A Clearance System for Prostaglandin D₂, a Sleep-Promoting Factor, in Cerebrospinal Fluid: Role of the Blood-Cerebrospinal Barrier Transporters

Masanori Tachikawa, Kazuhiro Tsuji, Reiji Yokoyama, Takanori Higuchi, Go Ozeki, Ayane Yashiki, Shin-ichi Akanuma, Kazuyuki Hayashi, Akio Nishiura, and Ken-ichi Hosoya

Department of Pharmaceutics, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama, Japan (M.T., K.T., R.Y., T.H., G.O., A.Y., S.A., K.Ho.); Division of Membrane Transport and Drug Targeting, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan (M.T.); and Ono Pharmaceutical Co., Ltd, Osaka, Japan (K.Ha., A.N.)

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ABSTRACT

Although the level of prostaglandin (PG) D₂ in cerebrospinal fluid (CSF) affects the action of D-type prostanoid receptors that promote physiological sleep, the regulatory system of PGD₂ clearance from the CSF is not fully understood. The purpose of this study was to investigate PGD₂ elimination from the CSF via the blood-CSF barrier (BCSFB). The in vivo PGD₂ elimination clearance from the CSF was 16-fold greater than that of inulin, which is considered to reflect CSF bulk flow. This process was inhibited by the simultaneous injection of unlabeled PGD₂. The characteristics of PGD₂ uptake by isolated choroid plexus were, at least partially, consistent with those of PG transporter (PGT) and organic anion transporter 3 (OAT3). Studies using an oocyte expression system showed that PGT and OAT3 were able to mediate PGD₂ transport with a Michaelis-Menten constant of 1.07 and 7.32 M, respectively. Reverse transcription-polymerase chain reaction and immunohistochemical analyses revealed that PGT was localized on the brush-border membrane of the choroid plexus epithelial cells. These findings indicate that the system regulating the PGD₂ level in the CSF involves PGT- and OAT3-mediated PGD₂ uptake by the choroid plexus epithelial cells, acting as a pathway for PGD₂ clearance from the CSF via the BCSFB.

Introduction

Prostaglandin (PG) D₂ is an endogenous sleep-promoting substance that regulates physiological sleep, both non-rapid eye movement and rapid eye movement sleep, through D-type prostanoid receptor 1 (DP₁ receptor) (Huang et al., 2007). Because the DP₁ receptors are localized predominantly in the arachnoid trabecular cells in the ventral surface of the rostral basal forebrain (Mizoguchi et al., 2001), which faces the CSF, the CSF concentration of PGD₂ will be a key determinant of the action of DP₁ receptors. Indeed, infusion of PGD₂ into the lateral ventricle increases the amount of non-rapid eye movement sleep in a dose-dependent manner in wild-type mice but not DP₁ receptor knockout mice (Mizoguchi et al., 2001). In unanesthetized conscious rats, the CSF concentration of PGD₂ exhibits a significant circadian fluctuation with a peak (1197 pg/ml; 3.4 nM) at 2:00 PM and a minimum (438 pg/ml; 1.2 nM) at 6:00 AM (Pandey et al., 1995). These lines of evidence imply that regulatory systems for the generation and/or elimination of PGD₂ in the CSF produce a circadian rhythm.

The rates of generation and elimination of PGD₂ in the CSF seem to be well balanced to maintain PGD₂ levels in the CSF. It has been proposed that PGD₂ is generated from PGH₂ mainly by lipocalin-type prostaglandin D synthetase (L-PGDS) and released into the CSF (Qu et al., 2006). PGD₂ is also transported from the circulating blood to the brain across the blood-brain barrier (Suzuki et al., 1986). However, a clearance system for PGD₂ from the CSF would be essential for regulating PGD₂ levels in the CSF. In the brain, it is unlikely that prostaglandins are effectively inactivated, because...
cause there is little expression and activity of 15-hydroxyprostaglandin dehydrogenase, the rate-limiting enzyme for prostaglandin catabolism, in the adult brain (Alix et al., 2008). It is thus conceivable that the primary pathway for removing PGD$_2$ from the CSF is the CSF-to-blood vectorial transport across the blood-CSF barrier (BCSFB), which is formed by the complex tight junctions of choroid plexus epithelial cells in the ventricle (Hosoya et al., 2004). In support of this notion, it has been reported that the choroid plexus takes up PGF$_2\alpha$ via a saturable transport process (DiBenedetto and Bito, 1986).

Cellular transport of PGs is mediated by several transporters: organic anion transporter (OAT) (OAT/SLC22) (Kimura et al., 2002), organic cation transporter (OCT) (OCT/SLC22) (Kimura et al., 2002), and organic anion transporting polypeptide (oatp) (OATP/SLCO) (Kanai et al., 1995; Cattori et al., 2002), and organic anion transporting polypeptide (oatp) (OATP/SLCO) (Kimura et al., 2002), and organic anion transporting polypeptide (oatp) (OATP/SLCO) (Kanai et al., 1995; Cattori et al., 2001). Among high-affinity transporters for PGs with peptide (oatp) (OATP/SLCO) (Kanai et al., 1995; Cattori et al., 2002), organic cation transporter (OCT) (OCT/SLC22) (Kimura et al., 2002), prostaglandin transporter (PGT) (PGT/SLCO2A1) (Kis et al., 2006) are expressed in the choroid plexus. OAT3 recognizes PGF$_2\alpha$ with a $K_m$ value of 345 nM (Kimura et al., 2002), whereas PGT transports various prostaglandins, including PGF$_2\alpha$, PGD$_2$, and PGE2, with a $K_m$ value of 70 to 104 nM (Kanai et al., 1995). Because there is an overlap in substrates between PGT and OAT3, a combination of these transporters may be responsible for PGD$_2$ transport by using an expression system involving Xenopus laevis oocytes.

**Materials and Methods**

**Animals.** Adult male ddY mice (25–30 g), Wistar rats (260–280 g), and female Hartley guinea pigs (300–350 g) were purchased from Japan SLC (Hamamatsu, Japan). Mature female X. laevis oocytes were purchased from Kato-S-Science (Chiba, Japan). They were maintained in a controlled environment, and all experiments were approved by the Animal Care Committee of the University of Toyama. To minimize the diurnal variations, the transport analyses were conducted from noon to 7:00 PM.

**Reagents.** [5,6,8,9,12,14,15-3H(N)]PGD$_2$ ($^{3}$HPGD$_2$; 188.8 Ci/mmol) was obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). [Carboxyl-14C]inulin ([14C]inulin; 1.9 mCi/g) was obtained from Moravek Biochemicals (Brea, CA). [1-14C]butanol ([14C]butanol; 5.0 mCi/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals were commercial products of analytical grade.

In **In Vivo PGD$_2$ Efflux from the CSF after Intracerebroventricular Administration.** $[^{3}]$HPGD$_2$ elimination from the CSF after intracerebroventricular administration was studied by using the method described previously (Tachikawa et al., 2008a,b). Male Wistar rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg body weight), and their heads were fixed in a stereotaxic apparatus (Narishige, Tokyo, Japan). A hole was drilled in the skull, 1.5 mm to the left and 0.5 mm posterior to the bregma, into which a needle was fixed as a cannula for injection. A cerebrovascular dose of $[^{3}]$HPGD$_2$ (0.4 μCi/rat) and [14C]inulin (0.005 μCi/rat) dissolved in 10 μl of extracellular fluid buffer containing 122 mM NaCl, 25 mM NaHCO$_3$, 3 mM KCl, 0.4 mM K$_2$HPO$_4$, 1.4 mM CaCl$_2$, 1.2 mM MgSO$_4$, 10 mM d-glucose, and 10 mM HEPES was administered to the left lateral ventricle through the cannula. At designated times, CSF (50–100 μl) was withdrawn by cisternal puncture. The remaining radioactivity in the CSF specimens was determined by using a liquid scintillation spectrophotometer (LSC-5000; Aloka, Tokyo, Japan). The kinetic parameters for the elimination of $[^{3}]$HPGD$_2$ and [14C]inulin were determined from Eq. 1 by using the nonlinear least-square regression analysis program MULTI (Yamaoka et al., 1986):

$$C_{CSF}(t) = V_{d}/V_r \times \exp(-k_a \times t) \quad (1)$$

where $C_{CSF}(t)$, $V_r$, and $k_a$ are the CSF concentration at time $t$, the volume of CSF, and the elimination rate constant, respectively. As indicated in Fig. 1a, the $y$-intercept was expressed as percentage of dose per milliliter of CSF. Because the $y$-intercept values of $[^{3}]$HPGD$_2$ and [14C]inulin in Fig. 1a were ~500% dose/ml CSF, the volume of CSF in which [14C]inulin can be initially distributed was determined to be approximately 200 μl. This volume is in close agreement with the total CSF volume in rats as reported previously (Cserr and Berman, 1978). An apparent elimination clearance was determined by multiplying $k_a$ by $V_d$ ($k_a \times V_d$).

**HPLC Analysis.** At 2 min after intracerebroventricular administration of $[^{3}]$HPGD$_2$ (2 μCi/rat), the CSF was collected via cisternal puncture. The CSF was mixed with acetonitrile and acetic acid each at a concentration of 45% (v/v) and 0.1% (v/v), respectively. The mixture was centrifuged at 12,000 g for 5 min at 4°C, and the supernatant was collected. Then, 20 μl was subjected to HPLC analysis. The HPLC system consisted of a pump (EP-300; Eicom, Kyoto, Japan) and a column oven (ATC-300; Eicom). The HPLC analytical column was an Inertsil ODS-2 column (5 μm, 4.6 × 150 mm; GL Sciences, Tokyo, Japan). The mobile phase (acetonitrile/acetic acid/water, 45:0.1:55, v/v/v) was pumped through the column at a rate of 1.0 ml/min at 20°C. Samples of eluate were collected in scintillation counting vials, and the $^{3}$H radioactivity in each fraction (1.0 ml) was determined in a liquid scintillation counter (LSC-5000; Aloka).

**PGD$_2$ Uptake Using Freshly Isolated Rat Choroid Plexus.** The uptake of $[^{3}]$HPGD$_2$ by rat choroid plexus was examined by using the centrifugal filtration method described previously (Tachikawa et al., 2008a,b). The rats were decapitated, and the choroid plexus was isolated from the lateral ventricles and incubated at 37°C for 1 min in 300 μl of extracellular fluid buffer. Incubation medium containing both $[^{3}]$HPGD$_2$ and [14C]butanol in the presence or absence of inhibitors was added to initiate uptake. The final concentrations of incubation medium were 30 nM for $[^{3}]$HPGD$_2$ and 300 μM for [14C]butanol. The radioactivity in the specimens was determined by using a liquid scintillation spectrophotometer (LSC-5000; Aloka). The distribution volume of $[^{3}]$HPGD$_2$ per microliter of isolated choroid plexus (dpm/μl choroid plexus) divided by the medium concentration of $[^{3}]$HPGD$_2$ (dpm/μl). The volume (μl) of the isolated choroid plexus was calculated as the amount of $[^{3}]$HPGD$_2$ accumulated per microliter of isolated choroid plexus (dpm/μl choroid plexus) divided by the medium concentration of [14C]butanol (dpm/μl).

**Uptake Using PGT- and OAT3-Expressing X. laevis Oocytes.** Using T7 RNA polymerase, capped cRNA was transcribed from NotI-linearized pGEM-HEN containing an open reading frame of mouse PGT and rat OAT3 cDNAs as described elsewhere (Mori et al., 2003). Defolliculated oocytes were injected with 25 nl of water or the capped cRNA (50–100 ng) and incubated at 18°C in freshly prepared standard oocyte saline solution containing 100 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, 5 mM HEPES, 25 μg/ml gentamicin, 2.5 mM pyruvic acid, and 1% bovine serum albumin, pH 7.5. The standard oocyte saline solution used to incubate the oocytes was replaced daily with fresh solution. Experiments were performed after incubation for 4 to 6 days. An uptake study using PGT- and OAT3-expressing X. laevis oocytes was performed as described previously (Mori et al., 2003). The oocytes injected with PGT cRNA or OAT3 cRNA were
preincubated with 500 μl of ND96 solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.4, for 20 min at 20°C before the uptake experiment. The uptake experiment was initiated by replacing the ND96 solution with 200 μl of the same solution containing 1.2 nM [³H]PGD₂ and terminated by the addition of ice-cold ND96 solution after incubation for a designated time at 20°C. Oocytes were then washed four times with ice-cold ND96 solution and solubilized in 5% standard oocyte saline solution, and the accumulated radioactivity was determined in a liquid scintillation counter (LSC-5000; Aloka). For the inhibition study, the ND96 solution and solubilized in 5% standard oocyte saline solution, and the accumulated radioactivity was determined in a liquid scintillation counter (LSC-5000; Aloka). For the inhibition study, the ND96 solution and solubilized in 5% standard oocyte saline solution, and the accumulated radioactivity was determined in a liquid scintillation counter (LSC-5000; Aloka). For the inhibition study, the ND96 solution and solubilized in 5% standard oocyte saline solution, and the accumulated radioactivity was determined in a liquid scintillation counter (LSC-5000; Aloka). For the inhibition study, the ND96 solution and solubilized in 5% standard oocyte saline solution, and the accumulated radioactivity was determined in a liquid scintillation counter (LSC-5000; Aloka). For the inhibition study, the ND96 solution and solubilized in 5% standard oocyte saline solution, and the accumulated radioactivity was determined in a liquid scintillation counter (LSC-5000; Aloka). For the inhibition study, the ND96 solution and solubilized in 5% standard oocyte saline solution, and the accumulated radioactivity was determined in a liquid scintillation counter (LSC-5000; Aloka). For the inhibition study, the ND96 solution and solubilized in 5% standard oocyte saline solution, and the accumulated radioactivity was determined in a liquid scintillation counter (LSC-5000; Aloka).

Kinetic Analyses. The kinetic parameters for PGD₂ uptake by Xenopus oocytes expressing PGT and OAT3 were obtained from Eq. 2:

\[ V = \frac{(V_{\text{max}} \times S)}{K_m + S} \]  

where \( V \) is the uptake rate of PGD₂, \( S \) is the PGD₂ concentration in the medium, \( K_m \) is the Michaelis-Menten constant, and \( V_{\text{max}} \) is the maximum uptake rate. To obtain kinetic parameters, the equation was fitted by using the iterative nonlinear least-squares regression analysis program MULTI (Yamaoka et al., 1986).

RT-PCR Analysis. Total RNA was isolated from mouse choroid plexus and rat brain and lung by using TRIzol reagent (Invitrogen, Carlsbad, CA) as described by the manufacturer. The RNA was reverse-transcribed by using oligo(dT) primer and ReverTraAce (Toyobo Engineering, Osaka, Japan). PCR was performed by using ExTaq DNA polymerase (Takara, Kyoto, Japan) with the following thermal cycle program: 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final period of 72°C for 10 min. The sequences of the specific primers for mouse PGT (GenBank accession number AF323958) were as follows: the sense sequence was 5’-CATCTTTCTGTCGACGCTACTTCGGC-3’ and the antisense sequence was 5’-CACATGCTGTGGTCTCCTTCTT-3’. The PCR products were separated by electrophoresis on an agarose gel in the presence of ethidium bromide and visualized under ultraviolet light. The molecular identity of the resultant product was confirmed by sequence analysis using a DNA sequencer (ABI PRISM 3100; Applied Biosystems, Foster City, CA).

Antibodies. Polyclonal antibodies to PGT and L-PGDS were raised against amino acid residues 122 to 161 of mouse PGT (GenBank accession number AF323958) and amino acid residues 150 to 190 of mouse L-PGDS (GenBank accession number NP032989). The polypeptides were expressed as glutathione transferase fusion proteins using the pGEX4T-2 plasmid vector (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The fusion protein was purified with glutathione-Sepharose 4B (GE Healthcare), emulsified with Freund’s complete adjuvant (Difco, Detroit, MI), and injected subcutaneously into female Hartley guinea-pigs at 2-week intervals. Two weeks after the sixth injection, affinity-purified antibodies were prepared, first using protein G-Sepharose (GE Healthcare) and then using antigen peptides coupled to cyanogen bromide-activated Sepharose 4B (GE Healthcare). For the preparation of affinity media, polypeptides free of glutathione transferase were obtained by elution of the cleaved polypeptide after in-column thrombin digestion of fusion proteins bound to glutathione-Sepharose 4B.

Immunoblotting. Mouse lung and choroid plexus were homogenized in a buffer containing 10 mM HEPES, 1 mM EDTA, 1 mM EGTA, 320 mM sucrose, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), pH 7.4. Mouse brain was homogenized in a radioimmunoprecipitation assay buffer containing 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 1% nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, and a protease inhibitor cocktail (Sigma-Aldrich). The homogenates were centrifuged at 10,000g for 15 min. The supernatants were then obtained as whole lysate protein samples or further centrifuged at 100,000g for 60 min to obtain a crude membrane fraction from the...
pellets. The protein concentration was determined by the Bradford method using a DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Protein samples (50 μg per lane) were fractionated by SDS-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. The blotted membrane was incubated with an affinity-purified PGT antibody at 0.1 μg/ml or affinity-purified L-PGDS antibody at 0.5 μg/ml in Tris-buffered saline (25 mM Tris-HCl, pH 8.0 and 125 mM NaCl, pH 7.4) containing 0.1% Tween 20 and 4% skimmed milk, and visualized with an enhanced chemiluminescence kit (GE Healthcare).

**Immunohistochemistry.** Preparation of brain paraffin sections (5 μm) using a sliding microtome (SM2000R; Leica Wetzlar, Nussloch, Germany) and immunohistochemistry were performed as described previously (Tachikawa et al., 2004). In brief, for immunofluorescence, sections were immunoreacted overnight with guinea pig antibody to PGT or L-PGDS (2 μg/ml) singly or in combination with rabbit glucose transporter 1 (GLUT1) antibody (0.5 μg/ml; Sakai et al., 2003) and mouse Na⁺, K⁺-ATPase antibody (2 μg/ml; Millipore Corporation, Billerica, CA). Subsequently, they were incubated with species-specific Alexa Fluor 488-conjugated (Invitrogen) and Cy3-conjugated (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) secondary antibodies for 2 h. Photographs were taken with a confocal laser scanning microscope (TCS-SP5; Leica).

**Statistical Analysis.** Unless otherwise indicated, all data are presented as the mean ± S.E.M. The kinetic parameters are presented as the mean ± S.D. An unpaired, two-tailed Student's t test was used to determine the significance of differences between two groups. One-way analysis of variance followed by the modified Fisher’s least-squares difference method was used to assess the statistical significance of differences among means of more than two groups.

**Results**

**Elimination of PGD₂ from Rat CSF after Intracerebroventricular Administration.** Figure 1a shows the residual CSF concentration of [³H]PGD₂ and [¹⁴C]inulin after intracerebroventricular administration as a function of time. [³H]PGD₂ was eliminated from the CSF with a higher rate constant of 0.604 ± 0.118 min⁻¹ than that of [¹⁴C]inulin (0.0397 ± 0.0241 min⁻¹), a reference compound for CSF turnover and diffusion into the brain interstitial space through the ependymal space. The half-lives of [³H]PGD₂ and [¹⁴C]inulin elimination from the CSF were estimated to be 1.15 and 17.5 min, respectively. The elimination clearance of PGD₂ from the CSF (124 μl/min) was approximately 16-fold higher than that of inulin (7.80 μl/min). The elimination clearance of inulin was close to the CSF bulk flow rate (2.9 μl/min) obtained by Suzuki et al. (1985). The simultaneous injection of unlabeled PGD₂ with [³H]PGD₂ at a CSF concentration of 100 μM resulted in a 3.4-fold increase in the residual concentration of [³H]PGD₂ (240% dose/ml CSF) compared with that in the absence of PGD₂ (101% dose/ml CSF) at 20 min.

Fig. 1a, b, and c shows representative HPLC chromatograms of [³H] radioactivity in the injectate of [³H]PGD₂ and the CSF at 2 min after intracerebroventricular administration of [³H]PGD₂, respectively. One major peak that corresponds to intact [³H]PGD₂ was detected at the elution time of 7.5 min in the injectate. At 2 min after intracerebroventricular administration of [³H]PGD₂, two major peaks were detected in the CSF at the same elution time as intact [³H]PGD₂ and the earlier elution time (3.5 min). According to Eguchi et al. (1992), the additional peak was presumably considered to correspond to PGF₂α. Because the sum of radioactivity eluted from 6.5 to 9.5 min, which corresponds to intact [³H]PGD₂, showed 73.2% of the total radioactivity, most of the [³H]PGD₂ administrated in the CSF would undergo elimination as the intact form.

**Characteristics of PGD₂ Uptake by Isolated Rat Choroid Plexus.** To examine whether PGD₂ was eliminated from the CSF via the BCSFB, a study of [³H]PGD₂ uptake by the isolated rat choroid plexus was performed. [³H]PGD₂ uptake by the isolated choroid plexus exhibited a time-dependent increase linearly for up to 1 min of incubation with an initial uptake rate of 4.47 ± 0.56 μl/min · μl choroid plexus (Fig. 1d). To characterize the transporters involved in [³H]PGD₂ uptake by the isolated choroid plexus, the inhibitory effects of various compounds on the [³H]PGD₂ uptake were investigated (Table 1). PGD₂ and PGB₁ produced 78 and 85% inhibition of the [³H]PGD₂ uptake at a concentration of 1 mM. Bromocresolgreen, an inhibitor of PGT, inhibited the uptake by 79% at a concentration of 1 mM. The uptake was also inhibited by OAT3 substrates and/or inhibitors, such as dehydroepiandrosterone-3-sulfate (DHEAS), indomethacin, benzylenepicillin, and probenecid, by 23 to 75%. In contrast, 1-lactic acid had little effect on the uptake. These results suggest the involvement of PGT and OAT3 in PGD₂ uptake by the isolated rat choroid plexus.

**Characteristics of PGT and OAT3-Mediated [³H]PGD₂ Uptake in Mouse PGT-Expressing and Rat OAT3-Expressing Xenopus Oocytes.** Although the [³H]PGD₂ uptake study using the isolated choroid plexus suggests the involvement of PGT and OAT3, the contribution of each transporter to the PGD₂ uptake remains unknown. To determine the characteristics of PGT- and OAT3-mediated PGD₂ transport, [³H]PGD₂ uptake was studied with PGT- and OAT3-expressing oocytes (PGT/oocytes and OAT3/oocytes, respectively). [³H]PGD₂ was taken up in a time-dependent manner by PGT/oocytes (Fig. 2a) and OAT3/oocytes (Fig. 3a), and the uptake by these oocytes was several-fold higher than in water-injected oocytes. As shown in Figs. 2b and 3b, PGD₂ uptake by PGT/oocytes and OAT3/oocytes exhibited saturable kinetics with a Kₘ of 1.07 ± 0.32 μM and a Vₘₐₓ of 9.36 ± 0.71 pmol/(h · oocyte), and a Kₘ of 7.32 ± 1.27 μM and a Vₘₐₓ of 2.14 ± 0.28 pmol/(h · oocyte), respectively. The inhibitory effects of various compounds on PGT- and OAT3-mediated [³H]PGD₂ uptake were examined (Table 2). Bromocresolgreen, at a concentration of

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<td>1-Lactic acid</td>
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* P < 0.01, significantly different from control.
1 mM, produced a marked inhibition of the PGT-mediated \[^{3}H\]PGD\(_{2}\) uptake, whereas it had lesser effect on the OAT3- mediated uptake. In contrast, benzylpenicillin, at a concentration of 1 mM, significantly inhibited OAT3-mediated \[^{3}H\]PGD\(_{2}\) uptake, whereas it had no effect on PGT-mediated \[^{3}H\]PGD\(_{2}\) uptake. DHEAS, at a concentration of 1 mM, had little effect on PGT-mediated \[^{3}H\]PGD\(_{2}\) uptake.

**Expression and Localization of PGT in the Choroid Plexus.** RT-PCR gave one amplified product at the expected size of 255 bp in the mouse choroid plexus as well as the brain and lung used as a positive control for PGT expression (Fig. 4). The nucleotide sequences of the products were identical with that of mouse PGT (GenBank accession number AB103401). The expression and localization of PGT protein in the choroid plexus were characterized with guinea pig PGT antibody. Immunoblot analysis showed a single band at ∼55 to 65 kDa, which was detected in the lung used as a positive control for PGT expression (Fig. 5, a and b). One possible explanation for the difference in the molecular size of PGT between the lung and choroid plexus is the glycosylation between the lung and choroid plexus PGT. By using immunohistochemical techniques, the PGT antibody labeled the choroid plexus (Fig. 6a). PGT immunofluorescence was intense on the CSF side membrane of the choroid plexus (Fig. 6c). The bands and immunohistochemical staining were abolished with the use of antibody with preabsorbed antigen peptides (100 μg/ml), indicating the specific- ity of PGT antibody (Figs. 5a and 6d). By double immunofluorescence for Na\(^+-\)K\(^+-\)ATPase, a brush-border membrane marker of choroid plexus epithelial cells, the PGT immunoreactivities showed extensive overlapping with those of Na\(^{+}\), K\(^{-}\)-ATPase (Fig. 6e). In contrast, they were negative for GLUT1, a basolateral membrane marker of choroid plexus epithelial cells (Fig. 6f). These results indicate that PGT is localized on the brush-border membrane of choroid plexus.
epithelial cells. Guinea pig L-PGDS antibody recognized a single band at 26 kDa (Fig. 5c), which is consistent with previous results (Urade et al., 1989). The band was abolished with the use of antibody with preabsorbed antigen peptides (100 μg/ml), indicating the specificity of L-PGDS antibody (Fig. 5c). The L-PGDS antibody strongly labeled the leptomeninges (Fig. 6b).

Discussion

In the present study, our findings indicate that a regulatory system of the PGD₂ level in the CSF involves PGT- and OAT3-mediated PGD₂ uptake by the choroid plexus epithelial cells, acting as a pathway for PGD₂ elimination from the CSF.

[³H]PGD₂, after intracerebroventricular administration, was removed from the CSF most likely as the intact form with a 16-fold greater elimination clearance than that of [¹⁴C]inulin, which is considered to reflect the CSF bulk flow (Fig. 1a). The rapid elimination of [³H]PGD₂ from the CSF was inhibited by simultaneous injection of unlabeled PGD₂ at a CSF concentration of 100 μM (Fig. 1a), suggesting active efflux transport of PGD₂ from the CSF. This is in close agreement with a previous report demonstrating active elimination of PGF₂α, from rabbit CSF using ventriculo-cisternal perfusions (Bito et al., 1976). To evaluate the role of the BCSFB in PGD₂ elimination from CSF, [³H]PGD₂ uptake by the freshly isolated rat choroid plexus was investigated (Fig. 1d; Table 1). [³H]PGD₂ undergoes concentrative uptake by the choroid plexus, and this is inhibited by unlabeled PGD₂ (Fig. 1d). This indicates that the BCSFB contributes to the carrier-mediated efflux transport of PGD₂ from the CSF. The elimination clearance of PGD₂ from the CSF via the BCSFB was estimated from the initial uptake rate of PGD₂ by the isolated choroid plexus and found to be 26.8 μl/min per rat [4.47 μl/(min·μl choroid plexus) × 6 μl (the volume of total rat choroid plexus per rat); Ogawa et al., 1994]. The PGD₂ efflux transport via the BCSFB makes a 21.6% contribution to the total PGD₂ elimination clearance from the CSF in vivo (124 μl/min per rat). Because the elimination clearance of inulin from the CSF was 7.80 μl/min per rat, i.e., 6.29% of the total PGD₂ elimination clearance from the CSF, the contribution of the CSF bulk flow and diffusion into the brain interstitial space seems to be limited. It has been reported that Slo2b1 (moat1) transports PGD₂ and is expressed in the brain parenchymal cells (Nishio et al., 2000). It would raise another possibility that Slo2b1-mediated uptake by brain parenchymal cells is a potential elimination pathway of PGD₂ in the CSF.

The effects of several inhibitors on [³H]PGD₂ uptake by the isolated choroid plexus were examined to identify the transporters involved (Table 1). Uptake studies with PGT- and OAT3-expressing Xenopus oocytes showed that bromoresolgreen and DHEAS inhibit both PGT- and OAT3-mediated [³H]PGD₂ uptake, whereas benzylpenicillin inhibits only OAT3-mediated [³H]PGD₂ uptake (Table 2). The inhibition ratio of bromoresolgreen and DHEAS for the [³H]PGD₂ uptake (75–80%) by the choroid plexus is greater than that of benzylpenicillin (54%), each at a concentration of 1 mM (Table 1). Furthermore, although the Kᵢ value of probenecid for OAT3 was reported to be 4.43 to 20 μM (Sugiyama et al., 2001; Nagata et al., 2002), probenecid inhibited [³H]PGD₂ uptake by the choroid plexus only by 23% at a concentration of 100 μM (Table 1). Because probenecid at a concentration of 1 mM had little inhibitory effect on PGT-mediated [³H]PGD₂ uptake (Table 2), it is likely that both PGT and OAT3 are mediators of PGD₂ uptake by the choroid plexus. In support of this notion, the present study indicates that PGT is localized on the brush-border membrane of choroid plexus epithelial cells (Fig. 6). It should also be noted that DHEAS, PGE₂, and indomethacin are transportable substrates (Cattori et al., 2001) and/or inhibitors (Kusuhara et al., 2003), respectively, of oatp1a5 (Slo2b1), which is localized on the brush-border membrane of choroid plexus epithelial cells (Ohtsuki et al., 2001).
et al., 2003, 2004). It is thus intriguing in the future to study the contributions of OAT3, PGT, and oatp1a5.

The $K_m$ values of PGD$_2$ transport mediated by PGT (1.07 μM; Fig. 2b) and OAT3 (7.32 μM; Fig. 3b) are almost three orders of magnitude greater than the CSF concentration of PGD$_2$ (1–3 nM; Pandey et al., 1995) and PGE$_2$ (1.2 nM; Gao et al., 2009). This suggests that PGT and OAT3 mediate PGD$_2$ uptake by the choroid plexus epithelial cells without saturation, thus producing continuous removal of PGD$_2$ from the CSF. Because the circadian fluctuation of the PGD$_2$ concentration in the CSF ranges from 1 to 3 nM (Pandey et al., 1995), this may not be caused by the changes in the function of PGT- and OAT3-mediated PGD$_2$ elimination from the CSF at the BCSFB. It has been reported that the inhibition of PGD$_2$-generating enzyme L-PGDS results in a reduction in brain PGD$_2$ content and suppression of sleep in rats, indicating the crucial role of this enzyme in the regulation of physiological sleep (Qu et al., 2006). Immunohistochemical analysis reveals that L-PGDS is highly expressed in the leptomeninges facing the CSF (Fig. 6), which is consistent with previous reports (Urade et al., 1993). Taking these findings into consideration, it seems likely that the circadian fluctuation in the PGD$_2$ level in the CSF depends on the local production of PGD$_2$, presumably via L-PGDS.

There are two types of CSF-to-blood efflux transport at the BCSFB: 1) influx transport across the brush-border (apical) membrane of choroid plexus epithelial cells from the CSF into the cells and 2) subsequent efflux transport across the basolateral membrane from the cells into the blood. The present findings suggest that PGT and OAT3 on the brush-border membrane are involved in PGD$_2$ uptake from the CSF into choroid plexus epithelial cells. It has been reported that polarized apical localization of PGT in Madin-Darby canine kidney cells induces a 100-fold increase in apical-to-basal PGE$_2$ transport (Endo et al., 2002). Chan et al. (2002) have reported that PGT-mediated uptake of PGs is coupled to the efflux of intracellular lactate in PGT-overexpressing HeLa cells, which raises the possibility that PGT-mediated concentrative PGD$_2$ transport in the CSF-to-choroid plexus epithelial cells direction would be produced by the outward lactate gradient, although the lactate gradient in the epithelial cells remains to be determined. Furthermore, the polarized choroid plexus epithelial cells produce transcellular transport of PGE$_2$ in the apical-to-basolateral direction without inactivation of PGE$_2$ in the cells (Khuth et al., 2005). In this regard, PGT and OAT3 on the brush-border membrane of choroid plexus epithelial cells would be a key determinant of the CSF-to-blood PGD$_2$ efflux transport across the BCSFB. On the other hand, multidrug resistance-associated protein 4 (MRP4)/ATP-binding cassette transporter C4 is localized on the basolateral membrane of choroid plexus epithelial cells (Leggas et al., 2004) and mediates active efflux transport of prostaglandins, such as PGE$_1$ and PGE$_2$, out of the cells (Reid et al., 2003). Thus, MRP4 would be a potent candidate transporter for mediating PGD$_2$ efflux transport from the choroid plexus epithelial cells into the blood, although further studies are needed to clarify whether PGD$_2$ is a substrate of MRP4.

The continuous inhibition of the clearance of PGD$_2$ at the BCSFB may modify the PGD$_2$ level in the CSF, thus affecting physiological sleep. Kis et al. (2006) have reported that lipopolysaccharide stimulation diminishes the luminal mem-

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**Fig. 6.** Immunofluorescence with guinea pig PGT and L-PGDS antibodies. a$_1$ and a$_2$, PGT immunofluorescence (green) in the choroid plexus. b, intense L-PGDS immunolabeling (red) on the arachnoid membrane. c, intense PGT immunoreactivities (green) on the CSF side membrane (arrowheads) of the choroid plexus epithelial cells. d, abolished PGT immunostaining in the choroid plexus with preabsorbed antibody. Nuclei were stained with 4,6-diamidino-2-phenylindole (blue, a$_1$ and b) or propidium iodide (red, c and d). e$_1$ to e$_3$, PGT (red, e$_1$ and e$_3$) overlapped with Na$^+$, K$^+$-ATPase (green, e$_2$ and e$_3$) on the brush-border membrane (arrowheads) of choroid plexus epithelial cells. f$_1$ to f$_3$, lack of PGT immunostaining (red, f$_1$ and f$_3$) in GLUT1-positive basolateral membrane (green, f$_2$) of choroid plexus epithelial cells. Arrowheads and arrows indicate the brush-border membrane and the basolateral membrane, respectively. Scale bars: a$_1$, a$_2$, b, and d; 50 μm; c, e$_1$ to e$_3$, and f$_1$ to f$_3$; 10 μm.
branе localization of PGT in the cerebral endothelial cells. If this were true for PGT localization in the choroid plexus epithelial cells, PGT-mediated elimination of PGD2 from the CSF might be reduced under inflammatory conditions. Some commonly used nonsteroidal anti-inflammatory drugs, such as indomethacin and ibuprofen, have been shown to inhibit the transport of PGF2α, in the choroid plexus (Bito and Sal- vador, 1976). The present study indicates that indomethacin and diconfenc inhibit OAT3-mediated PGD2 transport (Table 2). Furthermore, we have found that ceftmozolyl, cefazolin, ceftriaxone, and cefotaxime, administered intravenously to mice, inhibit [3H]PGD2 efflux transport from the brain across the blood-brain barrier most likely because of the inhibition of MRP4 (Akanuma et al., 2010). Thus, the inhibitory effect on the PGT- and OAT3-mediated PGD2 transport should be taken into consideration in the development of new drugs.

In conclusion, PGT and OAT3 at the BCSFB represent the primary mechanisms for the continuous removal of locally produced PGD2 from the CSF, thereby regulating PGD2 lev- els in the CSF. The present findings thus provide novel insight into the mechanisms of PGs’ clearance from the CSF and may be helpful in the development of new therapeutic targets for insomnia.

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Authorship Contributions

Participated in research design: Tachikawa, Tsuji, Akanuma, Hayashi, Nishirui, and Hosoya.

Conducted experiments: Tachikawa, Tsuji, Yokoyama, Higuchi, Ozeki, Yashiki, and Akanuma.

Contributed new reagents or analytic tools: Hayashi and Nishirui.

Performed data analysis: Tachikawa, Tsuji, Yokoyama, Higuchi, Ozeki, Yashiki, and Akanuma.

Wrote or contributed to the writing of the manuscript: Tachikawa, Akanuma, and Hosoya.

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Address correspondence to: Dr. Ken-ichi Hosoya, Department of Pharmaceutical Sciences, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan. E-mail: hosoyak@pha.u-toyama.ac.jp