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Functional characterization of adenosine transport across the BBB in mice

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Abstract

We investigated transport characteristics of adenosine across the blood–brain barrier (BBB) in mice. Uptake clearance across the BBB was measured by using an in situ mouse brain perfusion technique and cultured mouse brain capillary endothelial cell line (MBEC4 cells). Nucleoside transporter was cloned by RT-PCR and expressed on *Xenopus laevis* oocyte. Both in situ and in vitro studies revealed that the adenosine uptake is concentration-dependent, Na^+-independent and *S*-(*p*-nitrobenzyl)-6-thioinosine (NBMPR)-sensitive. The K_t values of in situ and in vitro studies were 31.7 ± 13.8 μM and 11.9 ± 2.84 μM, respectively. A good correlation was found for the inhibitory effects of nucleoside analogs to adenosine uptake between in situ and in vitro studies. RT-PCR revealed the expression of RNA of mouse equilibrative nucleoside transporter (mENT1) in mouse brain capillary and MBEC4 cells. In mENT1 expressed on *X. laevis* oocyte, K_t value of adenosine transport was 6.9 ± 2.7 μM (and comparable to those in situ and in vitro studies). In conclusion, we characterized the adenosine transport across the BBB in mice by using in situ brain perfusion technique and MBEC4 cells and found that these transports share common characteristics with mENT1-mediated transport. Transport of adenosine across the BBB in mice may be attributable to mENT1.

Keywords: Adenosine; Equilibrative nucleoside transporter; Blood–brain barrier

1. Introduction

In humans, nucleotides play physiologically important roles in neurotransmission, preservation of genetic information, and enzymatic actions (Shryock and Blardinelli, 1997). Although most mammalian cells obtain

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nucleotides by de novo biosynthesis and salvage pathway, the brain lacks the de novo biosynthetic pathway (Calra and Giacomini, 1995), suggesting that the transport of nucleosides and nucleobases from blood into the brain is physiologically essential. Since both nucleotides and nucleosides are hydrophilic, they are unlikely to cross the blood–brain barrier (BBB) by passive diffusion (Griffith and Jarvis, 1996). Therefore, it is expected that specific transport proteins for nucleosides exist at the BBB.

Mammalian nucleoside transporters are classified into sodium-dependent (concentrative) and -independent (equilibrative) types. Equilibrative transporters are subdivided into two types, i.e., *S*-(*p*-nitrobenzyl)-6-thioinosine (NBMPR)-sensitive (*es* transporters) and NBMPR-insensitive (*ei* transporters). Concentrative transporters are typically insensitive to NBMPR and are subdivided into three types: N1 is purine nucleoside and thymidine-sensitive, N2 is pyrimidine and adenosine-sensitive, and N3 is both purine- and pyrimidine-selective (Griffith and Jarvis, 1996). This concentrative transport is coupled to the flux of sodium ions.

Recently, various nucleoside transporters have been identified by molecular cloning (Yao et al., 1996; Griffiths et al., 1997a,b; Wang et al., 1997; Yao et al., 1997; Patel et al., 2000; Kiss et al., 2000) and their tissue distribution is becoming clear. Although the functional existence of nucleoside transporter at the BBB has been demonstrated (Pardridge et al., 1994), their detailed mechanism and characteristics remain unclear. In this study, we aimed to investigate the transport characteristics and mechanisms of adenosine at the BBB in mice using an in situ brain perfusion technique and mouse brain capillary endothelial cell line (MBEC4 cells) in culture.

2. Materials and methods

2.1. Reagents and animals

[^{14}C]Sucrose (495 mCi/mmol) and [^{3}H]adenosine (18.5 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA, USA). Hypoxanthine, guanine, thymine, uracil, adenosine, guanosine, inosine, uridine, and thymidine were purchased from Nacalai Tesque (Kyoto, Japan). Adenine and *S*-(*p*-nitrobenzyl)-6-thioinosine (NBMPR) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Solvable was obtained from Packard Instruments (Downers Grove, IL, USA). All other chemicals used in the experiments were of analytical grade. Male ddY mice (20–25 g) were purchased from Seac Yoshitomi (Fukuoka, Japan).

2.2. In situ mouse brain perfusion

Surgery was performed according to our previous report (Murakami et al., 2000). In brief, mice were anesthetized with pentobarbital sodium (Dainippon Pharmaceutical, Osaka, Japan). With the aid of a microscope, the right common carotid artery was exposed and the right pterygopalatine artery was ligated. Then the right external carotid artery was catheterized for retrograde infusion with 10 cm of polyethylene tubing (SP-10, Natume, Tokyo, Japan) filled with heparinized saline. The opening of the catheter was placed 2–3 mm above the bifurcation of the common carotid artery. The perfusion fluid containing radiolabeled drug was infused into the right external carotid artery at a constant rate (1.0 mL/min) and the right common carotid artery was ligated immediately. The perfusion was terminated by decapitation. The right cerebral hemisphere was immediately excised and weighed. Samples were digested at 50 °C in 1.5 mL of Solvable in a sealed container. After cooling, they were treated with 200 μL of 30% H_2O_2, neutralized by addition of 100 μL of 6 M HCl and prepared for scintillation counting by addition of 15 mL of scintillation cocktail (Clearsol I, Nacalai Tesque, Kyoto, Japan). Immediately after the decapitation, a 20 μL sample of perfusate was prepared for scintillation counting by addition of 15 mL of scintillation cocktail. The radioactivities of the brain or perfusate samples were measured with a liquid scintillation counter (LS6500, Beckman Instruments, Fullerton, CA, USA). Krebs–Henseleit buffer containing 118.0 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 2.5 mM $CaCl_2$ (pH 7.4) was used as perfusion fluid. Prior to experiments, 10 mM D-glucose was added and the perfusate was oxygenated for 10 min with 95% O_2–5% CO_2. Radiolabeled test compound and [^{14}C]sucrose were added to the perfusion fluid and the mixture was equilibrated at 37 °C by using a water bath. In the Na^+-free study, NaCl in the perfusate was replaced by choline chlo-

ride, and $NaHCO_3$ was replaced by $KHCO_3$. In the inhibition study, prior to pH adjustment, inhibitors were added to the perfusion fluid.

2.3. In vitro uptake study by MBEC4 cells

Mouse brain capillary endothelial cell line (MBEC4 cells) established previously (Tatsuta et al., 1992) were routinely grown in 75 cm^2 plastic T-flasks at 37 °C in a 5% CO_2–95% air atmosphere. The cell line was maintained in Dulbecco's modified Eagle's medium (Nikken Bio Medical Lab., Kyoto, Japan) supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. For the uptake study, MBEC4 cells were seeded at a density of 4×10^4 cells/mL on four-well multi dishes (Nunc, Denmark). Three days later, the cells were rinsed three times with 1 mL of incubation buffer containing 141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM D-glucose and 10 mM HEPES (pH 7.4). The uptake study was initiated by adding 250 μL of incubation buffer containing radiolabeled test compound (10 nM). The cells were incubated at 37 °C for the time designated. To terminate uptake, the cells were washed twice with ice-cold incubation buffer. For the quantitation of the radioactivity associated with the cells, the cells were solubilized with 200 μL of 3N NaOH. After neutralization with 100 μL of 6N HCl, the 150 μL sample was placed in a scintillation vial, and 4 mL of scintillation cocktail was added. In the Na^+-free study, NaCl in the buffer was replaced with *N*-methylglucamine. The concentration of test compound was measured with a liquid scintillation counter. Cellular protein content was measured by Lowry's method (Lowry et al., 1951).

2.4. Extraction of RNAs

Adult male mice were anesthetized with pentobarbital sodium. Krebs–Henseleit buffer was perfused for a minute and right cerebral hemisphere was sampled. Brain samples of 29 mice were homogenated and capillaries were isolated using 30 μm nylon mesh. Mouse liver was sampled from an anesthetized adult male mouse. Total RNAs were extracted from brain capillaries and liver by the use of RNeasy Mini kit (QIAGEN GmbH, Hilden, Germany). Total RNA from MBEC4 cells was extracted using AGPC method (Chomczynski and Sacchi, 1987). In brief, proteins in MBEC4 cells were solubilized with guanidine thiocyanate. Acid phenol was added. After centrifugation, water-phase was collected and total RNA was obtained by ethanol precipitation. mRNA was extracted from total RNA by mRNA Purification Kit (Amersham Biosciences, Buckinghamshire, UK).

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

RNA was reverse-transcribed into cDNA by reverse transcriptase (SuperScript™II RNase H^- Reverse Transcriptase; Invitrogen Corp., Carlsbad, CA, USA). The PCR reaction (94 °C for 3 min; 30 cycles of 94 °C for 1 min; 59 °C for 1 min; 72 °C for 2 min; and 72 °C for 10 min) was conducted on a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA, USA). Sense pimer was 5′-TACCCCCAGCAAGAGCCAGAG-3′ and antisense primer was 5′-CTGGCAGGGAAGGAAGTGAGC-3′. PCR products were extracted from the agarose gels, purified with a GFX™ PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Buckinghamshire, UK) and used as a template for cycle sequencing with a BigDye Terminator Cycle Sequencing Kit (PE Biosystems, Foster City, CA, USA). The sequence was determined using an ABI 373 DNA Sequencer (PE Biosystems, Foster City, CA, USA).

2.6. Uptake study by mENT1-expressed oocyte

Xenopus laevis female frogs were obtained from Seac Yoshitomi (Fukuoka, Japan). Ovary lobes were removed from the frog and treated with collagenase (type II; Sigma Chemical, St. Louis, MO, USA) for 30–60 min at 18 °C in Ca^{2+}-free buffer (88.0 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 15.0 mM Tris–HCl, 0.3 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$; pH 7.6). Healthy oocytes were selected and incubated overnight in modified Barths' saline (88.0 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 15.0 mM Tris–HCl, 0.3 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 μg/mL benzylpenicillin potassium, 10 μg/mL streptomycin sulfate; pH 7.6) at 18 °C. Capped RNA of mENT1 (50 ng) or water was microinjected into oocytes under microscope. Uptake experiment was performed 4 days after injection. Groups of 4–6

oocytes were incubated in 400 μL of the uptake buffer (100 mM NaCl or choline chloride, 2.0 mM KCl, 1.0 mM $MgSO_4$, 1.0 mM $CaCl_2$, and 10 mM HEPES; pH 7.4) containing radiolabeled drugs at 18 °C. The uptake was terminated by washing the oocytes for three times in ice-cold buffer. Each washed oocyte was transferred into scintillation vials and dissolved in 200 μL of 10% sodium lauryl sulfate. Four hours later, 4 mL of scintillation cocktail was added. The concentration of test compound was measured with a liquid scintillation counter.

2.7. Data analysis

The uptake clearance at the BBB (CL_{pf}, μL/g/s) was calculated from Eq. (1), where T is perfusion time (30 s), C_{brain} the brain concentration of tracer (pmol/g) corrected for vascular volume, C_{pf} the concentration of tracer in the perfusate (pmol/mL) and F_{pf} cerebral perfusion fluid flow (71.3 μL/g/s, Murakami et al., 2000). The uptake clearance by MBEC4 cells (CL_{MBEC}) and oocyte (CL_{oocyte}) were calculated from Eq. (2), where C_{cell} is the amount of tracer transferred into MBEC4 cells for 5 min (pmol/mg/5 min) or oocytes for 30 min (pmol/mg/30 min), and C_{buffer} the concentration of tracer in the buffer (pmol/mL). To estimate the kinetic parameters, the observed uptake clearance was fitted to the following Eq. (3) incorporating both saturable and non-saturable components, using the nonlinear least-squares regression analysis program, MULTI (Yamaoka et al., 1981), where J_{max} is the maximum uptake rate for the saturable component, K_t the Michaelis constant (μM), k_d the first-order constant for the non-saturable component, and C concentration of test compounds.

$$CL_{pf} = -F_{pf} \times \ln\left(1 - \frac{C_{brain}/C_{pf}}{F_{pf}/T}\right) \quad (1)$$

$$CL_{MBEC} \text{ or } CL_{oocyte} = \frac{C_{cell}}{C_{buffer}} \quad (2)$$

$$CL = \frac{J_{max}}{K_t + C} + k_d \quad (3)$$

All of the data are expressed as the mean ± S.E. The difference between means was considered to be significant when the p-value was less than 0.05 (Student's two-tailed t-test). For comparison of in situ–in vitro inhibitory effects, p-values were determined by multiple regression analysis.

3. Results

We evaluated the uptake at 30 s in the in situ experiments, since the brain-to-perfusate concentration ratio of adenosine was proportional to the lapse of time for 60 s. As shown in Fig. 1, the uptake of adenosine became saturated as the concentration was increased, and was not affected by Na^+. Although we did not investigate any other Na^+-dependent transport processes as a control study, it is quite conclusive that the majority of the uptake of adenosine is Na^+-independent. Using Eq. (3), the K_t-, J_{max}-, and k_d-values for Na^+-independent uptake were estimated to be 31.7 ± 13.8 μM, 15.7 ± 6.3 pmol/g/s, and 0.00117 ± 0.00162 μL/g/s, respectively. In addition, CL_{pf} was remarkably decreased by 1 μM NBMPR (Fig. 2).

In the study of adenosine uptake from the apical side of MBEC4 cells, the uptake was Na^+-independent and proportional to the lapse of time for 5 min (data not shown). Therefore, the uptake of adenosine on MBEC4 cells was evaluated at 5 min in the absence of Na^+. As shown in Fig. 3, CL_{MBEC} became saturated as the concentration was increased. Kinetic parameters (K_t, J_{max}, and k_d)

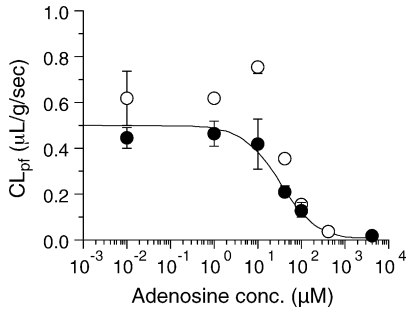

Fig. 1. Concentration dependence of the in situ uptake of adenosine at various concentrations (10 nM–4 mM) in the presence (open symbols) or absence (closed symbols) of Na^+ for 30 s. The concentration of [^{3}H]adenosine was 10 nM. Data are the mean ± S.E. (n = 3 mice). Kinetic parameters (J_{max}, K_t, and k_d) for the uptake in the absence of Na^+ were 15.7 ± 6.3 pmol/g/s, 31.7 ± 13.8 μM, and 0.00117 ± 0.00162 μL/g/s, respectively.

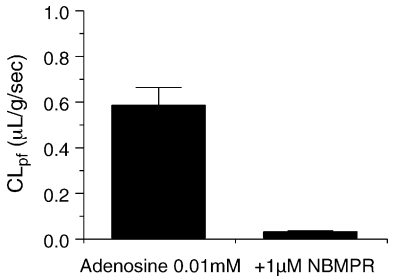

Fig. 2. Effect of NBMPR on the in situ uptake of adenosine for 30 s. The concentration of [^{3}H]adenosine was 10 nM. Data are the mean ± S.E. (n = 3 mice).

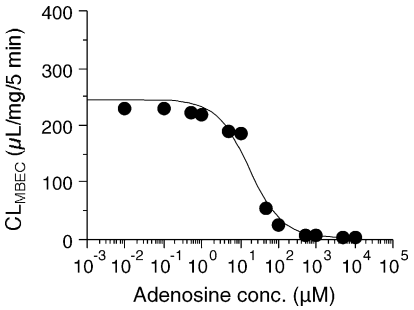

Fig. 3. Concentration dependence of adenosine uptake at various concentrations (10 nM–4 mM) in MBEC4 cells in the absence of Na^+ for 5 min. Data are the mean ± S.E. for four experiments. Kinetic parameters (J_{max}, K_t, and k_d) were 3230 ± 271 pmol/mg/5 min, 11.9 ± 2.84 μM, and 1.97 ± 0.00932 μL/mg/5 min, respectively.

were 11.9 ± 2.84 μM, 3230 ± 271 pmol/mg/5 min, and 1.97 ± 0.0932 μL/mg/5 min, respectively. NBMPR suppressed CL_{MBEC} in a concentration-dependent manner with a K_i-value of 2.29 ± 0.73 μM (Fig. 4).

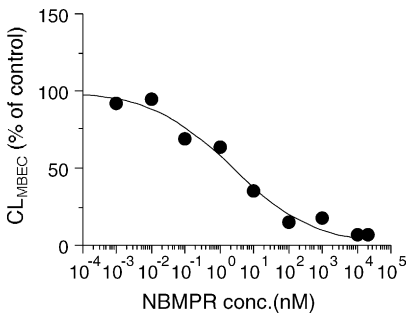

Fig. 4. Effect of NBMPR (1 pM–20 μM) on the uptake of adenosine in MBEC4 cells for 5 min. Data are the mean ± S.E. for four experiments.

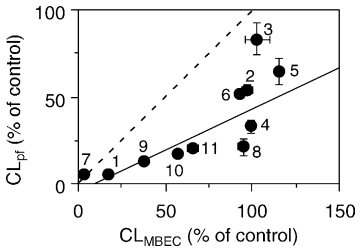

Fig. 5. Comparison of effects of various inhibitors on adenosine uptake between in vitro and in situ studies. Each point represents the mean ± S.E. for three (in situ) or four (in vitro) experiments. The dashed lines show 1:1 lines. The solid lines show least-square fit of the equation $Y = A + B \times X$ to the data. The values of A, B, correlation coefficient $(r)^2$, p are −6.43, 0.567, 0.813, 0.0023, respectively. (1 NBMPR, 2 adenine, 3 guanine, 4 hypoxanthine, 5 thymine, 6 uracil, 7 adenosine, 8 guanosine, 9 inosine, 10 thymidine, 11 uridine).

We investigated the inhibitory effects of eleven nucleoside analogs (1 μM for NBMPR, 250 μM for guanine, and 1 mM for others) on the adenosine uptake. The uptake of adenosine was remarkably decreased by both purine and pyrimidine nucleosides, but not by nucleobases. A good correlation was observed between the inhibitory effects in situ and in vitro ($p < 0.01$, $r^2 = 0.662$, Fig. 5).

RT-PCR was performed using specific primers for mENT1 and template extracted from brain capillary and MBEC4 cells. Agarose gel electrophoresis of the PCR products gave fragments of the expected size (1.5–1.6 kbp) for mENT1 in both objects (Fig. 6). Sequences of the products were identical among the brain capillary, MBEC4 cells, and liver. The sequence was identical to those for mENT1 (accession number:

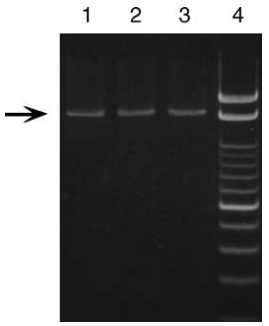

Fig. 6. RT-PCR analysis of equilibrative nucleoside transporters in mouse brain capillary and MBEC4 cells. Lane 1: mouse brain capillary; lane 2: MBEC4 cells; lane 3: mouse liver (positive control) and lane 4: size marker.

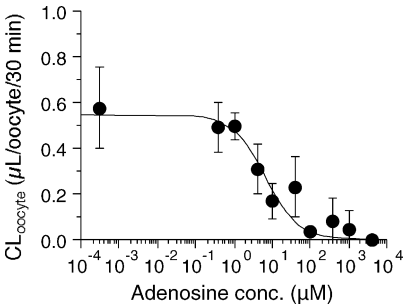

Fig. 7. Concentration dependence of adenosine uptake by recombinant mENT1 expressed in *X. laevis* oocytes. Uptake was assayed at 18 °C for 30 min in Na^+-free buffer containing 0.3 nM [^{3}H]adenosine. Data are mean ± S.E. ($n = 4$).

AF131212) with the exception of two bases without amino acid substitution.

Compared to water-injected oocyte, uptake of adenosine into mENT1-expressed oocyte was increased significantly. Since the uptake of adenosine into oocytes was linear up to 60 min, following studies were assayed at 30 min. Uptake of adenosine was Na^+-independent and NBMPR-sensitive ($K_i = 0.90 \pm 0.0038$ nM). In the absence of Na^+, the uptake of adenosine was concentration-dependent with K_t-value of 6.9 ± 2.7 μM (Fig. 7).

4. Discussion

The transport systems of nucleosides in mammalians are classified into sodium-dependent (concentrative) and sodium-independent (equilibrative) types, and are further classified based on their substrate selectivity, as noted in the introduction. Our results in the perfusion study showed that the transport of adenosine at the BBB was Na^+-independent and NBMPR-sensitive, which classified as an *es* transporter.

Several investigators have reported the transport of nucleosides at the BBB (Spector, 1982; Wu and Phillis, 1982; Pardridge et al., 1994). By using rat brain perfusion technique, it was demonstrated that both a Na^+-dependent nucleoside transporter and an *ei* transporter exist on the blood side of the BBB, while an *es* transporter exists on the brain side of the BBB (Pardridge et al.,1994), which is contrary to our results. On the other hand, Chishty et al. (2003) have reported in rat brain endothelial (RBE4) cells that the majority of the uptake of adenosine into RBE4 cells are Na^+-independent and consist of both NBMPR-sensitive and -insensitive components. A speculative explanation for the discrepancy from the Pardridge's results is that they used 10 nM NBMPR, while we used 1 μM for the experiment on NBMPR inhibition. Although the K_i-value of NBMPR for *es* transporters was reported to range from 0.1 to 10 nM (Baldwin et al., 1999), 10 nM NBMPR may not be high enough since the inhibitory potency may depend upon the tissue or method employed. Indeed, Chishty et al. (2003) have reported that the uptake of adenosine into rat brain endothelial cells was partially inhibited by NBMPR with IC_{50}-value of 20 ± 8 nM. Assuming that the affinity of NBMPR for the *es* transporter on the BBB is low (e.g. 20–100 nM), the discrepancy can be accounted for NBMPR sensitivity.

With respect to the localization of the *es* transporter, our result of the MBEC4 cells study demonstrated that it exists, at least, on the apical side of BBB. Since the intercellular junction of MBEC4 cells is not tight enough to investigate the transcellular transport (or uptake) of hydrophillic drugs with small molecular size, such as adenosine from the brain side, we did not investigate the functional expression of *es* transporter at the brain side of the BBB using MBEC4 cells. Nevertheless, the characteristics of adenosine transport shown in the in situ brain perfusion study and MBEC4 cells were quite similar in many respects, such as Na^+-independency, NBMPR-sensitivity, K_t-values and inhibitory effects of nucleosides and nucleobases. There is some disagreement in the inhibitory properties between in situ and in vitro. For example guanosine inhibited the in situ brain uptake of adenosine, but did not affect its uptake into MBEC4 cells. One possible explanation is the difference in the contribution of mENT1, which has reported to be inhibited by guanosine. Therefore, it may possible that the contribution of mENT1 is greater in the in situ than in MBEC4 cells. However, taking into account that the in situ brain uptake was more potently inhibited by all the drugs investigated in comparison with the uptake into MBEC4 cells, the inhibitory potency may have merely varied with the experimental condition.

cDNAs of mammalian nucleoside transporters have been cloned from human, rat, and mouse tissues and have found to encode proteins with functional characteristics of N1, N2, *es*, and *ei* nucleoside transporters.

Tissue distribution of these proteins has been identified. However, as for the BBB, only rCNT2 was demonstrated to express (Li et al., 2001). In our in situ perfusion and in vitro uptake studies, the uptake of adenosine at BBB was Na^+-independent, NBMPR-sensitive, and exhibited broad substrate specificity to recognize a variety of purine and pyrimidine nucleosides, suggesting that the uptake of adenosine is attributable to mENT1. RT-PCR analysis also demonstrated that the expression of mRNA of mENT1 in mouse brain capillary and MBEC4 cells. In comparison with the water-injected oocytes, the uptake of adenosine by mENT1-injected oocytes was obviously high, suggesting that mENT1 transports adenosine. Moreover, the K_t-value was comparable to that obtained from the in situ brain perfusion and in vitro uptake studies. These results are consistent with previous findings. Human ENT1 has been demonstrated to transport adenosine (Ward et al., 2000). Kiss et al. (2000) have reported that the mENT1-mediated uptake of uridine was inhibited by adenosine with K_i-value of 47 μM, which is in good agreement with the K_t-values in the present study obtained from the in situ and in vitro experiments. Therefore, mENT1 is considered to play a major role in the transport of adenosine at the apical membranes of brain capillary endothelial cells.

Another member of ENT family, ENT2, has been known to transport adenosine. However, NBMPR thoroughly attenuated the adenosine transport both in situ and in vitro, suggesting that ENT2 may not contribute to the transport of adenosine across the BBB. Recently, other members of ENT family, ENT3 and ENT4, have been identified (Baldwin et al., 2004). While Baldwin et al. (2004) have described that the substrate specificity and inhibitor sensitivity of human ENT3 are similar to those of human ENT2, the transport properties of mouse ENT3 and mouse ENT4 has not been characterized. Contribution of these isoforms to the transport of adenosine across the BBB cannot be thoroughly excluded and additional studies are needed to clear this issue.

In conclusion, we characterized the adenosine transport across the BBB in mice by using in situ brain perfusion technique and MBEC4 cells, and found that these transports share common characteristics with mENT1-mediated transport. Transport of adenosine across the BBB in mice may be attributable to mENT1.

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