JPET Fast Forward. Published on March 1, 2010 as DOI: 10.1124/jpet.109.165332

Involvement of multidrug resistance-associated protein 4 in efflux transport of prostaglandin E_2 across mouse blood-brain barrier and its inhibition by intravenous administration of cephalosporins

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JPET Fast Forward. Published on March 1, 2010 as DOI: 10.1124/jpet.109.165332
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Running Title:

Cephalosporins inhibit MRP4-mediated BBB efflux of PGE_2

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Number of text pages – 31

Number of figures – 4

Number of tables – 3

Number of references – 40

Number of words in abstract – 227

Number of words in introduction – 528

Number of words in discussion – 1393

List of Abbreviations

15-PGDH, NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase; BBB, blood-brain barrier; BCECs, brain capillary endothelial cells; BCRP, breast cancer resistance protein, BEI, brain efflux index; CNS, central nervous system; COX, cyclooxygenase; DMSO, dimethylsulfoxide; ECF, extracellular fluid; HPLC, High-performance liquid chromatography; IC_{50}, median inhibitory concentration; ID_{50}, median inhibitory dose; MRP, multidrug resistance-associated protein; NSAIDs, nonsteroidal anti-inflammatory drugs; Oat3, organic anion transporter 3; Oatp1a4, organic anion transporting polypeptide 1a4; PGE_2, prostaglandin E_2; Pgt, prostaglandin transporter; P-gp, P-glycoprotein; PGES, prostaglandin E synthase

Recommended section

Metabolism, Transport, and Pharmacogenomics

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Abstract

Prostaglandin E_2 (PGE_2) acts as a modulator of synaptic signaling and excitability in the brain. Because PGE_2 is hardly inactivated enzymatically in adult brain, its brain level is considered to be controlled by efflux transport across the blood-brain barrier (BBB). The purpose of the present study was to clarify the efflux transport of PGE_2 at the BBB and the interaction of various drugs with this process. [^{3}H]PGE_2 was eliminated from brain across the BBB with a half-life of 16.3 min and the elimination was inhibited by 3 mM unlabeled PGE_2. Multidrug-resistance-associated protein 4 (MRP4/*ABCC4*) was reported to be localized at the luminal membrane of the BBB. MRP4-expressing membrane vesicles showed significant uptake of [^{3}H]PGE_2 and the uptake was inhibited by cefmetazole with an IC_{50} value of 10.2 μM. At the concentration of 20 μM, several drugs, including cefazolin, cefotaxime, ceftriaxone, and ketoprofen, significantly inhibited [^{3}H]PGE_2 uptake into MRP4-expressing membrane vesicles. Using the brain efflux index method, pre-administration of cefmetazole, cefazolin, ceftriaxone and cefotaxime was found to inhibit [^{3}H]PGE_2 efflux from brain across the BBB. Furthermore, intravenous administration of cefmetazole dose-dependently reduced [^{3}H]PGE_2 elimination across the BBB (ID_{50} = 120 mg/kg). These results indicate that PGE_2 is eliminated from the brain by MRP4-mediated efflux transport at the BBB, and peripheral administration of cefmetazole decreases the efflux transport of PGE_2 at the BBB; this interaction may influence brain function.

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Introduction

Prostaglandin E_2 (PGE_2) is a main product of the cyclooxygenase (COX) pathway. Two COX isoenzymes, COX-1 and COX-2, convert arachidonic acid released by phospholipases A_2 to prostaglandin H_2, which in turn is metabolized by terminal prostaglandin E synthase (PGES) into PGE_2 (Ivanov and Romanovsky, 2004). In the brain, COX-2 is constitutively expressed in various neuronal cells, especially in hippocampal and cortical glutamatergic neurons (Ikeda-Matsuo et al., 2005). PGE_2 in the brain acts as a modulator of synaptic signaling and excitability, both physiologically and pathologically (Chen and Bazan, 2005). In addition, COX-2 and PGES are upregulated by inflammatory stimuli, and specifically induce fever via hypothalamic E-type prostanoid receptor activation (Sugimoto and Narumiya, 2007). Thus, PGE_2 in the brain plays an important role in the modulation of neuronal signaling and neuro-inflammatory responses.

The level, and thus the biological activity, of PGE_2 is controlled by the balance between synthesis and inactivation of PGE_2 in each organ. In peripheral organs, the first step of the inactivation process is mediated by NAD^+-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH). Although COX-2 and PGES-positive neuronal and glial cells exist in the brain, 15-PGDH activity is barely detectable in cerebral cortex of adult brain (Nakano et al., 1972; Alix et al., 2008). Therefore, PGE_2 may be eliminated through a brain-to-blood efflux process at the blood-brain barrier (BBB) to control its level in the brain.

The BBB is formed by tight-junctions of brain capillary endothelial cells (BCECs). PGE_2 (pKa ~ 5) exists predominantly in charged form at physiological pH, and cannot easily cross the BBB by passive diffusion. Although it is still unknown whether PGE_2 in the brain is eliminated via a carrier-mediated process at the BBB, it was reported that PGE_2 is transported by organic anion transporters, such as organic anion transporter 3 (Oat3/*SLC22A8*), prostaglandin transporter (Pgt/*SLCO2A1*) and multidrug resistance-associated protein 4 (MRP4/*ABCC4*) (Kimura et al., 2002; Reid et al., 2003; Kobayashi et al., 2004). It has also

been shown that organic anion transporting polypeptide 1a4 (Oatp1a4/*SLCO1A4*), Oat3 and/or MRP4 are involved in brain-to-blood efflux transport at the BBB (Leggas et al., 2004; Ohtsuki and Terasaki, 2007). Therefore, these transporters are likely to be involved in the brain-to-blood efflux transport of PGE_2 at the BBB.

Cephalosporins and non-steroidal anti-inflammatory drugs (NSAIDs) are used to treat numerous infectious diseases and to suppress auto-immune responses such as fever (Molavi, 1991; Kim et al., 2009), but have adverse effects such as inhibitory-neuronal signal attenuation and encephalitis (Schliamser et al., 1991; Sunden et al., 2003). These drugs are substrates or inhibitors for organic anion transporters, including Oat3 and MRP4 (El-Sheikh et al., 2007; Nozaki et al., 2007). Since PGE_2 is also related to these central nervous system (CNS) symptoms (Phillis et al., 2006), it was hypothesized that PGE_2 elimination across the BBB might be inhibited by the above drugs, resulting in alterations of brain function through increased brain levels of PGE_2.

The purpose of this study was to elucidate the mechanism of PGE_2 elimination from brain across the BBB by using the brain efflux index (BEI) method in mice, and to evaluate the effect of various drugs on *in vitro* MRP4-mediated PGE_2 transport and *in vivo* PGE_2 elimination from brain across the BBB.

Materials and methods

Animals

Adult male C57BL/6J mice (20-30 g) were purchased from Japan SLC (Hamamatsu, Japan). All experiments conformed to the provisions of Animal Care Committee, University of Toyama and were approved by the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University.

Reagents

Prostaglandin E_2, [5,6,8,11,12,14,15-^{3}H(N)]- ([^{3}H]PGE_2; 185.6 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Inulin carboxyl, [carboxyl-^{14}C]- ([^{14}C]inulin; 1.9 mCi/g) was purchased from Moravek Biochemicals, Inc. (Brea, CA). Amphotericin B, ampiroxicam, clarithromycin, dimethylsulfoxide (DMSO), dipyridamole, fluconazole, itraconazole, kanamycin sulfate, mefenamic acid, meloxicam, miconazole, salicylic acid, sulpyrine monohydrate, sodium taurocholate and vancomycin hydrochloride were obtained from Wako Pure Chemicals Industries (Osaka, Japan). Acetaminophen, cefaclor, cefazolin sodium salt, cefmetazole sodium salt, cefotaxime sodium salt, cefsulodin sodium salt hydrate, ceftriaxone disodium salt, cephalexin hydrate, diclofenac, ibuprofen, indomethacin, loxoprofen, sodium salt, ketoprofen, minocycline hydrochloride and ursodeoxycholic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Cefotiam was purchased from The United States Pharmacopeial Convention, Inc. (Kansas City, MO). Cefdinir and celecoxib were purchased from Toronto Research Chemicals Inc. (North York, Canada). Control vesicles and human MRP4-expressing Sf9 membrane vesicles were purchased from GenoMembrane (Yokohama, Japan). All other chemicals were commercial reagent-grade products.

Transport studies with MRP4-expressing membrane vesicles

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Control vesicles and MRP4-expressing membrane vesicles were derived from insect Sf9 cells. Uptake experiments were performed using the rapid-filtration technique as described previously with minor modifications (Reid et al., 2003; Uchida et al., 2007). In brief, it was carried out in medium containing 5 μg membrane vesicles, 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 4 mM ATP, 10 mM phosphocreatine, 100 μg/mL creatine phosphokinase and 0.2 μCi [^{3}H]PGE_2 with or without inhibitor at the indicated concentration in a total volume of 55 μL. The reactions were carried out at 37°C and stopped by addition of 400 μL ice-cold stop solution (0.25 M sucrose, 10 mM Tris-HCl (pH 7.4) and 100 mM NaCl). Immediately, the samples were passed through 0.45 μm MF-Millipore™ membrane filters (HAWP, Millipore Corporation, Billerica, MA) under vacuum. The filters were washed three times with 3 mL ice-cold stop solution. The radioactivity derived from [^{3}H]PGE_2 that was retained on the filter was determined by liquid scintillation counting using ACSII (GE Healthcare). The protein content was determined with a DC-Protein Assay kit (Bio-Rad, Hercules, CA), with bovine serum albumin as a standard. The uptake value was expressed as the vesicle/medium ratio calculated by dividing the amount taken up into the membrane vesicles by the substrate concentration in the uptake medium.

The median inhibitory concentration (IC_{50}) value of cefmetazole was estimated from the following equation (Eqn. 1), using the nonlinear least-squares regression analysis program MULTI:

$$v = V_{min} + (V_{max} - V_{min})/[1 + ([I]/IC_{50})^n] \qquad \text{(Eqn. 1)}$$

where v, V_{min}, V_{max}, [I] and n are the uptake rate, the minimum uptake rate, the maximum uptake rate, the concentration of cefmetazole and Hill's constant, respectively.

PGE_2 efflux studies from brain across the BBB using brain efflux index (BEI) method in mice

In vivo mouse brain elimination experiments were performed using the intracerebral microinjection technique (Akanuma et al., 2008). In brief, a C57BL/6J mouse was anesthetized by intraperitoneal administration of pentobarbital (50 mg/kg body weight) and

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placed in a stereotaxic frame (SR-5M; Narishige, Tokyo, Japan). The applied solution, consisting of [^{3}H]PGE_2 (96 nCi) and [^{14}C]inulin (4.8 nCi) dissolved in 0.3 μL extracellular fluid (ECF) buffer (122 mM NaCl, 25 mM $NaHCO_3$, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, 10 mM D-glucose and 10 mM HEPES, pH 7.4) with 0.1% ethanol in the presence or absence of unlabeled compounds, was administered into the secondary somatosensory cortex 2 (S2) region of the brain. The needle was left in the injection configuration for an additional 4 min to prevent reflux of the injected solution along the injection track, before being slowly retracted. [^{14}C]Inulin is an impermeable marker used to normalize the actual injection volume, as the injection volume is small (0.3 μL). In a pre-administration study, the inhibitor solution (10 μL) at the indicated concentration in ECF buffer with or without 0.25% DMSO was injected into the S2 region 5 min prior to administration of the applied solution. As a control, ECF buffer with or without 0.25% DMSO was injected. In an intravenous administration study, 200 μL of the Ringer-HEPES buffer (141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$ and 10 mM HEPES-NaOH, pH 7.4) containing each compound was injected into the jugular vein 15 min prior to administration of the applied solution. At a designated time, the mouse was decapitated and the left and right cerebrum and cerebellum were excised. Each tissue was dissolved in 2 mL 2 N NaOH at 55°C for 3 h, and then mixed with 14 mL Hionic-Fluor (PerkinElmer Life & Analytical Sciences). The radioactivity was measured in a liquid scintillation counter equipped with an appropriate crossover correction for ^{3}H and ^{14}C (LSC-5100, Aloka, Tokyo, Japan).

The BEI value was defined according to Eqn. 2 and the percentage of [^{3}H]PGE_2 remaining in the ipsilateral cerebrum (100-BEI) was determined using Eqn. 3 (Kakee et al., 1996):

$$\text{BEI (\%)} = \frac{\text{Test substrate undergoing efflux at the BBB}}{\text{Test substrate injected into the brain}} \times 100 \qquad \text{(Eqn. 2)}$$

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$$100\text{-BEI}\ (\%) = \frac{\begin{array}{c}(\text{Amount of } [^3\text{H}]\text{PGE}_2 \text{ in the brain} \\ / \text{ amount of } [^{14}\text{C}]\text{inulin in the brain})\end{array}}{\begin{array}{c}(\text{Concentration of } [^3\text{H}]\text{PGE}_2 \text{ in injectate} \\ / \text{ concentration of } [^{14}\text{C}]\text{inulin in injectate})\end{array}} \times 100 \quad \text{(Eqn. 3)}$$

The apparent elimination rate constant (k_{eff}) was determined by fitting a semilogarithmic plot of 100-BEI, i.e., the percentage remaining in the ipsilateral cerebrum, versus time, using the nonlinear least-squares regression analysis program MULTI. To evaluate the inhibitory effect on $[^3\text{H}]\text{PGE}_2$ efflux across the BBB, the BEI value of $[^3\text{H}]\text{PGE}_2$ at 40 min was determined in the presence or absence of various drugs.

The median inhibitory dose (ID_{50}) value of cefmetazole was obtained from Eqn. 4, using the nonlinear least-squares regression analysis program MULTI:

$$BEI = BEI_{min} + (BEI_{max} - BEI_{min})/[1 + ([I]/ID_{50})^n] \quad \text{(Eqn. 4)}$$

where BEI_{min}, BEI_{max}, [I] and n are the minimum of BEI, the maximum of BEI, the intravenous administration dose of cefmetazole and Hill's constant, respectively.

High-performance liquid chromatography (HPLC) analysis

To examine ^{3}H-labeled compounds in the plasma and brain after microinjection, 1 μCi of $[^3\text{H}]\text{PGE}_2$ was administered to the S2 region of mouse brain. At 5 min after administration of $[^3\text{H}]\text{PGE}_2$, blood was collected via the jugular vein. Immediately thereafter, the mouse was decapitated and the ipsilateral cerebrum was obtained. Plasma was obtained by centrifugation at 7,800 × g for 5 min at 4°C. For deproteinization, 250 μL plasma was mixed with 1.0 mL of ethanol. The mixture was centrifuged at 15,000 × g for 20 min at 4°C, and the supernatant was collected. The brain sample was homogenized in 10 volumes of ethanol. The homogenate was centrifuged at 15,000 × g for 20 min at 4°C, and the supernatant was collected. Each sample was dried under nitrogen. The residue was dissolved in 25 μL of the mobile phase (acetonitrile: acetic acid: water = 45: 0.1: 55 (v: v: v)). Then, 20 μL was subjected to HPLC analysis according to the reported method (Eguchi et al., 1992; Alix et al.,

2008). The HPLC system consisted of a pump (EP-300, Eicom, Kyoto, Japan), a column oven (ATC-300, Eicom) and UV monitor (NOD-10, Eicom). The HPLC analytical column was an Inertsil ODS-2 (5 μm, 4.6 × 150 mm, BL Sciences, Tokyo, Japan). The mobile phase 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-5200, Aloka).

Data analysis

Unless otherwise indicated, all data represent the mean ± S.E.M.. The parameters determined by the least-squares regression analysis (k_{eff}, IC_{50} and ID_{50}) are presented as the mean ± S.D.. Statistical significance of differences between the means was determined by using the unpaired two-tailed Student's *t*-test for two groups and by one-way analysis of variance followed by Dunnett's test for more than two groups.

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Results

Efflux transport of [^{3}H]PGE$_2$ in the cerebral cortex across the BBB

Figure 1A shows the time-profile of the percentage of [^{3}H]PGE$_2$ remaining in the ipsilateral cerebrum after microinjection into the S2 region of the mouse brain. The percentage of [^{3}H]PGE$_2$ remaining decreased in a time-dependent manner, indicating that [^{3}H]PGE$_2$ was eliminated from mouse brain. The apparent efflux rate constant across the BBB (k_{eff}) of [^{3}H]PGE$_2$ calculated from the slope was $4.23 \times 10^{-2} \pm 0.27 \times 10^{-2}$ min^{-1} (mean ± S.D.). No radioactivity associated with this efflux transport process was detected in the contralateral cerebrum or cerebellum (data not shown). In addition, the percentage of [^{3}H]PGE$_2$ remaining in the brain at 40 min after microinjection was increased by co-administration of 3 mM unlabeled PGE$_2$ (Figure 1B). Figure 2 shows typical HPLC chromatograms of ^{3}H-radioactivity in the injectate (Figure 2A), ipsilateral cerebrum (Figure 2B) and jugular venous plasma (Figure 2C) at 5 min after intracerebral microinjection of [^{3}H]PGE$_2$. The peak of radioactivity was detected at the elution time of 8 min in all samples, and the sum of radioactivity eluted from 6 min to 10 min, which was considered to correspond to intact PGE$_2$, was 90.7%, 87.1% and 48.1% of the total radioactivity in the injectate, cerebrum and plasma, respectively. This result suggests that [^{3}H]PGE$_2$ in the cerebral cortex is predominantly transported in intact form to the circulation across the BBB.

Cis-inhibitory effect of drugs on MRP4-mediated [^{3}H]PGE$_2$ transport

It was considered that MRP4 is involved in PGE$_2$ efflux transport from inside of brain capillary endothelial cells to circulating blood, and drugs inhibited MRP4-mediated PGE$_2$ transport at the intra-cellular side, which is known as cis-inhibition. Thus, the cis-inhibitory effect of various drugs on MRP4-mediated PGE$_2$ transport was assessed by means of uptake study using inside-out human MRP4-expressing membrane vesicles. The uptake of [^{3}H]PGE$_2$ into MRP4-expressing membrane vesicles increased linearly for 10 min

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and was significantly greater than that into control vesicles (Figure 3A). Cefmetazole is a selective substrate for human MRP4 with a K_m value of 28.5 μM and is not a substrate/inhibitor for other ATP-binding cassette transporters expressed at the BBB, such as P-glycoprotein (P-gp/*ABCB1*) and rat breast cancer resistance protein (BCRP/*ABCG2*) (Okumura et al., 2007; Uchida et al., 2007). The concentration-dependent inhibitory effect of cefmetazole on human MRP4-mediated [^{3}H]PGE_2 transport was examined at 5 min, and the IC_{50} value was determined to be 10.2 ± 3.2 μM (Figure 3B; mean ± S.D.).

Inhibitory effects of drugs, including antibiotics and NSAIDs, were also examined at the concentration of 20 μM (Table 1). Among cephalosporins, cefazolin, cefotaxime, ceftriaxone, cefotiam and cefdinir significantly inhibited MRP4-mediated [^{3}H]PGE_2 transport by more than 35%, whereas cephalexin, cefsulodin and cefaclor did not. Other antibiotics and antifungals exhibited no effect. Among inhibitors of organic anion transporters, dipyridamole significantly inhibited MRP4-mediated [^{3}H]PGE_2 transport by 57%, while taurocholate and ursodeoxycholate were not inhibitory. Among NSAIDs, indomethacin strongly inhibited MRP4-mediated [^{3}H]PGE_2 transport by 81% and ibuprofen, ketoprofen, acetaminophen and celecoxib significantly inhibited the transport by more than 30%, whereas salicylate, loxoprofen, mefenamic acid, diclofenac, sulpyrine, meloxicam and ampiroxicam were not inhibitory.

Effect of intracerebral pre-administration of drugs on [^{3}H]PGE_2 elimination from the brain

The effect of drugs on [^{3}H]PGE_2 elimination from the brain across the BBB was investigated by pre-administration into the brain. As shown in Table 2, cefmetazole, cefazolin, cefotaxime, ceftriaxone and benzylpenicillin decreased the BEI values of [^{3}H]PGE_2 by 39%, 32%, 51%, 39% and 29%, respectively, indicating that these drugs inhibited brain-to-blood efflux transport of [^{3}H]PGE_2 at the BBB. Cefotiam, cefsulodin, itraconazole and clarithromycin had no significant effect. Among NSAIDs, indomethacin and ketoprofen decreased the BEI values of [^{3}H]PGE_2, while celecoxib did not. In addition, dipyridamole did

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not inhibit [^{3}H]PGE_2 efflux from brain across the BBB. The BEI value in the case of pre-administration of ECF buffer with or without 0.25% DMSO was not significantly different from that in the case of no pre-administration treatment (Figure 1 and Table 2).

Effect of intravenous administration of drugs on [^{3}H]PGE_2 elimination from the brain

The inhibitory effect of intravenous administration of cefmetazole on [^{3}H]PGE_2 efflux at the BBB was examined to clarify the effect of cefmetazole in circulating blood on PGE_2 elimination (Figure 4). The BEI value of [^{3}H]PGE_2 was significantly reduced by administration of 100 mg/kg, 200 mg/kg and 1,000 mg/kg cefmetazole and the estimated ID_{50} value was 120 ± 18 mg/kg (mean ± S.D.).

Table 3 shows the inhibitory effect of intravenous administration of various drugs on [^{3}H]PGE_2 elimination from the brain. Among antibiotics, 200 mg/kg cefmetazole and 200 mg/kg cefazolin significantly reduced the BEI value of [^{3}H]PGE_2 by 25% and 19%, respectively. Cefotaxime, ceftriaxone, cefalexin, cefaclor and cefsulodin had no significant effect at the dose of 200 mg/kg. In addition, 40 mg/kg dipyridamole significantly decreased the BEI value of [^{3}H]PGE_2 by 28%, while 10 mg/kg and 100 mg/kg taurocholate, 100 mg/kg ursodeoxycholate, 20 mg/kg diclofenac and 40 mg/kg ketoprofen had little effect (Table 3).

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Discussion

The present study indicates that PGE_2 is eliminated from the mouse cerebral cortex via a carrier-mediated process at the BBB (Figures 1 and 2). In addition, intracerebral and/or intravenous administration of cefmetazole and cefazolin, which inhibit MRP4-mediated [^{3}H]PGE_2 transport *in vitro* (Figure 3 and Table 1), reduces [^{3}H]PGE_2 efflux from brain across the BBB (Figure 4; Tables 2 and 3).

Using the BEI method, it was shown that [^{3}H]PGE_2 injected into mouse cerebral cortex was eliminated with a half-life of 16.3 min and the elimination of [^{3}H]PGE_2 was inhibited by co-administration of unlabelled PGE_2 (Figure 1). Moreover, [^{3}H]PGE_2 was suggested to cross the BBB predominantly in the intact form, since as much as 48.1% of the total ^{3}H radioactivity in the jugular venous plasma was found to be associated with intact PGE_2 (Figure 2), and it has been reported that [^{3}H]PGE_2 in the circulating blood after systemic administration is extensively metabolized in 5 min in rats (Eguchi et al., 1992). The other radioactivity in the plasma could be due mainly to metabolites generated in peripheral tissues. Overall, it appears that the BBB plays a role in modulating the activity of PGE_2 through the elimination of intact PGE_2 from the brain interstitial fluid to circulating blood via a carrier-mediated process.

PGE_2 is a substrate of MRP4, Oat3 and Pgt, which were reported to be expressed at the BBB (Pucci et al., 1999; Reid et al., 2003; Nilwarangkoon et al., 2007). We recently found that the protein expression level of MRP4 in mouse brain capillaries is 10.3-12.5% of that of P-gp, 33.1-39.6% of that of BCRP, and similar to that of Oat3 protein (Kamiie et al., 2008). A pre-administration study demonstrated that [^{3}H]PGE_2 elimination from mouse brain was inhibited by cefmetazole, which is a high-affinity substrate for MRP4 (Table 2). In addition, [^{3}H]PGE_2 was taken up into human MRP4-expressing membrane vesicles and cefmetazole inhibited [^{3}H]PGE_2 uptake into the vesicles with an IC_{50} value of 10.2 μM (Figure 3). This value is similar to the K_m value of human MRP4-mediated cefmetazole transport (Uchida et

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al., 2007). These results suggest that MRP4 is involved in the brain-to-blood efflux transport of [^{3}H]PGE_2 at the BBB

Oatp1a4 and OAT3 are involved in brain-to-blood efflux transport at the BBB, while Pgt is thought to be involved in blood-to-brain influx transport (Kis et al., 2006; Ohtsuki and Terasaki, 2007). Cefmetazole was reported to be a substrate of rat Oatp1a4 (Nakakariya et al., 2008), but there is no report that PGE_2 is a substrate for Oatp1a4. Furthermore, cefotiam inhibits human Oat3-mediated estrone sulfate transport (IC_{50} value = 213 μM) and cefsulodin is a substrate for rat Oatp1a4 (Ueo et al., 2005; Nakakariya et al., 2008). Since the dilution effect of compounds at the microinjection site should have been minimal in this study, 5 mM cefotiam and cefsulodin injected in the brain should have been sufficient to inhibit Oat3 and Oatp1a4 at the BBB. However, in a pre-administration study, cefotiam and cefsulodin did not inhibit [^{3}H]PGE_2 elimination from brain across the BBB (Table 2), suggesting that Oat3 and Oatp1a4 do not play major roles in the brain-to-blood efflux transport of [^{3}H]PGE_2 at the BBB.

At the BBB, MRP4 plays a role in excreting compounds from BCECs to circulating blood. To cause a cis-inhibitory effect on MRP4-mediated PGE_2 transport, drugs would have to be taken up from brain interstitial fluid and maintained at a high concentration in BCECs. Cefazolin, cefotaxime, ceftriaxone and indomethacin inhibited MRP4-mediated [^{3}H]PGE_2 accumulation in membrane vesicles, and pre-administration of these drugs into the brain also decreased [^{3}H]PGE_2 elimination from the brain (Table 2). In contrast, pre-administration of cefotiam, dipyridamole and celecoxib did not significantly decrease [^{3}H]PGE_2 elimination (Table 2), though these drugs inhibited the uptake of [^{3}H]PGE_2 into the membrane vesicles (Table 1). These differences in inhibitory effect may be due to differences in the uptake process from brain interstitial fluid into the BCECs.

It is important to clarify the effect of peripheral administration of drugs in order to understand drug interactions with [^{3}H]PGE_2 elimination from the brain. Intravenous administration of cefmetazole inhibited the efflux from brain across the BBB with an ID_{50}

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value of 120 mg/kg in mice (Figure 4). Although the cefmetazole dosage (14.3-28.6 mg/kg) is lower in clinical cases, it is considered that cerebral PGE_2 elimination across the BBB may be inhibited by cefmetazole administration in humans, because total body clearance of cefmetazole in humans (approximately 1.46 mL/min/kg) is lower than that in mice (approximately 26.2 mL/min/kg) (Shindo et al., 1978; Komiya et al., 1981; Ko et al., 1989). Moreover, in patients with renal failure who receive cefmetazole, there is a decline of total body clearance and the concentration of cefmetazole remains high (Tajima et al., 2006). Under this condition, it is possible that a high plasma concentration of cefmetazole would reduce [^{3}H]PGE_2 efflux from brain and aggravate excitatory and inflammatory responses, such as fever and seizure.

Intravenous administration of cefazolin also inhibited [^{3}H]PGE_2 efflux from brain across the BBB (Table 3). Cefazolin and cefmetazole are substrates of rat Oatp1a4, whereas cefotaxime, ceftriaxone and ketoprofen are not (Nakakariya et al., 2008). Oatp1a4 has been reported to be localized at the abluminal and luminal membranes of rat BBB, and to be involved in transport in both directions at the BBB (Gao et al., 1999). Therefore, cefazolin and cefmetazole are likely to be concentratedly taken up into BCECs via Oatp1a4 at the luminal membrane of the BBB, leading to cis-inhibition of MRP4-mediated PGE_2 transport at the luminal membrane of the BBB. On the other hand, intravenous administration of cefotaxime, ceftriaxone and ketoprofen, which have not been identified as Oatp1a4 substrates, had little effect on [^{3}H]PGE_2 elimination from the brain (Table 3), even though intracerebral pre-administration of these drugs inhibited [^{3}H]PGE_2 efflux transport (Table 2). This may be because their concentrations in the BCECs were not sufficiently high to inhibit the MRP4-mediated [^{3}H]PGE_2 transport, due to the lack of a transport process for these drugs at the luminal membrane of BCECs.

The [^{3}H]PGE_2 elimination from brain across the BBB was inhibited by intravenous administration of dipyridamole (Table 3). *In vitro* MRP4-mediated [^{3}H]PGE_2 transport was strongly inhibited by dipyridamole (Table 1). However, dipyridamole did not inhibit

JPET Fast Forward. Published on March 1, 2010 as DOI: 10.1124/jpet.109.165332
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[^{3}H]PGE_2 elimination in a pre-administration study (Table 2). It is possible that the blood-to-cell transport process for dipyridamole is different from the brain-to-cell transport process at the BBB, although the mechanism(s) remains to be established.

Antibiotics and NSAIDs are administered peripherally for the remediation and palliative treatment of infectious and inflammatory conditions. It is known that β-lactam antibiotics, including cefmetazole and cefazolin, inhibit $GABA_A$ receptor response in rat cerebral cortex and cause seizure (Schliamser et al., 1991; Fujimoto et al., 1995). PGE_2 in the brain enhances excitatory neuronal response and induces a pain response (Oliveira et al., 2009). In addition, the brain concentration of PGE_2 is increased in inflammation induced by endotoxin administration (Inoue et al., 2002). Because PGE_2 elimination from brain across the BBB is inhibited by cefmetazole or cefazolin administration (Tables 2 and 3), the concentration of PGE_2 in the brain might be increased and the increased level of PGE_2 might have an additive or synergistic effect on neuroexcitatory response. Moreover, many studies have attested to the importance of brain PGE_2, including COX function, in the development of neurodegenerative diseases, such as Alzheimer's disease (Takadera et al., 2002; Hoshino et al., 2007), and the PGE_2 concentration in cerebrospinal fluid is increased under these conditions (Montine et al., 1999). The present study has shown that PGE_2 in the brain is eliminated across the BBB and the elimination process could serve to control PGE_2 activity (Figure 1). Hence, drugs which do not inhibit PGE_2 elimination from brain might be more appropriate for the treatment of these diseases, and assessment of inhibitory effect on MRP4-mediated PGE_2 transport could be a valuable tool in drug development.

In conclusion, this study has provided the first evidence that PGE_2 in mouse brain is eliminated by a carrier-mediated process at the BBB, and MRP4 is involved in this elimination. Furthermore, intravenous administration of cefmetazole, cefazolin and dipyridamole inhibited the elimination, and might therefore affect CNS function via a resulting increase in the brain level of PGE_2. To ensure that drugs have no adverse effect in inflammatory and infectious diseases associated with high levels of PGE_2, it may be necessary

to assess their inhibitory effect on PGE_2 transport mediated by MRP4.

Acknowledgement

The authors thank Mr. Yasuo Uchida for valuable discussions.

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Footnotes

This study was supported in part by Grants-in-Aid for Young Scientists (Start-up) [KAKENHI: 20890079], for Young Scientists (B) [KAKENHI: 20790127] and for Scientific Research (B) [KAKENHI: 21390042] from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT), and a grant from the Japan Society for the Promotion of Science (JSPS). This study was also supported in part by the Industrial Technology Research Grant Program from New Energy and Industrial Technology Development Organization (NEDO) of Japan.

Legends for figures

Figure 1 *In vivo* efflux transport of [^{3}H]PGE_2 from mouse brain across the BBB. (A) Time-course of [^{3}H]PGE_2 in the ipsilateral cerebrum after intracerebral microinjection in the presence of [^{14}C]inulin as an internal reference. A mixture of [^{3}H]PGE_2 (96 nCi) and [^{14}C]inulin (4.8 nCi) dissolved in 0.3 μL ECF buffer containing 0.1% ethanol was injected into the S2 region of mouse brain. Each point represents the mean ± SEM (n = 4-5). (B) Self-inhibitory effect of unlabeled PGE_2 on the percentage of [^{3}H]PGE_2 remaining in the ipsilateral cerebrum at 40 min after intracerebral microinjection. A mixture of [^{3}H]PGE_2 and [^{14}C]inulin dissolved in 0.3 μL ECF buffer containing 3 mM PGE_2 was injected. Each column represents the mean ± S.E.M. (n = 3-4). $^{**}p < 0.01$, significantly different from the control.

Figure 2 Typical HPLC chromatograms of [^{3}H]PGE_2 in the injectate (A), ipsilateral cerebrum (B) and jugular venous plasma (C). An aliquot of [^{3}H]PGE_2 (1 μCi) was injected into the S2 region of mouse brain. Ipsilateral cerebrum was removed at 5 min and venous blood was collected from the ipsilateral jugular vein at 5 min after microinjection. HPLC analysis was performed at a flow rate of 1.0 mL/min. Each point represents the radioactivity measured in the corresponding fraction (1.0 mL/each).

Figure 3 Time-profile of [^{3}H]PGE_2 uptake (A) and concentration-dependent inhibitory effect of cefmetazole on [^{3}H]PGE_2 uptake (B) into MRP4-expressing membrane vesicles. Membrane vesicles (5 μg) were incubated at 37°C in the uptake medium containing 20 nM [^{3}H]PGE_2 and 4 mM ATP. (A) The [^{3}H]PGE_2 uptake into MRP4-expressing Sf9 membrane vesicles (closed circles) or control vesicles (mock, open circles) was examined for the indicated time. (B) [^{3}H]PGE_2 uptake was measured for 5 min in the presence of cefmetazole at the indicated concentration. MRP4-mediated transport was obtained by subtracting the transport rate in mock from that in MRP4-expressing Sf9 membrane vesicles. The values are

expressed as a percentage of the [^{3}H]PGE_2 uptake mediated by MRP4 in the absence of cefmetazole. The solid line was obtained by means of nonlinear least-squares regression analysis. Each point represents the mean ± S.E.M. (n = 3).

Figure 4 Dose-dependent inhibitory effect of cefmetazole *i.v.* administration on [^{3}H]PGE_2 elimination from mouse brain across the BBB. Various amounts of cefmetazole were administered intravenously 15 min prior to administration of [^{3}H]PGE_2. The BEI value was determined 40 min after intracerebral microinjection of [^{3}H]PGE_2. Each point represents the mean ± S.E.M. (n=3-4). *$p < 0.05$ and **$p < 0.01$, significantly different from control.

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Table 1 Effect of various compounds at 20 μM on [^{3}H]PGE_2 uptake into MRP4-expressing membrane vesicles.

Compound	No. Studied	Percentage of control
Control	15	100 ± 4
Antibiotics (cephems)		
Ceftriaxone	3	6.62 ± 5.05**
Cefotaxime	3	34.5 ± 6.4**
Cefdinir	3	36.1 ± 3.3**
Cefotiam	3	59.0 ± 3.8**
Cefazolin	3	63.2 ± 3.3**
Cephalexin	3	109 ± 3
Cefsulodin	3	106 ± 2
Cefaclor	5	100 ± 4
Other kinds of antibiotics		
Clarithromycin	3	79.6 ± 6.6
Kanamycin	3	97.7 ± 0.3
Minocycline	3	96.1 ± 4.4
Vancomycin	3	113 ± 7
Antifungals		
Amphotericin B	3	81.4 ± 3.3
Itraconazole	3	111 ± 9
Fluconazole	3	98.0 ± 2.3
Miconazole	3	108 ± 7
NSAIDs		
Indomethacin	3	18.9 ± 1.8**
Ketoprofen	3	49.1 ± 2.4**
Ibuprofen	3	53.3 ± 2.6**
Celecoxib	3	62.4 ± 4.4**
Acetaminophen	3	70.7 ± 3.0**
Mefenamic acid	3	80.9 ± 0.3
Loxoprofen	3	85.0 ± 6.3
Sulpyrine	3	89.1 ± 4.7
Salicylate	3	88.5 ± 6.7
Meloxicam	3	94.2 ± 4.9
Diclofenac	3	94.8 ± 0.7
Ampiroxicam	3	103 ± 2

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Table 1 (*continued*)

Organic anion transporter inhibitors		
Dipyridamole	3	42.7 ± 4.2**
Ursodeoxycholate	3	77.1 ± 4.2
Taurocholate	3	101 ± 2

Membrane vesicles (5 μg) were incubated at 37°C in the uptake medium containing 20 nM [^{3}H]PGE_2 and 4 mM ATP. [^{3}H]PGE_2 uptake was measured for 5 min in the presence of each inhibitor at the concentration of 20 μM. MRP4-mediated transport was obtained by subtracting the transport rate in mock from that in MRP4-expressing Sf9 membrane vesicles. The values are expressed as a percentage of the [^{3}H]PGE_2 uptake mediated by MRP4 in the absence of inhibitors. Each value represents the mean ± S.E.M.. **$p < 0.01$, significantly different from control.

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Table 2 Effect of pre-administration of various compounds at 5 mM on [^{3}H]PGE_2 elimination from brain across the BBB in mice.

Compound	No. Studied	Brain efflux index (BEI, %)	Percentage of control
Antibiotics and antifungals			
Control	7	65.3 ± 1.6	100 ± 3
Cefmetazole	6	40.2 ± 3.2	61.5 ± 4.8**
Cefazolin	5	44.5 ± 4.3	68.2 ± 6.6**
Cefotaxime	3	32.0 ± 2.0	49.0 ± 3.0**
Ceftriaxone	4	39.9 ± 7.0	61.0 ± 10.7**
Benzylpenicillin	4	46.5 ± 4.7	71.2 ± 7.1**
Cefotiam	3	63.9 ± 4.7	97.9 ± 5.2
Cefsulodin	4	58.3 ± 3.4	89.3 ± 5.2
Control (0.25% DMSO)	8	62.6 ± 3.5	100 ± 6
Clarithromycin	5	55.3 ± 5.7	88.3 ± 9.1
Itraconazole	7	51.4 ± 3.8	82.1 ± 6.1
Inhibitors of organic anion transporters and NSAIDs			
Control (0.25% DMSO)	8	62.6 ± 3.5	100 ± 6
Indomethacin	3	13.0 ± 5.6	20.8 ± 9.0**
Ketoprofen	3	30.1 ± 4.4	48.1 ± 7.1**
Dipyridamole	3	61.4 ± 5.4	98.1 ± 8.6
Celecoxib	3	63.9 ± 5.7	102 ± 9

Each compound or ECF buffer (control) was administered at 5 min prior to administration of [^{3}H]PGE_2. Clarithromycin, itraconazole, indomethacin, ketoprofen, dipyridamole or celecoxib was administered with ECF buffer containing 0.25% DMSO. As a control for these compounds, ECF buffer containing 0.25% DMSO was injected. The BEI value was determined 40 min after intracerebral microinjection of [^{3}H]PGE_2. Each value represents the mean ± S.E.M.. **$p < 0.01$, significantly different from control.

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Table 3 Effect of *i.v.* administration of various compounds on [^{3}H]PGE_2 elimination from brain across the BBB in mice.

Compound	Injected amount	No. Studied	Brain efflux index (BEI, %)	Percentage of control
Control		18	66.7 ± 2.0	100 ± 3
Antibiotics (cephems)				
Cefmetazole	200 mg/kg	6	50.3 ± 5.5	75.4 ± 8.2**
Cefazolin	200 mg/kg	4	53.7 ± 5.6	80.6 ± 8.4*
Cefotaxime	200 mg/kg	4	76.4 ± 2.7	115 ± 4
Ceftriaxone	200 mg/kg	3	66.0 ± 1.2	98.9 ± 1.8
Cefalexin	200 mg/kg	4	70.2 ± 1.9	105 ± 3
Cefaclor	200 mg/kg	4	70.8 ± 2.7	106 ± 4
Cefsulodin	200 mg/kg	6	66.8 ± 2.2	100 ± 3
Inhibitors of organic anion transporters and NSAIDs				
Dipyridamole	40 mg/kg	4	47.8 ± 12.4	71.6 ± 18.6*
Taurocholate	10 mg/kg	5	60.2 ± 5.6	90.3 ± 8.5
	100 mg/kg	5	58.5 ± 5.1	87.7 ± 7.6
Ursodeoxycholate	100 mg/kg	4	64.7 ± 5.8	97.1 ± 8.7
Ketoprofen	40 mg/kg	4	66.5 ± 5.3	99.7 ± 7.9
Diclofenac	20 mg/kg	4	74.4 ± 4.7	111 ± 7.1

Each compound or Ringer-HEPES buffer (control) was administered via the jugular vein at 15 min prior to administration of [^{3}H]PGE_2. The BEI value was determined 40 min after intracerebral microinjection of [^{3}H]PGE_2. Each value represents the mean ± S.E.M.. *$p < 0.05$ and **$p < 0.01$, significantly different from control.

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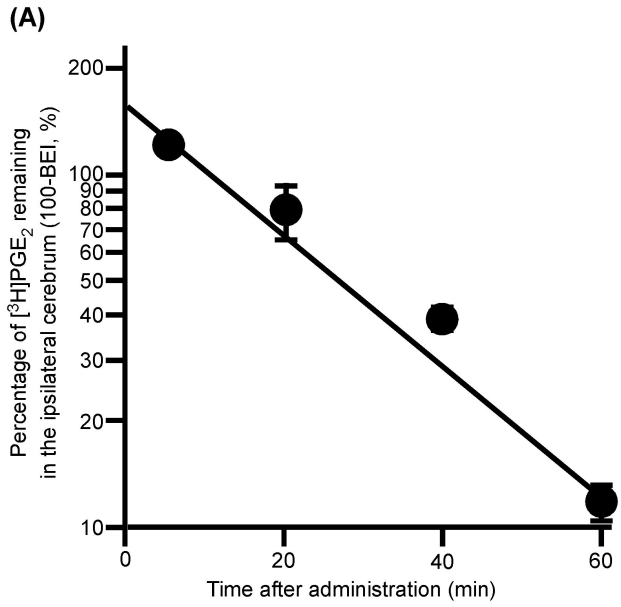

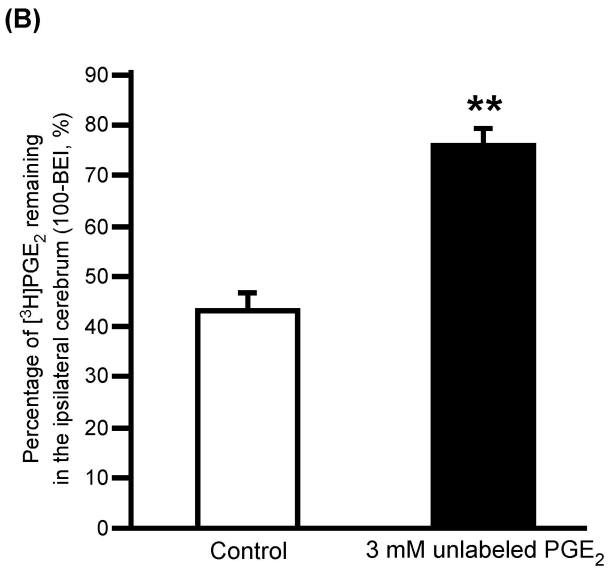

Figure 1

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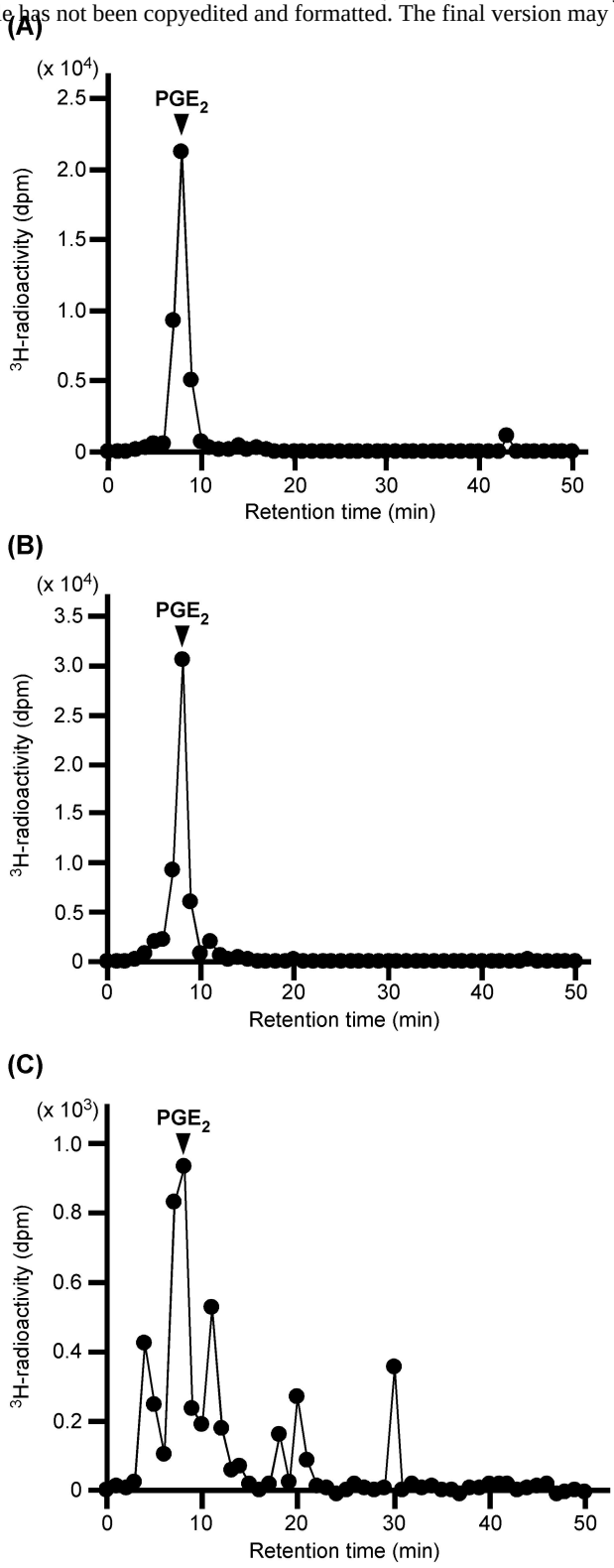

Figure 2

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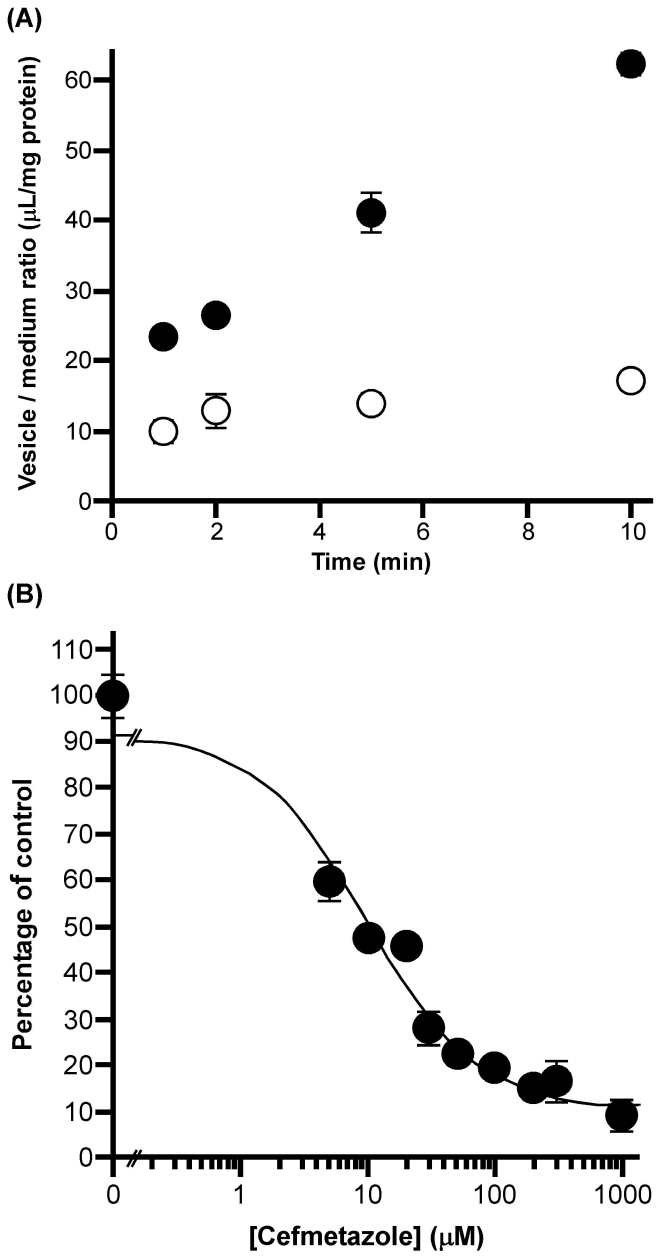

Figure 3

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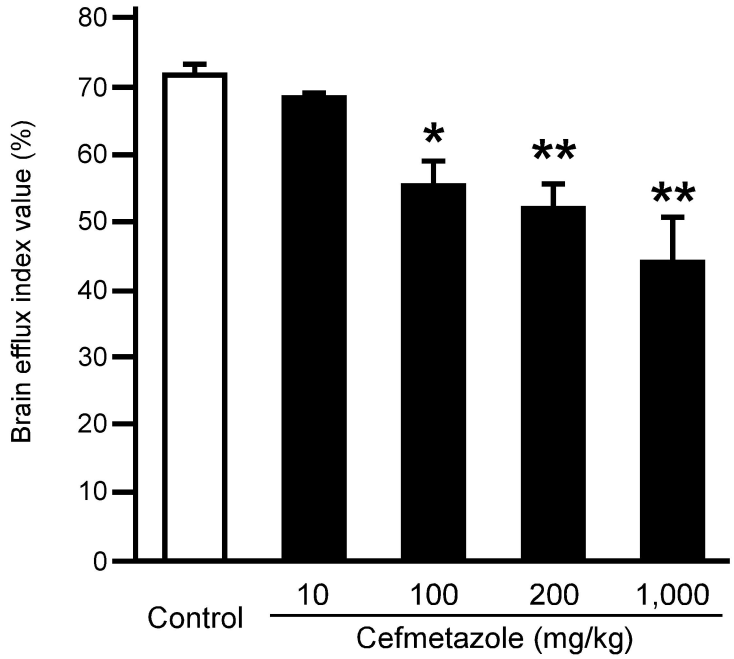

Figure 4