Involvement of Multidrug Resistance Associated Protein 1 (Mrp1) in the Efflux Transport of 17β Estradiol-D-17β-Glucuronide ($E_2$17βG) across the Blood-Brain Barrier

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Purpose. The purpose of present study is to investigate the involvement of multidrug resistance-associated protein 1 (Mrp1), Mrp2, and P-glycoprotein (Mdr1a) in the efflux transport of 17β–estradiol-D-17β-glucuronide ($E_2$17βG) across the blood–brain barrier (BBB).

Method. The expression of Mrp1 and Mrp2 at the BBB was investigated by RT-PCR and Western blot analyses. The time profiles of the remaining radioactivity of [^{3}H]$E_2$17βG in the brain were compared in wild-type, Mdr1a/Mdr1b and Mrp1 knockout mice and normal and Mrp2-deficient mutant rats [Sprague-Dawley and Eisai hyperbilirubinemic rats (EHBR), respectively] after intracerebral microinjection.

Results. RT-PCR and Western blot analyses revealed the expression of Mrp1 in isolated rat brain capillary; however, RT-PCR was unable to detect any expression of Mrp2. Significant elimination of $E_2$17βG was observed in wild-type mice at a rate constant of 0.007 min^{-1}, which was significantly decreased (0.004 min^{-1}) in Mrp1 knockout mice. In contrast, there was no difference in the efflux of $E_2$17βG from the brain in wild-type and Mdr1a/Mdr1b knockout mice and in normal and EHBR. No significant difference was observed in the accumulation of $E_2$17βG by brain slices prepared from wild-type and Mrp1 knockout mice.

Conclusion. Mrp1, but not Mrp2, is involved in the excretion of $E_2$17βG at the BBB and provides a barrier function by extruding conjugated metabolites into the blood.

KEY WORDS: blood-brain barrier; efflux transport; organic anion; Mrp.

INTRODUCTION

The blood–brain barrier (BBB) is formed by brain capillary endothelial cells (BCEC).

BCEC act as an interface between the central nervous system (CNS) and the blood circulation and are characterized by tightly sealed intracellular junctions (tight junction) and a paucity of fenestra and pinocytotic vesicles (1,2). Because of these characteristics, transcellular transport is the major transport route for the exchange of compounds between the brain extracellular fluid and blood circulation (1,2). In addition to these characteristics, metabolic enzymes and efflux transport act as detoxification systems in the brain and facilitate the elimination of xenobiotics from the brain (3–7). Glucuronidation is one of the detoxification mechanisms in the brain (8,9). The activity of 1-naphthol glucuronidation has been shown to be greater in BCEC than in brain homogenate (8,9). Certain types of xenobiotics are detoxified by this enzyme in the brain and/or BCEC and subsequently excreted into the blood. Involvement of transporters in the efflux of glucuronide conjugates from the brain has been suggested because the elimination of 1-naphthol-17β-glucuronide from the brain was saturated at higher injected doses (8). Rat organic anion-transporting polypeptide 2 (rOatp2; *Slc21a5*) has been identified on both the abluminal and luminal membrane of rat BCEC (10). Although rOatp2 was originally characterized as an uptake transporter for amphipathic organic anions, it can also mediate bidirectional transport (11). It is possible that rOatp2 is involved in both uptake and efflux across the BBB.

When 17β-estradiol-D-17β-glucuronide ($E_2$17βG), a substrate of rOatp2, was microinjected into the cerebral cortex, it was eliminated from the brain in a carrier-mediated manner (12). Digoxin, a high-affinity substrate for rOatp2, partially inhibited the elimination of $E_2$17βG across the BBB (approximately 40% inhibition), although taurocholate and probenecid inhibited it completely. In addition to rOatp2, involvement of other transporters has been suggested to account for the digoxin-resistant fraction (12). Two ABC transporters are candidates involved in the efflux transport of $E_2$17βG from the brain. Multidrug resistance-associated protein 1 (MRP1; *Abcc1*) is a primary active transporter, the substrates of which include anticancer drugs such as anthracyclines and vincristine and organic anions such as glutathione conjugates, glucuronide conjugates, and estrone sulfate (13–16). We have previously reported that a primary active transporter was involved in the uptake of glutathione conjugates such as 2,4-dinitrophenyl-S-glutathione (DNP-SG), leukotriene C_4 (LTC_4), and glutathione bimane (GS-B) by membrane vesicles prepared from immortalized mouse brain capillary endothelial cells (MBEC4), and we have demonstrated the expression of Mrp1 in this cell line by Northern and Western blot analyses (17,18). It has also been detected in isolated rat capillary by Western blot analysis (17,19) and in bovine brain capillaries and primary cultured bovine brain capillary endothelial cells by RT-PCR (20). The role of Mrp1 in the CNS was investigated by comparing the concentrations of etoposide in the plasma, brain, and cerebrospinal fluid in Mrp1/Mdr1a/Mdr1b triple-knockout mice and Mdr1a/Mdr1b double-knockout mice. Although the CSF concentration of etoposide was significantly increased in the triple-knockout mice, the concentrations in the plasma and brain were similar in the triple- and double-knockout mice (21). Sun *et al.* demonstrated an insignificant difference in the brain-to-plasma concentration ratio of fluorescein between wild-type and Mrp1 knockout mice at 0.5 or 2 h after its intravenous administration (22). These results suggest that the contribution of Mrp1 at the BBB is small or even zero, at least for the efflux transport of etoposide (21) and fluorescein (22). Mrp2 (*Abcc2*) is an isoform of Mrp1 that accepts glutathione and glucuronide conjugates and certain types of nonconjugated

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ABBREVIATIONS: ABC, ATP-binding cassette; BBB, blood–brain barrier; BCEC, brain capillary endothelial cells; DPDPE, [D-penicillamine^{2,5}]-enkephalin; EHBR, Eisai hyperbilirubinemic rats; $E_2$17βG, 17β estradiol-D-17β-glucuronide; Mdr, multidrug resistance; Mrp, multidrug resistance-associated protein; SD, Sprague-Dawley.

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amphipathic organic anions (23–25). Mrp2 is abundantly expressed in the liver and responsible for the biliary secretion of amphipathic organic anions (23–25). The luminal localization of positive signals by anti-Mrp2 antibody in the brain capillaries, which was absent in Mrp2-deficient mutant rats (TR^- rats), suggests that Mrp2 is involved in the excretion of organic anions through the BBB (26). The primary purpose of the present study is to examine the contribution of Mrp1 and Mrp2 to the efflux transport of $E_2$17βG across the BBB. The brain concentration of $E_2$17βG after microinjection into the cerebral cortex was compared between wild-type and *Mrp1* knockout mice and between normal and Mrp2-deficient mutant rats (EHBR). Because P-gp has been shown to accept $E_2$17βG as substrate (27), the involvement of P-gp (Mdr1a) was also examined by comparing the brain concentrations after microinjection into the cerebral cortex in wild-type and *Mdr1a/Mdr1b* knockout mice.

MATERIALS AND METHODS

Materials

[^{3}H]$E_2$17βG (44 Ci/mmole) and [^{14}C]carboxylinulin (2.5 mCi/g) were purchased from Perkin Elmer Life Sciences (Boston, MA). [^{3}H]$E_2$17βG and [^{14}C]carboxylinulin were stored at –20°C before use. Ketamine hydrochloride was purchased from Sankyo (Tokyo, Japan). Xylazine and ketamine hydrochloride were used as anesthetics. All other chemicals were commercially available, of reagent grade, and used without any further purification.

Animals

Male Sprague-Dawley (SD) and Eisai-hyperbilirubinemic rats (EHBR) (SLC, Shizuoka, Japan) weighing 240 to 270 g were used throughout this study and had free access to food and water. Male FVB mice, *Mrp1* knockout mice, and *Mdr1a/1b* knockout mice were purchased from Taconic Farms (Germantown, NY) and used throughout this study; they had free access to food and water. All experiments using animals in this study were carried out according to the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, The University of Tokyo).

Capillary Isolation

Rat brain capillaries were isolated using a modification of the procedure of Boado and Pardridge (28). All steps in the isolation procedure were carried out at 4°C in pregassed (95% O_2–5% CO_2) solutions. The tissue was kept on ice and in well-gassed buffers. Briefly, pieces of gray matter were gently homogenized in three volumes (v/w) of ECF buffer (122 mM NaCl, 25 mM $NaHCO_3$, 10 mM D-glucose, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, and 10 mM HEPES, pH 7.4), and after addition of dextran (final concentration 15%), the homogenate was centrifuged at 5800 × *g* for 10 min. The resulting pellet was resuspended in buffer B [103 mM NaCl, 25 mM $NaHCO_3$, 10 mM D-glucose, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM K_2HPO_4, 15 mM HEPES, 1 mM Na-pyruvate, 0.5% (w/v) BSA, pH 7.4] and then filtered through a 200-μm nylon mesh. The filtrate was passed over a column of glass beads, and, after washing with 500 ml buffer B, the capillaries adhering to the beads were collected by gentle agitation. The capillaries were centrifuged, and the pellet resuspended in ice-cold, ECF buffer.

RNA Isolation

Total RNA from rat brain capillaries was prepared by a single-step guanidium thiocyanate procedure using Isogen (Nippon Gene, Toyama, Japan).

Amplification of cDNA Fragments

cDNA fragments were amplified by RT-PCR with the total RNA prepared from rat brain capillaries as a template using the Takara RNA LA PCR kit (Takara Shuzo, Osaka, Japan). The following primers were designed and used to amplify rat Mrp1 and rat Mrp2 cDNA: forward primer, 5′-TATCTGCACTGGCTTCTAACTAT-3′; reverse primer, 5′-ATGATGACTGCGGCAATG-3′ for rMrp1; and forward primer, 5′-ATAGACCTGTCTCTTGCGC-3′; reverse primer, 5′-AAGAATCAGGAGGACAATCTGG-3′ for rMrp2, respectively. PCR was performed according to the following protocol: 96°C for 30 s, 55°C for 30 s, and 72°C for 1 min; 40 cycles. The sequences of the amplified fragments were conformed by DNA sequencing using a DNA seqeuncer (Model 377 DNA sequencer; Perkin Elmer, Foster City, CA).

Western Blot Analysis

The brain homogenate, brain capillaries, and crude liver membrane were diluted with 3 × Red Loading Buffer (Bio-Labs, Hertfordshire, UK). They were then boiled for 3 min and loaded onto an 8.5% SDS-polyacrylamide electrophoresis gel with a 3.75% stacking gel. Proteins were electroblotted onto a polyvinylidene difluoride membrane (Pall, NY) using a blotter (Trans-blot; Bio-Rad, Richmond, CA) at 15 V for 1 h. The membrane was blocked with Tris-buffered saline containing 0.05% Tween 20 (TBS-T) and 5% skimmed milk for 1 h at room temperature. After washing with TBS-T, the membrane was incubated with the antibodies (dilution 1:100). The membrane was allowed to bind a horseradish peroxidase-labeled antimouse IgG antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) for MRPr1, diluted 1:5000 in TBS-T for 1 h at room temperature, followed by washing with TBS-T.

Efflux of [^{3}H]$E_2$17βG from Mouse and Rat Brain after Microinjection into the Cerebral Cortex

The *in vivo* brain efflux experiments were carried out using the BEI method as described previously (29). The microinjection into the mouse cerebral cortex was carried out according to the previous report by Banks *et al.* (30). Mice and rats were anesthetized with intramuscular doses of ketamine (125 mg/kg) and xylazine (1.22 mg/kg). After exposure of the skull, a 1.5-mm hole was made in the skull 3.5 mm lateral to the bregma for the mouse, and a 2.5-mm hole was made in the skull 0.20 mm anterior and 5.5 mm lateral to the bregma for the rat, using a dental drill. A stereotactic frame (Narishige, Tokyo, Japan) was used to determine the injection site. The microinjection needle (330-μm diameter; Seieido Medical Industry, Tokyo, Japan) was inserted into the hole to a depth of 2.5 mm for the mouse and 4.5 mm for the

rat. [^{3}H]$E_2$17βG (0.05 μCi/mouse or rat) and [^{14}C]carboxylinulin (0.005 μCi/mouse or rat) dissolved in 0.5 μl ECF buffer were injected. After microinjection of drug into the cerebral cortex, aliquots of CSF were taken from the cisterna magna at appropriate intervals. Immediately after CSF sampling, mice were decapitated, and the left and right cerebrum and cerebellum were removed. The excised cerebrum was dissolved in 2.5 ml 2 N NaOH at 55°C for 1 h after measurement of the wet weight. The radioactivity associated with the brain specimens was determined in a liquid scintillation counter (LS 6000SE; Beckman Instruments., Fullerton, CA) after addition of 14 ml scintillation fluid (Hionic-fluor; Packard Instruments, Meriden, CT) to the scintillation vials. The 100-BEI (%) that represents the remaining percentage of drug in the ipsilateral cerebrum is described by the following equation:

$$100 - \text{BEI}\,(\%) = \frac{\dfrac{\text{Amount of Test Drug in the Brain}}{\text{Amount of Reference in the Brain}}}{\dfrac{\text{Amount of Test Drug Injected}}{\text{Amount of Reference Injected}}} \times 100$$

The elimination rate constant of the drug from the brain (k_{el}) can be obtained by fitting the 100-BEI (%) values vs. time. A nonlinear least-squares regression program (MULTI) was used for the calculation.

The Uptake of [^{3}H]$E_2$17βG by Brain Slices

The distribution volume of $E_2$17βG in the brain was determined by the *in vitro* brain slice uptake technique. Brain slices were prepared as reported previously with minor modification (29). After decapitation, mouse brains were immediately removed and dissected in ice-cold oxygenated ECF buffer. A hypothalamic slice 500 μm thick was cut using a brain microslicer (DTK-2000, Dosaka EM, Kyoto, Japan) and kept in oxygenated ECF buffer equilibrated with 95% O_2–5% CO_2. After preincubation for 5 min at 37°C, the brain slice (20–30 mg) was transferred to 5 ml oxygenated incubation medium containing 0.05 μCi/ml [^{3}H]$E_2$17βG and 0.01 μCi/ml [^{14}C]carboxylinulin at 37°C. At appropriate times, brain slices and parts of the incubation medium were obtained for the determination of drug concentrations.

HPLC Analysis of $E_2$17βG Metabolites after the BEI Study

The level of [^{3}H]$E_2$17βG metabolism in the brain after intracerebral injection was determined by high-performance liquid chromatography (HPLC). At 60 min after intracerebral injection of 0.05 μCi [^{3}H]$E_2$17βG, mice were decapitated, and their ipsilateral cerebrum was separated and stored at −80°C until analysis. Weighed ipsilateral cerebrum was homogenized with 2 volumes 0.9% NaCl, and homogenate was deproteinized using 2 volumes MeOH. After centrifugation, the supernatant specimens were evaporated and reconstituted with mobile phase. The HPLC system consisted of a Hitachi model 6000 series liquid chromatograph. Isocratic elution was performed on a YMC-Pack ODS C_{18} column (4.6 × 150 mm, YMC, Kyoto, Japan) with a mobile phase of 45% MeOH in 0.01M ammonium acetate buffer adjusted to pH 4.0 with glacial acetic acid) at a flow rate of 1.5 ml/min (31). Fractions were collected at 1 min intervals and radioactivity was determined in a liquid scintillation counter (LS 6000SE, Beckman Coulter, Inc., Fullerton, CA).

RESULTS

Expression of Transporters on the Blood–Brain Barrier

The results of RT-PCR using RNA prepared from isolated rat brain capillaries are shown in Fig. 1A. The expression of Mrp1 was confirmed in the cDNA prepared from the rat brain homogenate and brain capillaries. Although the band corresponding to Mrp2 was detected in the liver, where Mrp2 is most abundantly expressed, no expression of Mrp2 was observed in the cDNA prepared from the rat brain homogenate and brain capillaries. Figure 1B shows the results of Western blot analysis. Bands at 190 kDa were detected in the rat choroid plexus, isolated brain capillaries, and brain homogenate by anti-MRP1 monoclonal antibody (MRPr1).

The Elimination of [^{3}H]$E_2$17βG across the BBB after Intracerebral Microinjection

Figure 2A shows the time profiles of the elimination of $E_2$17βG from the brain after intracerebral microinjection in

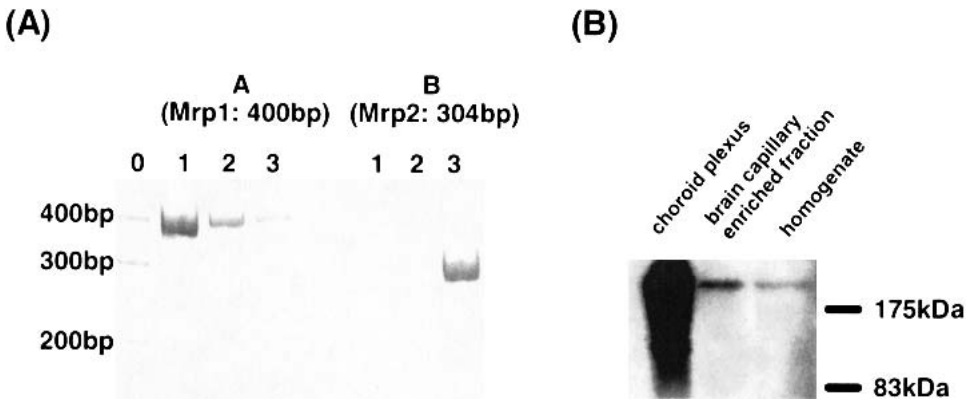

Fig. 1. RT-PCR analysis and Western blots. RNA samples were prepared from brain cortex (A1 and B1), isolated brain capillaries (A2 and B2), and liver (A3 and B3). PCR products stained with ethidium bromide were visualized under UV light. DNA size markers are shown in lane 0. Positive controls, G3PDH, are amplified from all these cDNA samples. B, Choroid plexus isolated from the third ventricle, isolated brain capillaries (50 μg protein), and brain homogenate (50 μg protein) were separated by SDS-PAGE (8.5% separating gel). Mrp1 was detected by MRPr1 antibody.

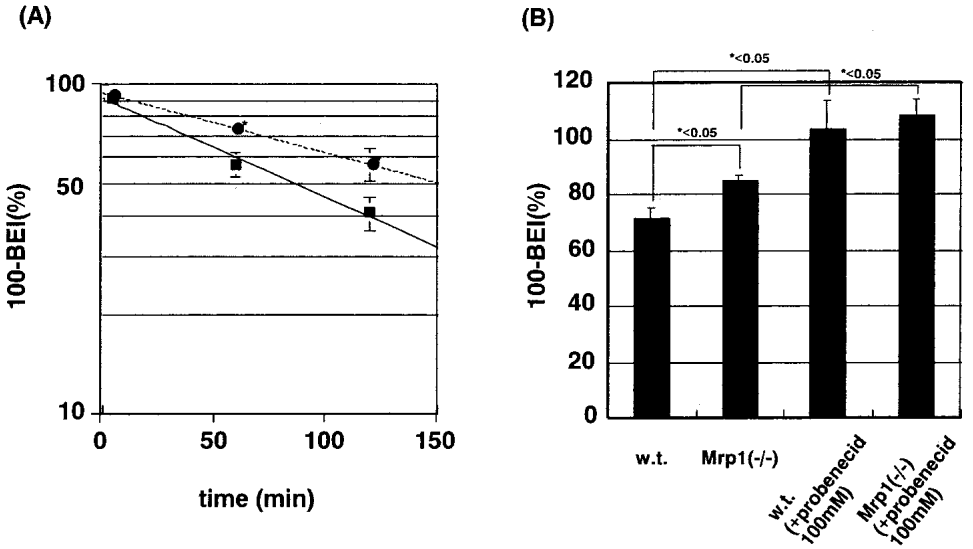

Fig. 2. Time profile of [^{3}H]$E_2$17βG in the ipsilateral cerebrum after intracerebral microinjection and inhibitory effect of probenecid on the efflux of [^{3}H]$E_2$17βG from the brain. A, A mixture of [^{3}H]$E_2$17βG (0.05 μCi/mouse) and [^{14}C]carboxylinulin (0.005 μCi/mouse) dissolved in 0.5 μl ECF buffer was injected into the mouse cerebrum, and then the animals were decapitated 5, 60, and 120 min after microinjection. Squares and circles represent the remaining percentage of [^{3}H]$E_2$17βG in control and *Mrp1* knockout mice, respectively. The slope of the solid line represents the elimination rate constant of a tracer amount of [^{3}H]$E_2$17βG obtained by nonlinear regression analysis. Each point represents the mean ± SE (n = 4). B, Inhibitory effect of 100 mM probenecid on the remaining percentage of [^{3}H]$E_2$17βG in the brain of the control and *Mrp1* knockout mice at 60 min. Each bar represents the mean ± SE (n = 4). *Significantly different from the control.

wild-type and *Mrp1* knockout mice. Approximately 60% of the administered dose of $E_2$17βG was eliminated from the ipsilateral cerebrum of wild-type mice within 120 min, whereas 45% of the administered dose of $E_2$17βG was eliminated from the ipsilateral cerebrum of *Mrp1* knockout mice within 120 min (Fig. 2A). The apparent elimination rate constants (k_{el}) of wild-type and *Mrp1* knockout mice were determined as 0.007 ± 0.001 and 0.004 ± 0.001 min^{-1}, respectively. No significant amount of $E_2$17βG or carboxylinulin was found in the contralateral cerebrum, cerebellum, or CSF compartment. Figure 2B shows the inhibitory effect of probenecid on the efflux of $E_2$17βG from the brain across the BBB. Probenecid (100 mM in the injectate) inhibited the efflux of $E_2$17βG from the brain of wild-type and *Mrp1* knockout mice.

The Distribution of $E_2$17βG into Brain Slices

Figure 3 shows the uptake of $E_2$17βG by brain slices from wild and *Mrp1* knockout mice. There was no significant difference in the uptake of $E_2$17βG by brain slices obtained from wild and *Mrp1* knockout mice (Fig. 3). Even in the presence of unlabeled $E_2$17βG (100 μM), the uptake was not saturated (Fig. 3).

The Elimination of $E_2$17βG across the BBB after Intracerebral Microinjection in Wild-Type and Mdr1a/1b Knockout Mice and Normal and EHBR

The 100-BEI values of $E_2$17βG at 120 min in wild-type and *Mdr1a/Mdr1b* double knockout mice were 25.8 ± 3.1% and 21.4 ± 1.6%, respectively, and this difference was not significant (Fig. 4A). Figure 4B shows the time profiles of the 100-BEI values of $E_2$17βG from the brain after intracerebral

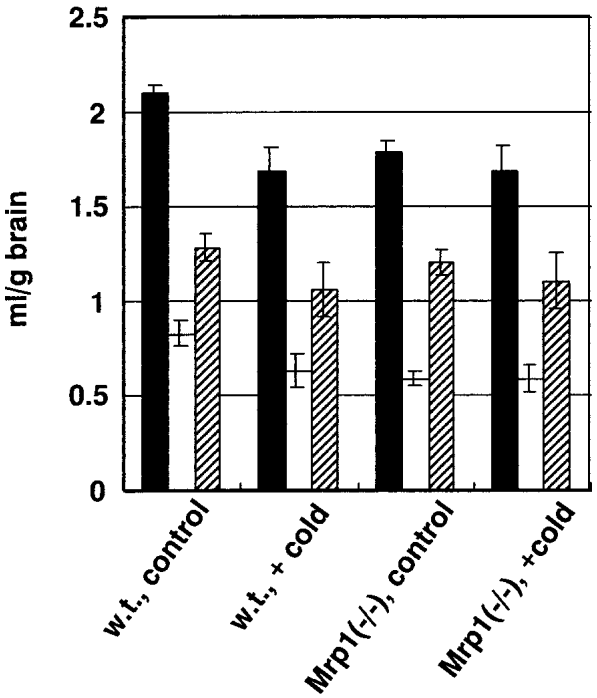

Fig. 3. Time profiles of the uptake of [^{3}H]$E_2$17βG into brain slices obtained from control and *Mrp1* knockout mice. Mouse brain slices were incubated with 0.05 μCi/ml [^{3}H]$E_2$17βG and 0.01 μCi/ml [^{14}C]carboxylinulin at 37°C. The radioactivity in the brain slices and incubation medium was measured at 120 min. Closed, open, and shaded bars represent the uptake of [^{3}H]$E_2$17βG, the uptake of [^{14}C]carboxylinulin, and the value for the uptake of [^{14}C]carboxylinulin subtracted from the uptake of [^{3}H]$E_2$17βG, respectively. Each bar represents the mean ± SE (n = 3).

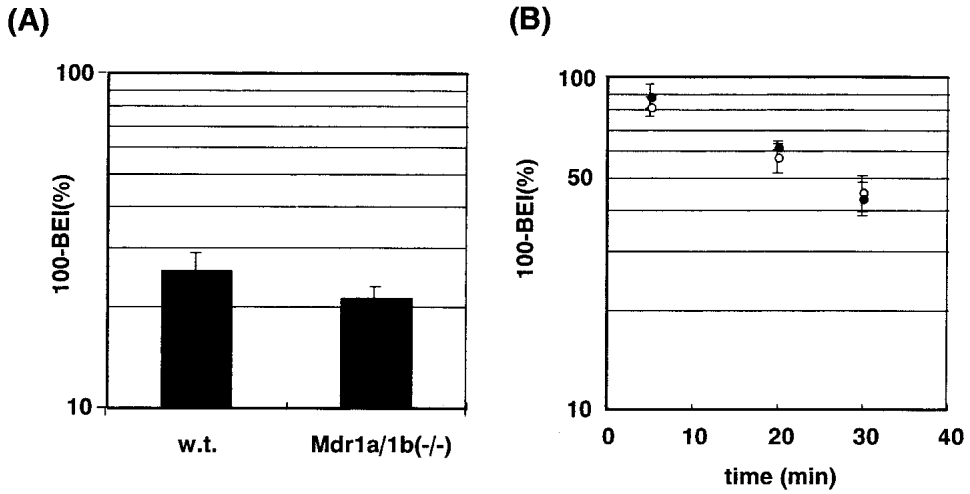

Fig. 4. Effect of Mdr1a/1b and Mrp2 on the efflux transport of [^{3}H]$E_2$17βG from the brain. A, A mixture of [^{3}H]$E_2$17βG (0.05 μCi/mouse) and [^{14}C]carboxylinulin (0.005 μCi/mouse) dissolved in 0.5 μl ECF buffer was injected into the mouse cerebrum, and then the animals were decapitated 120 min after microinjection. Each bar represents the mean ± SE (n = 4). B, A mixture of [^{3}H]$E_2$17βG (0.05 μCi/rat) and [^{14}C]carboxylinulin (0.005 μCi/rat) dissolved in 0.5 μl ECF buffer was injected into the rat cerebrum, and then the animals were decapitated 5, 20, and 30 min after microinjection. Closed circles (●) and open circles (○) represent the remaining percentage of [^{3}H]$E_2$17βG in SD and EHBR, respectively. Each point represents the mean ± SE (n = 4).

microinjection in SD and EHBR. Approximately 55% of the administered dose of $E_2$17βG was eliminated from the ipsilateral cerebrum of wild mice within 30 min. The apparent elimination rate constants (k_{el}) were determined as 0.028 ± 0.003 and 0.024 ± 0.001 min^{-1}, respectively. No significant amount of $E_2$17βG or [^{14}C]carboxylinulin was found in the contralateral cerebrum, cerebellum, or CSF compartment.

DISCUSSION

The contribution of Mrp1, P-gp (Mdr1a), and Mrp2 to the elimination of $E_2$17βG from the brain across the BBB was investigated in this study.

The expression of Mrp1 in the BBB was confirmed by RT-PCR and Western blot analysis (Fig. 1). A band was detected at the expected size by primers designed for rat Mrp1 when cDNA prepared from brain homogenate, isolated brain capillaries, and liver was used as a template (Fig. 1A). Anti-hMRP1 monoclonal antibody (MRPr1) detected a band in the choroid plexus, isolated rat capillaries, and brain homogenate at 190 kDa (Fig. 1B). These results suggest the expression of Mrp1 in the BCEC. In contrast, no band was detected in isolated rat brain capillaries or brain homogenate by primers designed for rat Mrp2 (Fig. 1A). This result contradicts the previous results using real-time PCR in which Mrp2 mRNA was detected in isolated brain capillaries (26) but consistent with the result in bovine brain capillaries in which Mrp1, Mrp4, and Mrp5 were detected by RT-PCR (20). The reason for this discrepancy remains unknown. The isoform expressed in the BBB may depend on the rat strain. Furthermore, the role of Mrp1 in the BBB was examined *in vivo* by comparing brain concentrations after microinjection into the cerebral cortex in wild-type and Mrp1 knockout mice.

$E_2$17βG disappeared from the brain after intracerebral microinjection with a rate constant of 0.007 min^{-1} in wild-type mice (Fig. 2), which is about fivefold slower than in rats. Because no radioactivity was detected in the contralateral cerebrum, cerebellum, or CSF compartment, the elimination from the brain is suggested to be across the BBB. Simultaneously administered probenecid inhibited the disappearance of $E_2$17βG from the brain completely, suggesting that transporters are involved in the efflux transport of $E_2$17βG across the BBB as reported in rats (Fig. 2B). The elimination of $E_2$17βG from the brain was significantly reduced in Mrp1 knockout mice (Fig. 2A). Two possibilities can account for this result: (a) reduced efflux transport activity at the BBB because of lack of Mrp1-mediated efflux and (b) increased distribution volume. To exclude the second possibility, the uptake of $E_2$17βG by brain slices from wild-type and Mrp1 knockout mice was compared (Fig. 3). The uptake of $E_2$17βG by brain slices from wild-type was three times greater than that of carboxylinulin, a marker for adherent water space. The total uptake of $E_2$17βG by brain slices was slightly smaller in Mrp1 knockout mice; however, this was because of the difference in the adherent water space because the uptake of $E_2$17βG by brain slices, corrected by the distribution of carboxylinulin, was comparable (Fig. 3). Therefore, it is most likely that Mrp1 is involved in the efflux transport of $E_2$17βG across the BBB.

A significant reduction in the brain concentration of $E_2$17βG was still observed in Mrp1 knockout mice, and this was inhibited by probenecid completely (Fig. 2B). Such complete inhibition by probenecid suggests the involvement of additional transporter(s) in the BBB. This has been also suggested by the observation that administration of probenecid caused a 1.5-fold increase in the brain-to-plasma concentration ratio of fluorescein in Mrp1 knockout mice (22). Although P-gp and Mrp2 were the other candidate transporters involved in the excretion process on the luminal membrane, there was no significant difference in the elimination of $E_2$17βG from the brain after microinjection into the cerebral

cortex between wild-type and Mdr1a/Mdr1b double knockout mice and between SD and EHBR. The finding in SD and EHBR was consistent with the result of PCR analysis, suggesting that Mrp2 does not play any role in the efflux transport across the BBB. However, *in vivo* results alone cannot exclude the possible involvement of P-gp. If the uptake process is rate limiting for allover elimination, lack of P-gp–mediated efflux may hardly affect the elimination time profiles in the elimination of $E_2$17βG from the brain. The contribution would be smaller than that of Mrp1 because a significant delay was observed in the elimination of $E_2$17βG in Mrp1 knockout mice.

RT-PCR analysis revealed the expression of other members of the MRP family (MRP4 and MRP5) in isolated bovine brain capillaries (20). MRP4 has been shown to accept $E_2$17βG as a substrate (32), although whether MRP5 accepts $E_2$17βG as substrate remains unknown. In addition to these ABC transporters, rOatp2 is expressed on the luminal membrane (10). These transporters may be involved in the excretion of conjugated metabolites in the BCEC in addition to Mrp1. Further studies are required to investigate the expression and localization of ABC transporters in the BCEC and to evaluate their contribution to the total efflux across the BBB.

The present study suggests that Mrp1 removes conjugated metabolites formed inside the BCEC as previously reported using immortalized mouse brain capillary endothelial cells (MBEC4 cells) (18). A similar detoxification system has been observed in primary cultured choroid epithelial cells, which act as a barrier (blood–CSF barrier) between the blood and CSF compartment (4). A large part of the intracellularly formed 1-naphthol-17β glucuronide was excreted into the basal side (blood side) when the choroid epithelial cells, cultured on a porous membrane, were incubated in the presence of 1-naphthol (4). Because Mrp1 has been shown to be localized along the basolateral membrane of the primary cultured choroid epithelial cells (33), Mrp1 may, at least partly, account for the excretion of amphipathic organic anions across the blood-side membrane in the CP.

Uptake and efflux transporters enable polarized cells to carry out efficient unidirectional transcellular transport of their common substrates. When Oatp2 and Mrp2 were expressed on the basolateral and apical membrane of MDCK II cells, respectively, the transcellular transport of their common substrates, such as $E_2$17βG and pravastatin, from basal to apical side was significantly enhanced compared with their single-gene expression systems and vector-transfected cells (34). Because Mrp1 mediates unidirectional efflux from inside the cells (13–16), *in vivo* results using Mrp1 knockout mice suggest luminal localization of Mrp1. Furthermore, rOatp2 and Mrp1 may be involved in the vectorial transport from the brain- to blood-side across the BBB and partially account for the efflux transport of $E_2$17βG across the BBB.

In conclusion, Mrp1 is partially involved in the elimination of $E_2$17βG from the brain at the BBB and provides an efficient efflux system for conjugated metabolites at the BBB.

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