyma.⁹,¹⁰ Thus, it is probable that the elimination of PGD₂ from the brain plays a major role as the PGD₂ clearance pathway in the brain. Our previous report indicated that PGD₂ in the cerebrospinal fluid is eliminated across the blood–cerebrospinal fluid barrier (BCSFB), which is formed by choroid plexus epithelial cells.¹¹ Similarly, in the brain parenchyma, the blood–brain barrier (BBB) is formed by brain capillary endothelial cells, and has the ability of efflux transport of PGE₂, as suggested from our previous studies.¹²,¹³ Considering the local elimination of PGD₂, which is produced in brain parenchyma via hematopoietic PGDS in the microglia, it is possible that BBB-mediated PGD₂ transport plays an important role in cerebral PGD₂ clearance.

At the BBB several transporters are known to play a role in brain-to-blood clearance and restriction of cerebral distribution of compounds/drugs. Mori et al. elucidated that organic anion transporter 3 (Oat3/solute carrier (Slc) 22a8) is expressed on the abluminal membrane of the rat BBB and is involved in cerebral elimination of homovanillic acid.¹⁴,¹⁵ The organic anion transporting polypeptide 1a4 (Oatp1a4/Slc01a4) is reportedly localized on both abluminal and luminal membranes of the BBB in rats.¹⁶ Furthermore, Oatp1a4

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Oat3 and Pgt accept PGD2 as a substrate. These transporters, thus far, it has been elucidated that both BBB-mediated PGD2 elimination and the alteration of these molecules at the BBB in inflammation will lead to understanding the role of PGD2 transport at the BBB in the pathogenesis of neural inflammation.

The objective of this study was, therefore, to investigate efflux transport of PGD2 across the BBB under inflammatory conditions in addition to normal ones. In order to investigate in vivo [3H]PGD2 elimination across the BBB, a rat intracerebral injection method, called brain efflux index (BEI) study, was utilized. The inhibitory effect of substrates of organic anion transporters on in vivo BBB-mediated PGD2 elimination was investigated in addition to mRNA expression levels of these organic anion transporters using rat capillaries isolated by nylon meshes. In vitro [3H]PGD2 transport activities were investigated using transporter-expressing Xenopus (X) oocytes.

MATERIALS AND METHODS

Animals and Statement of Experimental Ethics

The animal experiments described in this manuscript were approved as #A2017PHA-5 and #A2017PHA-6 by the Animal Care Committee of the University of Toyama. Wistar/ST rats (male, approx. 200 g/rat) and X. frogs (female, 140–220 g) were purchased from Japan SLC (Hamamatsu, Japan) and Kato-S-Science (Chiba, Japan), respectively. The rats were maintained with a 12/12h dark/light cycle in a controlled environment: humidity, approx. 45%; temperature, approx. 23°C. The frogs were maintained with a 12/12h dark/light cycle in a controlled environment: humidity, approx. 50%; temperature, approx. 15°C.

Reagents

Unlabeled PGD2, lipopolysaccharides (LPS) from Escherichia coli 0111:B4, and pentobarbital sodium salt solution were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.), Merck (Darmstadt, Germany), and Kyorin Seiyaku (Tokyo, Japan), respectively. Digoxin, [3H(G)]-([3H]dihydroxyin, 29.8 Ci/mmol), prostaglandin D2, [5,6,8,9,12,14,15-3H(N)]-([3H]PGD2; 131 Ci/mmol), and prostan- glandin E2, [5,6,8,11,12,14,15-3H(N)]-([3H]PGE2; 186 Ci/mmol) were purchased from PerkinElmer, Inc. (Waltham, MA, U.S.A.). Mannitol, n-[-1-14C]-([14C]d-mannitol; 55 mCi/mmol), a BBB-impermeable marker, was obtained from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade, and obtained from Thermo Fisher Scientific (Waltham, MA, U.S.A.), Merck, FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), or Nakalai Tesque (Kyoto, Japan).

Lipopolysaccharide Administration

LPS powder was dissolved in 0.9% NaCl solution. The LPS solution was administered to the rats via intraperitoneal injection at 4.0 mg/kg, and the animals were maintained for 24 h in the controlled environment.

Intracerebral Micro-Administration

For the evaluation of rat BBB-mediated PGD2 elimination, the intracerebral microinjection was performed using the BEI method, as previously described.12,13 The injection solution (0.5 µL; 122 mM NaCl, 25 mM NaHCO3, 10 mM d-glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES), 3 mM KCl, 0.4 mM K2HPO4, 1.4 mM CaCl2, 1.2 mM MgSO4; pH 7.4) contained [14C]d-mannitol (a BBB-impermeable marker; 0.005 µCi/rat, 182 µM) and [3H]PGD2 (0.1 µCi/rat, 1.53 µM), including or excluding inhibitors. 3H- and 14C-derived radioactivities were measured by liquid scintillation counting (AccuFLEX LSC7400, Aloka, Tokyo, Japan). The BEI value was defined as equation 1 and the percentage of [3H]PGD2 remaining in the ipsilateral cerebrum (100-BEI) was determined using equation 2:

\[
BEI(%) = \frac{[{}^{3}H]PGD_{2} \text{ undergoing BBB mediated efflux transport}}{[{}^{3}H]PGD_{2} \text{ injected into the cerebrum}} \times 100
\] (1)

\[
100 - BEI(%) = \frac{-[{}^{3}H]PGD_{2}/BBB}{-\text{impermeable marker in the brain}} \times 100
\] (2)

HPLC

HPLC, as previously described,12,26 was used to examine PGD2 and 3H-labeled compounds in plasma and brain samples after microinjection. Briefly, [3H]PGD2 (1 µCi/rat) was administered to rat Par2 region. Blood was collected via the jugular vein, 5 min after [3H]PGD2 administration. Plasma was obtained as supernatant following centrifugation (7800 × g, 4°C, 5 min). Immediately thereafter, the ipsilateral cerebrum was collected. For deproteinization, the brain sample was homogenized in 10 volumes of ethanol, and plasma was mixed with 4 volumes of ethanol. These samples were centrifuged (15000 × g, 4°C, 20 min), the supernatant was dried under N2, and then the residue was dissolved in the mobile phase (25 μL; acetoniitrile:acetic acid:water, 45:0.1:55 (v:v:v)). The HPLC conditions were as previously published.26 The 3H-derived radioactivity in each eluent was determined using AccuFLEX LSC7400 (Aloka).

Isolation of Rat Tissues Including Brain Capillaries

Rats were anesthetized by intraperitoneal pentobarbital sodium (50 mg/kg) injection after which whole liver, kidney, and cerebral choroid plexus were removed and rinsed with...
phosphate-buffered saline (–). Tissues were minced and used for isolation of total RNA.

Rat brain capillaries were isolated as previously published, with minor modifications.\(^2\)^ Briefly, four brains were collected from anesthetized rats into ice-cold isolation solution (137 mM NaCl, 10 mM D-glucose, 8.1 mM Na\(_2\)HPO\(_4\), 2.7 mM KCl, 2.0 mM sodium pyruvate 1.5 mM KH\(_2\)PO\(_4\), 1.8 mM CaCl\(_2\); pH 7.4). After removing the white matters and meninges, the brains were manually homogenized in isolation solution with 40 strokes, mixed with dextran (final concentration, 16%; Thermo Fisher Scientific), and centrifuged at 4500 \(\times g\) for 20 min, at 4°C. The pellet was suspended in 1% bovine serum albumin-containing isolation solution and filtered through a 210 \(\mu\)m nylon mesh (Kyoshin Rikoh, Tokyo, Japan). The filtrate was passed over a 30 \(\mu\)m pluriStrainer (pluriSelect Life Science, Leipzig, Germany), and the rat brain capillaries trapped onto the CellStrainer membrane were collected in 1% bovine serum albumin-containing isolation solution and filtered through a 10 \(\mu\)m nylon mesh (Kyoshin Rikoh, Tokyo, Japan). The capillary fraction was then washed twice with isolation solution and used for total RNA isolation.

**mRNA Expression Analyses** Total RNA was isolated from tissues using TRIzol reagent (Thermo Fisher Scientific). cDNA was synthesized with oligo dT primer from 100 ng total RNA using ReverTra Ace (Toyobo, Osaka, Japan). To confirm the expression of mRNA, non-quantitative PCR using DNA polymerase (ExTaq; TaKaRa Bio, Shiga, Japan) was performed with primers shown in Table 1, with the following reaction condition: 35 cycles of 95 °C for 30 s, 60–62 °C for 30 s and 72 °C for 45 s. Samples without reverse transcriptase in the reaction mixture were use as negative controls. PCR products were separated by electrophoresis on 2% agarose gels containing 0.6 \(\mu\)g/mL ethidium bromide, and were visualized using ultraviolet light. The mRNA expression levels in tissues were analyzed using quantitative real-time PCR (RT-qPCR) analysis with the same aforementioned primers (Table 1) and SYBR Premix ExTaq II (TaKaRa Bio) using a MX3005P QPCR system (Agilent Technologies, Santa Clara, CA, U.S.A.).

**Uptake Study Using X. Oocytes** Capped complementary RNA (cRNA) of rat Oatpl4a4 was prepared as previously described,\(^2\) and injected into X. oocytes (58 ng per oocyte). Nuclease-free water (23 nL/oocyte) was injected as a control. The oocytes were kept for 4 d in the culture medium (100 mM NaCl, 5.0 mM HEPES–NaOH, 2.5 mM pyruvic acid, 2.0 mM KCl, 1.8 mM CaCl\(_2\), 1.0 mM MgCl\(_2\), 1% bovine serum albumin, 25 \(\mu\)g/mL gentamicin sulfate; pH 7.5). Following the four-day incubation, these oocytes were treated with ND96 buffer composed of 96 mM NaCl, 5 mM HEPES–NaOH (pH 7.4), 2 mM KCl, 1.8 mM CaCl\(_2\), 1 mM MgCl\(_2\) for 5 min at 20°C. The uptake reaction was initiated by adding 200 \(\mu\)L of ND96 buffer containing \([\text{3H}]\text{PGD}_2\) (0.2 \(\mu\)Ci/mL, 1.53 nM), \([\text{3H}]\text{PGF}_2\alpha\) (0.2 \(\mu\)Ci/mL, 1.08 nM), or \([\text{3H}]\text{digoxin}\) (2\(\mu\)Ci/mL, 67.1 nM) at 20°C. The uptake reaction was terminated by washing oocytes 4 times with ND96 buffer at 4°C. In 5% sodium dodecyl sulfate/water at 15–25°C, oocytes were solubilized. The \(^3\)H-radioactivity in the samples was measured using AccuFLEX LSC-7400 (Aloka). The uptake activity was expressed as the distribution volume (\(\mu\)L/oocyte), namely oocyte/medium ratio, which was calculated by dividing the amount of \(^3\)H-labeled compound in the solubilized sample (\(\mu\)Ci/oocyte) by that in the uptake buffer (\(\mu\)Ci/mL).

**Data Explanation and Statistical Analyses** Kinetic parameters, such as \(k_d\), were expressed as the mean ± standard deviation (S.D.). Other data were presented as the mean ± standard error of the mean (S.E.M.). Statistical differences between the means of two or more groups were tested using the unpaired two-tailed Student’s \(t\)-test or one-way ANOVA, followed by Bonferroni multiple comparisons or Dunnett’s test, respectively.

**RESULTS**

**In Vivo Cerebral Elimination of PGD\(_2\) across the BBB in Normal and LPS-Treated Rats** Figure 1A shows a time-profile of \([\text{3H}]\text{PGD}_2\) 100-BEI values, \(i.e.,\) the percentage of \([\text{3H}]\text{PGD}_2\) remaining in the ipsilateral cerebrum after microinjection into the Par2 region. In normal rats (Fig. 1A, closed circles), the 100-BEI of \([\text{3H}]\text{PGD}_2\) decreased over time with a half-life of 13.0 ± 1.3 min. In addition, the \([\text{3H}]\text{PGD}_2\) 100-BEI value at 20 min was significantly (1.91-fold) increased by the co-administration of 3 mM unlabeled PGD\(_2\) (Fig. 1B). At 24 h after intraperitoneal 4.0 mg/kg LPS administration, \([\text{3H}]\text{PGD}_2\) 100-BEI values was time-dependently decreased (Fig. 1A, open squares). The half-life of this decrease was 55.5 ± 14.4 min. Regarding the recovery of \([\text{14C}]\text{mannitol}\) after intracerebral administration, no significant difference among samples at each time in either normal and LPS-treated rats was observed, where significance was analyzed using one-way ANOVA, followed by Bonferroni multiple comparisons (data not shown).

| Target    | GenBank accession # | Primer (upper, sense; lower, antisense) | Expected size |
|-----------|---------------------|------------------------------------------|---------------|
| Claudin-5 | NM_031701.2         | 5’-get acet gg tgt ggg gtc tgt-3’         | 483 bp        |
|           |                     | 5’-ccg gaa aga aca ggc aca gga-3’        |               |
| Oat3      | AB017446            | 5’-tgg ttc cag cgc cga tgt tgt-3’         | 372 bp        |
|           |                     | 5’-ccc cca acc aga gac cat-3’            |               |
| Oatpl4a4  | NM_131906           | 5’-tcc tgc tgc tgt ggt cta tgt cgg-3’     | 294 bp        |
|           |                     | 5’-aag aag aag aga aca gcc cca att-3’    |               |
| Mrp4      | NM_133411           | 5’-ggt acc ctc atc atc ggg ctc tgt aag-3’| 419 bp        |
|           |                     | 5’-gaat ctc ctc gta ctt aga cgg cca gga-3’|           |
| Pgt       | NM_022667           | 5’-ggt acc ctc atc atc ggg ctc tgt aag-3’| 443 bp        |
|           |                     | 5’-gaat ctc ctc gta ctt aga cgg cca gga-3’|           |
| \(\beta\)-Actin | NM_031144   | 5’-tca tga agg gtt acc gtc tgt cgc cgg-3’| 285 bp        |
|           |                     | 5’-cgg aca agg att tgc ggt gca cca tgt-3’|               |
is considered to correspond to intact PGD₂, the ratio of that in regard to the sum of radioactivity eluted from 4–8 min, which lateral cerebrum (Fig. 2B) and plasma (Fig. 2C) samples. With LPS-treated rats (open squares). Each point represents the mean ± S.E.M. (n = 3–4). The result of the non-linear least-square regression analysis is expressed by a solid line. (B) Effect of co-administration of 3 mM unlabeled PGD₂ on 100-BEI of [³H]PGD₂ at 20 min in normal rats. In this condition, the cerebral concentration of unlabeled PGD₂ was expected to be 65.2 µM, which was obtained by the PGD₂ concentration in the injected solution divided by 46.2, as a dilution factor.²⁰ Each column represents the mean ± S.E.M. (n = 3). Significant (p<0.01) differences from the control are indicated by a double asterisk (**).

HPLC Analysis of Brain and Plasma after Intracerebral [³H]PGD₂ Injection [³H]PGD₂ and metabolites thereof were examined using HPLC analysis of samples taken 5 min after injection of [³H]PGD₂ into the cerebral Par2 region of normal rats. The identity of [³H]PGD₂ was confirmed based on the retention time (6 min) of the [³H]-derived radioactivities (Fig. 2A). Based on retention time, [³H]PGD₂ was detected in ipsilateral cerebrum (Fig. 2B) and plasma (Fig. 2C) samples. With regard to the sum of radioactivity eluted from 4–8 min, which is considered to correspond to intact PGD₂, the ratio of that in the sample of the cerebrum (Fig. 2B) and plasma (Fig. 2C) to that in the injectate (Fig. 2A) was determined to be 99.0 and 47.1%, respectively.

Expression of PGD₂ Transporter mRNAs in Brain Capillaries in Normal Rats In order to examine the mRNA expression of organic anion transporters (Oat3, Oatplα4, Pgt, and Mrp4), which accept PGs as a substrate, RT-PCR analysis was performed on extracts from isolated brain capillaries from normal rats (Figs. 3A1, 4). Expression of Oat3, Mrp4, and Oatplα4 mRNAs was observed in isolated brain capillaries from normal rats, as well as in respective positive controls (Oat3 and Mrp4, rat kidney; Oatplα4, rat liver). However, Pgt mRNA expression was not detected in isolated rat brain capillaries, but only in rat choroid plexus, which was used as a positive control.

Alteration of PGD₂ Transporter mRNA Expression Level by LPS Administration The mRNA expression levels of PG transporters, which are expressed at the BBB in brain capillaries of LPS-treated rats (Fig. 3A2) were compared with expression levels in normal rats. No significant difference between the expression levels of claudin-5,²⁹ which is involved in the formation of tight-junctions at the BBB, was observed in isolated brain capillaries of normal and LPS-treated rats (Fig. 3B). As shown in Fig. 5, mRNA expression of Oat3, Oatplα4, and Mrp4 was significantly decreased by 59.4, 39.0, and 69.8%, respectively.

Inhibitory Effect of Transporter Inhibitors on [³H]PGD₂ Elimination across the BBB in Normal Rats The in vivo inhibition of BBB-mediated [³H]PGD₂ elimination by co-administration of substrates/inhibitors for the transporters was examined (Table 2), in order to elucidate which transporter(s) is/are responsible for PGD₂ elimination across the BBB. The BEI value of [³H]PGD₂ was significantly decreased by more than 44% 20 min after co-administration of 3 mM benzylpenicillin (Oat3 and Mrp4), 1 mM probenecid (Oat3, Oatplα4, and Mrp4), or 3 mM cefmetazole (Oatplα4 and Mrp4). A significant decrease of [³H]PGD₂ BEI value was however not observed following the co-administration of 0.1 mM digoxin, a specific substrate for Oatplα4, and 3 mM tetrathyammonium (TEA), which is not a substrate of Oat3, Oatplα4, or Mrp4.

PG Transport Study Using Rat Oatplα4-Expressing X. Oocytes To evaluate PG transport activities of rat Oatplα4, uptake studies using X. oocytes containing Oatplα4 cRNAs were performed (Fig. 6). Uptake of [³H]digoxin, a known substrate of rat Oatplα4,³⁰ in rat Oatplα4 cRNA-injected X. oocytes was significantly (4.21-fold) increased, compared with that of water-injected oocytes, indicating that rat Oatplα4 is functionally expressed in the X. oocytes. Uptake of [³H]PGD₂ and [³H]PGE₂ in rat Oatplα4-expressing X. oocytes was not significantly different from that in water-injected X. oocytes.

DISCUSSION

In this study it was demonstrated for the first time that intracerebral PGD₂ is eliminated across the rat BBB by carrier-mediated processes (Fig. 1). Peripheral LPS administration resulted in the decrease of this PGD₂ elimination and in the mRNA levels of several organic anion transporters (Figs. 1, 4). Data from the in vivo inhibition study (Table 2) suggest that Oat3 and Mrp4 at the rat BBB are involved in BBB-mediated PGD₂ elimination.

The BEI study showed that [³H]PGD₂, which was injected into the rat cerebral Par2 region, was eliminated with a half-life of 13 min (Fig. 1A), which corresponds to data from our previous report that showed a half-life of 14 min for PGE₂.
elimination from rat brain across the BBB. Thus, data suggest that the capacity of BBB-mediated clearance of PGD₂ is similar to that of PGE₂ in rats. In addition, HPLC analysis data, which showed that 5 min after [³H]PGD₂ intracerebral administration, 99% of intact [³H]PGD₂ was detected in the brain, suggest that cerebral [³H]PGD₂ crosses the rat BBB intact (Fig. 2). Eguchi et al. reported that following systemic administration in rats, [³H]PGD₂ was rapidly metabolized within 5 min with [³H]PGD₂ metabolites detected in samples of the circulating blood and the brain. In this study, approx. 50% of intracerebral administered [³H]PGD₂ was detected intact in circulating blood in rats 5 min after administration (Fig. 2C). It is possible that the other ³H-derived radioactivities are mainly derived from metabolites generated in the peripheral organs. Hence, it is considered that these metabolites could be detected in the samples of the rat brain which is collected at 20, 40, and 60 min in the study of the [³H]PGD₂ BEI study (Fig. 1A) if the HPLC analysis for [³H]PGD₂ is performed. However, the profile of one-compartmental [³H]PGD₂ elimination was observed in our study, indicating that the elimination property mainly reflects intact [³H]PGD₂ elimination across the rat BBB. Together these data suggest that the BBB takes part in controlling neural PGD₂ activities through the clearance of intact PGD₂ from the cerebral interstitial fluid into the blood.

The 100-BEI of [³H]PGD₂ was significantly increased by the co-administration of unlabeled PGD₂ (Fig. 1B), suggesting that cerebral PGD₂ elimination across the BBB is carrier-mediated. PGD₂ is reportedly a substrate of Oat3 and Pgt, and human MRP4 accepts PGE₂ as a substrate. Further- more, Taogoshi et al. reported that in in situ brain perfusion, cerebral PGE₁ transport was inhibited by digoxin, a selective substrate for Oatplα4. Among these transporters, mRNA expression of Oat3, Oatplα4, and Mrp4 at the rat BBB was detected in isolated rat brain capillaries (Figs. 3, 4), whereas Pgt mRNA expression in the isolated brain capillaries was not indicated in this study. Interestingly, Kis et al. reported that mRNA and protein expression of Pgt were detected in brain vascular endothelial cells. One possible explanation of this discrepancy could be relatively low expression levels (below the limits of detection) of Pgt mRNA in the BBB. It has been reported that Pgt is involved in PGD₂ and PGE₂ elimination.
from the cerebrospinal fluid across the BCSFB. Thus, in the BEI study, the leakage of the compounds to the cerebrospinal fluid after the intracerebral injection of 0.5 µL solution is reportedly negligible. Thus, it is suggested that Pgt is hardly involved in PGD₂ elimination from the cerebral cortex after the micro-injection. In summary, Oat3, Oatp1a4, and Mrp4 are proposed as candidate proteins that contribute to BBB-mediated PGD₂ efflux transport.

The aim of the in vivo inhibition study was to determine which molecules contribute to PGD₂ efflux transport across the BBB in rats. Oat3, Oatp1a4, and Mrp4 reportedly play a role in cerebral elimination and/or restriction of cerebral distribution of compounds and drugs across the BBB. Co-administration of probenecid, a known substrate or inhibitor of Oat3, Oatp1a4, and Mrp4, inhibited [³H]PGD₂ efflux transport across the rat BBB (Table 2), suggesting the involvement of these transporters in PGD₂ elimination across the BBB. Furthermore, co-administration of benzylpenicillin (substrate of Oat3 and Mrp4) and cefmetazole (substrate of Oatp1a4 and Mrp4) at an expected final cerebral concentration of approx. 65 µM, resulted in significant inhibition of [³H]PGD₂ elimination across the rat BBB (Table 2). Digoxin is reportedly a specific substrate of rat Oatp1a4, with a Kₘ of 0.24 µM, and therefore it was anticipated that digoxin co-administration at an expected final cerebral concentration of approx. 2.2 µM would inhibit Oatp1a4 at the rat BBB, however, [³H]PGD₂ elimination across the BBB was not significantly altered following digoxin administration (Table 2). In addition, our in vitro study using rat Oatp1a4-expressing X. oocytes revealed that rat Oatp1a4 did not transport PGD₂ (Fig. 6), as was reportedly observed for [³H]PGE₂, which is not a substrate of rat Oatp1a4. This therefore suggests that Oatp1a4 is not involved in PGD₂ elimination across the BBB. Our previous study demonstrated that rat Oat3 accepts PGD₂ as a substrate by using rat Oat3-overexpressing X. oocytes. A recent study determined that PGD₂ is transported via human Mrp4. In smooth muscle cells of both rats and humans, it was reported that MRP4 regulates proliferation by efflux transport of cAMP. Hence, it is highly possible that rat Mrp4 accepts PGD₂ as a substrate. Moreover, Oat3 and Mrp4 are reportedly predominantly localized on abluminal and luminal membranes of the BBB, respectively. Based on the above lines of evidence, it is probable that Oat3 and Mrp4 are major contributors to brain-to-blood vectorial transport of PGD₂ across the BBB.

Peripheral administration of 4 mg/kg LPS, a cell surface proteoglycan of Gram-negative bacteria, attenuates PGD₂ elimination across the rat BBB (Fig. 1A). Cherrington et al. reported that intraperitoneal LPS administration (4 mg/kg) induced acute inflammation and cholestasis, and altered the hepatic expression of several transporters, such as Oatp1a4 and Mrp2. Furthermore, in rodents peripheral LPS administration at a dose of 1–10 mg/kg induces severe inflammatory reactions, such as sepsis. Therefore, it is probable that BBB-
mediated PGD₂ elimination is decreased in severe Gram-negative bacterial infections. Several previous reports showed that peripheral LPS administration decreases the integrity of tight-junctions at the BBB. In contrast, protein expression of Claudin-5 and the cerebral distribution of BBB-impermeable compounds were reportedly not altered by peripheral LPS administration. In this study, recovery of [³⁵C]mannit, a BBB-impermeable compound, was not significantly varied at the examined times in normal and LPS-treated rats (data not shown). Moreover, the mRNA expression level of Claudin-5 in brain capillaries isolated from LPS-treated rats was not significantly changed compared with that in normal rats (Fig. 3C). This, thus, suggests that the attenuation of BBB-mediated PGD₂ elimination by the LPS administration is as a result of a reduction in the eflux transport capacity at the BBB of PGD₂, and not due to a change in tight-junction integrity at the BBB.

Our analysis of mRNA expression using isolated brain capillaries suggests that mRNA expression levels of Oat3, Oatplα4, and Mrp4 are significantly decreased following LPS administration (Fig. 5). The co-administration study results (Table 2) suggest that the attenuation of PGD₂ elimination across the BBB following LPS administration can, at least in part, be attributed to the reduction (> 59%) in Oat3 and Mrp4 transcript levels at the BBB. Regarding protein expression levels at the BBB during inflammation: our previous study showed a significant 26% reduction, compared with that of control, mice in Oat3 proteins, but not in Mrp4 proteins, in whole brain capillary lysate prepared from 3.0 mg/kg LPS-treated mice. Although there is no information of protein expression level of Oat3 and Mrp4 at the BBB after intraperitoneal administration of 4.0 mg/kg LPS in rats, it is highly possible that Oat3 and Mrp4 proteins at the BBB are down-regulated at 24 h after this LPS administration. Since it is considered that altered transcription, protein translation, and/or degradation profiles of Oat3 and Mrp4 at the mouse BBB during inflammation are different under normal conditions, analyses of the differences of these processes between mice and rats could be helpful in understanding the detail of the reduction in Oat3 and Mrp4 mRNA/protein levels in brain capillaries of rats and mice. Nevertheless, data from this study suggest that the attenuation of BBB-mediated PGD₂ elimination following peripheral LPS administration is, at least in part, attributable to the reduction in Oat3 and Mrp4 transcription at the BBB. In mice the PGD₂ contents in the brain reached approx. 50 ng/g approximately 40 pg/g. Indeed, hematopoietic PGDS is reportedly an enzyme inducible by LPS treatment. However, data from our in vivo study (Figs. 1A, 5) suggest that PGD₂ elimination across the BBB contributes to the increase in cerebral PGD₂ under inflammatory conditions. Based on these data it seems probable that the attenuation of BBB-mediated PGD₂ elimination might be applied in the treatment of central nervous system diseases, which are accompanied by inflammation. In human periodontal ligament stem cells, LPS treatment reportedly promotes the phosphorylation of glycogen synthase kinase (GSK) -3, which indicates GSK-3 activation. Harati et al. reported that expression of Oat3 mRNA in rat brain capillaries is upregulated by chemical inhibition of GSK-3. This inhibition of GSK-3 was also reported to increase Mrp4 mRNA and protein in human immortalized brain endothelial cells. Although the relationship between GSK-3 upregulation and Oat3/Mrp4 downregulation in rat brain capillaries after peripheral LPS administration requires further investigation, it should be considered that the inhibition of GSK-3 at the BBB normalizes BBB-mediated PGD₂ eflux transport, thus reducing the inflammatory responses induced by PGD₂ as occurs in inflammatory central nervous system diseases.

In conclusion, to the best of our knowledge, this is the first report of the elimination of intact cerebral PGD₂ across the

Table 2. Effect of Compound Co-administration on [³H]PGD₂ Efflux Transport across the Rat BBB

| Compound          | Injectate concentration (mM) | Expected concentration (µM) | BEI (%) | % of control |
|-------------------|-----------------------------|----------------------------|---------|-------------|
| Control           |                             |                           | 62.7±4.5| 100±7       |
| Benzylpenicillin  | 3                           | 64.9                      | 22.9±2.3| 36.4±3.6**  |
| Probenecid        | 1                           | 21.6                      | 27.8±4.0| 44.4±6.4**  |
| Cefmetazole       | 3                           | 64.9                      | 35.3±4.8| 56.4±7.6**  |
| Digoxin           | 0.1                         | 2.16                      | 49.2±3.5| 78.5±5.6    |
| TEA               | 3                           | 64.9                      | 58.8±1.6| 93.8±2.6    |

The BEI value was determined 20 min after intracerebral injection of 0.5 µL solution containing [³H]PGD₂ (0.1 µCi/head) and [¹⁴C]mannitol (0.005 µCi/head). Following dose administration, the cerebral concentration of each respective compound was calculated by dividing the compound concentration in the injectate by 46.2, which is the reported dilution factor. Each BEI value and percentage of control represents the mean ± S.E.M. (n = 3). Significant (p < 0.01) differences from the control are indicated by a double asterisk (**). TEA, tetraethylammonium.

![Fig. 6. Transport Study of [³H]PGD₂, [³H]PGE₂, and [³H]Digoxin Using Oatplα4 cRNA-Injected X. Oocytes](image)
BBB in rats. Furthermore, data from this study suggest the involvement of Oat3 and Mrp4 at the BBB in this PGD2 elimination. Under inflammatory conditions induced by peripheral administration of LPS, a decrease in BBB-mediated PGD2 elimination and in mRNA expression levels of Oat3 and Mrp4 was observed. These new data will aid in understanding the roles of these transporters at the BBB in cerebral elevation of PGD2-related neuro-reactions under pathological conditions, including inflammation.

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Conflict of Interest The authors declare no conflict of interest.

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