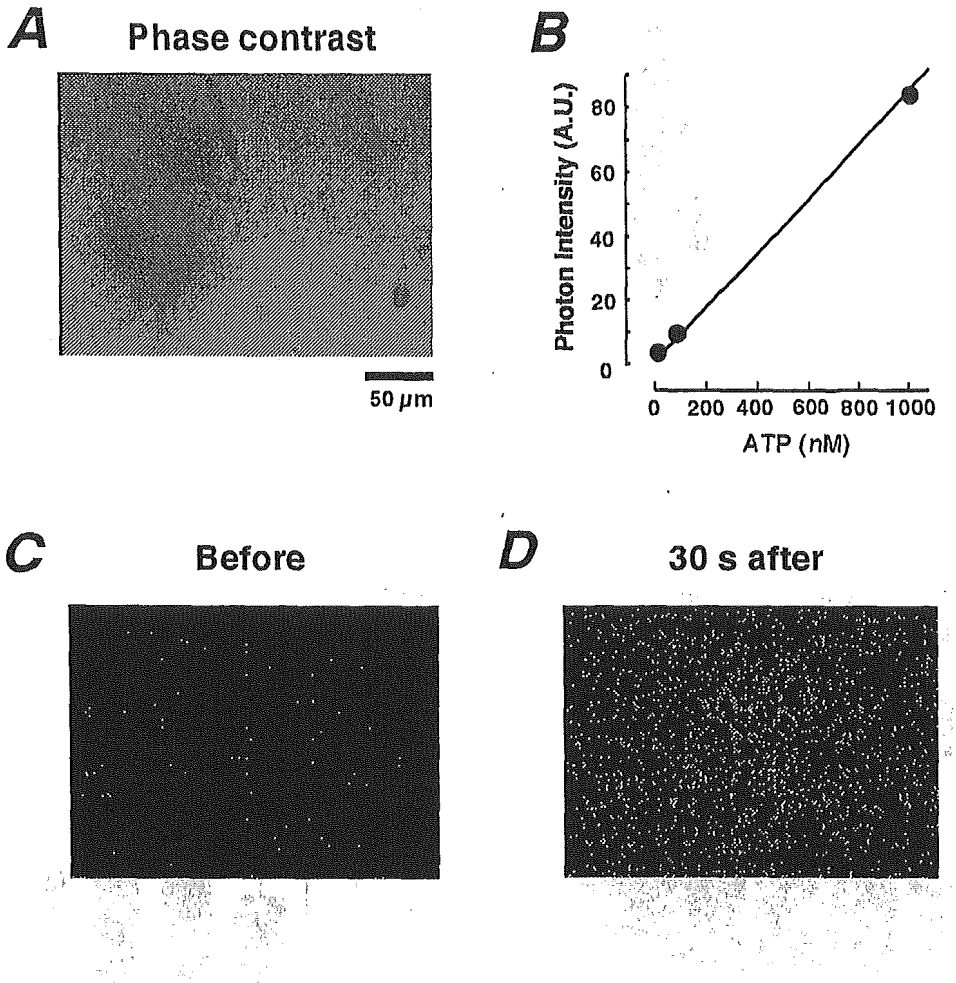

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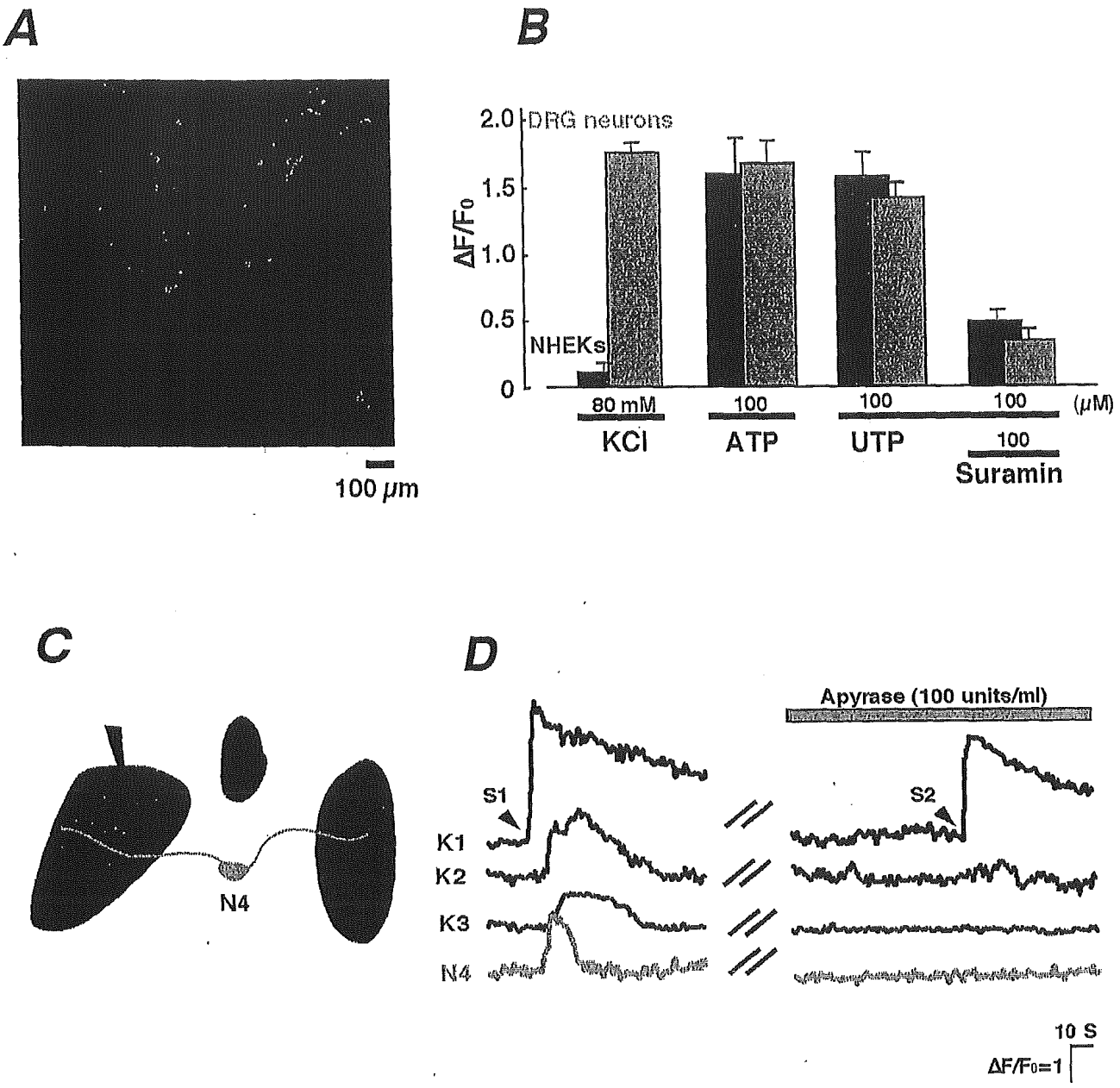

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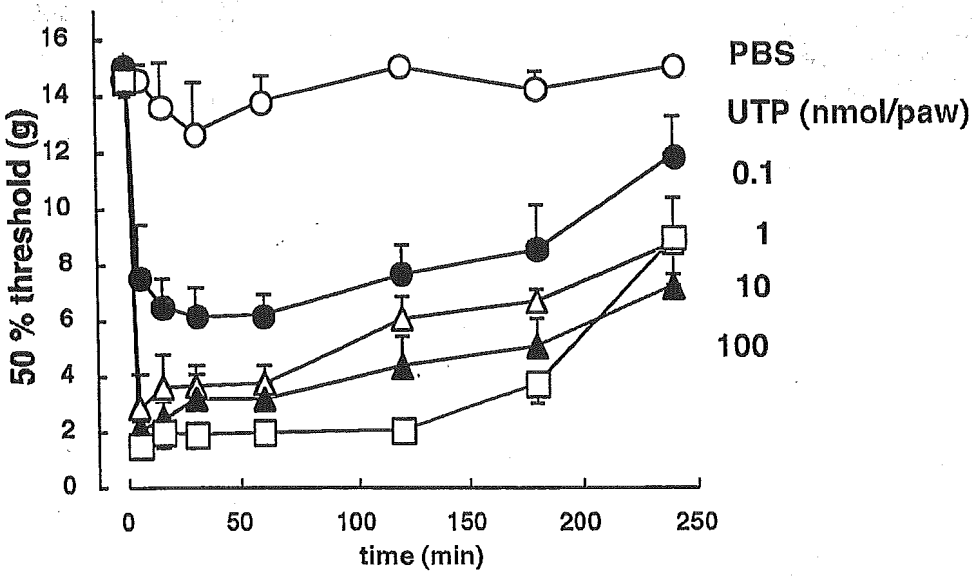

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Rapid Elimination of Cefaclor from the Cerebrospinal Fluid Is Mediated by a Benzylpenicillin-Sensitive Mechanism Distinct from Organic Anion Transporter 3

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ABSTRACT

The purpose of this study was to investigate the carrier-mediated elimination of cephalosporins from the cerebrospinal fluid (CSF) via the choroid plexus. Cefaclor and cefalexin are structural analogs with similar lipophilicity, differing by only one functional group (cefaclor, –Cl; cephalexin, $-CH_3$), and they are substrates of rat peptide transporter PEPT2 with similar transport activities. However, cefaclor was cleared from the CSF more rapidly than cefalexin after intracerebroventricular administration (the elimination rate constants were 0.11 and 0.050 min^{-1}, respectively). The elimination of cefaclor from the CSF was inhibited by benzylpenicillin, but not by glycylsarcosine (GlySar), whereas GlySar, but not benzylpenicillin, had an inhibitory effect on the elimination of cefalexin from the CSF. The uptake of cefaclor by the freshly isolated rat choroid plexus was saturable, with a K_m value of 250 μM, and the uptake clearance corresponding to saturable components accounts for the major part of the in vivo clearance from the CSF (17 versus 26 μl/min, respectively). The uptake of cefaclor by the choroid plexus was inhibited by benzylpenicillin, but not by GlySar. However, the inhibitory effect of benzylpenicillin was weaker than expected from its own K_m value, and furthermore, organic anion transporter (Oat)3 substrates (cimetidine or *p*-aminohippurate) had no effect. These results suggest that cefaclor and cefalexin are eliminated from the CSF by different transporters, and rapid elimination of cefaclor from the CSF is accounted for by a benzylpenicillin-sensitive mechanism distinct from Oat3. A slight modification of a single chemical group of cephalosporins can greatly affect the contribution of the transporters involved, and their duration in the CSF.

The β-lactam antibiotics display a broad spectrum of antibacterial activity with a relatively low risk of allergic and toxic reactions, and cephalosporin antibiotics are prescribed widely throughout the world for the treatment of various infections, including bacterial meningitis (Dancer, 2001). Despite the availability of effective antimicrobial therapy, the morbidity and mortality associated with bacterial meningitis still remain significantly high, especially in developing countries (Tunkel and Scheld, 1997). As in other body sites, the bactericidal activity of cephalosporins in cerebrospinal fluid (CSF) predominantly depends on the length of time during which their concentrations exceed the minimum bactericidal concentrations of the infecting organisms. After intravenous administration, despite the similarity in their structures, various β-lactam antibiotics exhibit a noticeable difference in their distribution, which cannot be explained by their physicochemical properties.

Two major factors are known to determine the pharmacokinetics of β-lactam antibiotics in the CSF. One is their ability to cross the barriers of the central nervous system, which may be influenced by molecular size, their degree of plasma protein binding, and ionization (Levin, 1980; Spector, 1987). Another factor is their rate of efflux from the CSF via an active transport system in the choroid plexus, a leaf-like, highly vascularized organ that protrudes into the ventricles and is comprised of fenestrated capillaries surrounded by a tightly joined monolayer of epithelial cells (Spector, 1990; Suzuki et al., 1997). Quantitative studies of the distribution of β-lactam antibiotics in the CSF, using cefodizime (Nohjoh et al., 1989) and imipenem (Suzuki et al., 1989b) in comparison with benzylpenicillin, have also indicated that the CSF concentration of β-lactam antibiotics is greatly affected by their active elimination via the choroid plexus.

Recent progress in molecular cloning of transporter genes has identified the transporters involved in the elimination of

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ABBREVIATIONS: CSF, cerebrospinal fluid; OAT/Oat, organic anion transporter; PEPT, peptide transporter; GlySar, glycylsarcosine; HPLC, high-performance liquid chromatography.

drugs from the CSF, namely, organic anion transporters [Oat3/*Slc22a8* (Nagata et al., 2002) and Oatp1a5/*Slco1a5* (Kusuhara et al., 2003)] and a peptide transporter (PEPT2/*Slc15a2*) (Novotny et al., 2000). Both Oat3 and Oatp1a5 are characterized by their broad substrate specificity; Oat3 accepts amphipathic and hydrophilic organic anions as well as H_2 receptor antagonists as substrates (Kusuhara et al., 1999; Nagata et al., 2002, 2004a,b), whereas Oatp1a5 accepts amphipathic organic anions as substrates. Kinetic studies using Oat3 substrates and inhibitors suggest that Oat3 plays a major role in the uptake of hydrophilic organic anions such as benzylpenicillin, *p*-aminohippurate, and 2,4-dichlorophenoxyacetate (Nagata et al., 2002, 2004b) as well as cationic drugs; H_2 receptor antagonists (Nagata et al., 2004a) via the isolated rat choroid plexus; but not an amphipathic organic anion, estradiol 17β glucuronide, the uptake of which is mainly accounted for by Oatp1a5 (Kusuhara et al., 2003). PEPT2 has been reported to recognize various di- and tripeptides as well as peptide-mimetic drugs, including some β-lactam antibiotics, that contain an α-amino group in their structures, such as cefadroxil and cefalexin (Ganapathy et al., 1995; Daniel and Kottra, 2004; Smith et al., 2004). Using PEPT2 knockout mice, cefadroxil uptake by the freshly isolated choroid plexus has been shown to be mainly mediated by PEPT2 and partly by Oat3 (Ocheltree et al., 2004). Therefore, Oat3 and PEPT2 play important roles in regulating the CSF concentration of β-lactam antibiotics.

We found that after intracerebroventricular administration, cefalexin and cefaclor were eliminated from the CSF with different rate constants, even though they are structural analogs with similar lipophilicity (their apparent isobutylalcohol-buffer partition coefficients at pH 7.3 are 0.129 and 0.08 for cefaclor and cefalexin, respectively; Suzuki et al., 1987), differing by only one functional group (cefaclor, –Cl; cephalexin, $-CH_3$) (Fig. 1). The present study is aimed at characterizing the transport mechanisms accounting for the difference in the efflux rates of cefalexin and cefaclor. An inhibition study was carried out using benzylpenicillin and GlySar and showed that their inhibitory effect differed for the elimination of cefalexin and cefaclor, suggesting that different pathways were involved in the elimination of cefalexin and cefaclor from the CSF.

Materials and Methods

Materials. [^{14}C]Mannitol (56.0 mCi/mmol) was purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK), and [^{3}H]GlySar (4 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Unlabeled benzylpenicillin and cefalexin were purchased from Wako Pure Chemicals (Osaka, Japan), and other cephalosporins were obtained from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were of analytical grade and were readily available from commercial sources. All cell culture media and reagents were purchased from Invitrogen (Carlsbad, CA), except for fetal bovine serum, which was obtained from Sigma-Aldrich.

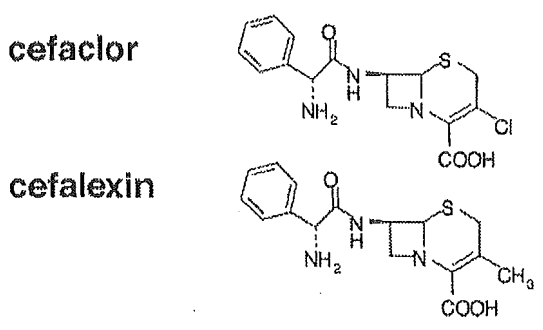

Fig. 1. Chemical structures of cefaclor and cephalexin.

Animals. Male Sprague-Dawley rats, weighing 220 to 240 g, were obtained from Japan SLC (Shizuoka, Japan), and experiments were carried out according to the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan).

Construction of the LLC-PK1 Cell Line Stably Expressing Rat PEPT2 (PEPT2-LLC). Full-length PEPT2 cDNA was isolated from rat kidney mRNA and, after ligation into T vector (Promega, Madison, WI), was subcloned into pcDNA3.1 vector (Invitrogen) using NotI, SpeI, and XbaI. The construct was introduced into LLC-PK1 cells by LipofectAMINE (Invitrogen) according to the manufacturer's protocol, and stably transfected cells were selected by adding G418 sulfate (Invitrogen) to the culture medium. Five weeks after transfection, different clones were seeded on 12-well culture plates, and the transport activity was tested for positive clones.

Cell Culture. LLC-PK1 cells expressing rat Oat3 (Oat3-LLC) have been established previously (Sugiyama et al., 2001). Oat3- and newly established PEPT2-LLC were cultured on culture dishes in M199 (Invitrogen) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 400 μg/ml G418 sulfate at 37°C with 5% CO_2 and 95% humidity. LLC-PK1 cells between passages 5 and 22 were used.

Transport Studies in cDNA-Transfected LLC-PK1 Cells. Cells were seeded in 12-well plates at a density of 1.2×10^5 cells/well for the transport studies. Twenty-four hours before beginning the assay, the cell culture medium was replaced with that containing 5 mM sodium butyrate to induce the expression of Oat3 and PEPT2.

Transport studies were carried out as described previously (Nagata et al., 2002). After cells were washed twice and preincubated with Krebs-Henseleit buffer at 37°C for 15 min, uptake was initiated by adding medium containing 1 μM unlabeled benzylpenicillin or GlySar in addition to 0.1 μCi of [^{3}H]benzylpenicillin or [^{3}H]GlySar. The Krebs-Henseleit buffer consisted of 118 mM NaCl, 23.8 mM $NaHCO_3$, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM $CaCl_2$, adjusted to pH 7.4. The uptake was terminated at designated time points by washing the cells twice with 1 ml of ice-cold Krebs-Henseleit buffer after removal of the incubation buffer. The cells were kept in 500 μl of 0.2 N NaOH overnight to allow lysis. After adding 100 μl of 1 N HCl, aliquots (400 μl) were transferred to scintillation vials containing 2 ml of scintillation fluid. The radioactivity associated with the cells and an aliquot of the incubation medium was determined using a liquid scintillation spectrophotometer (Amersham Biosciences, Inc., Piscataway, NJ) after the addition of scintillation fluid (Nacalai Tesque, Kyoto, Japan). Then, 50 μl of cell lysate was used to determine the protein concentration in each sample by the method of Lowry with bovine serum albumin as a standard.

Ligand uptake was given as the cell-to-medium concentration ratio determined as the amount of ligand accumulated in the cells divided by the medium concentration. Specific uptake was obtained by subtracting the uptake by vector-transfected cells from the uptake by cDNA-transfected cells. Inhibition studies were carried out by adding the desired concentrations of unlabeled inhibitors to the incubation medium.

Kinetic parameters were obtained using the following equation:

$$v = V_{max} \times S/(K_m + S) \quad (1)$$

where v is the uptake velocity of the substrate (picomoles per minute per milligram of protein), S is the substrate concentration in the medium (in micromolar), K_m is the Michaelis-Menten constant (in micromolar), and V_{max} is the maximum uptake rate (picomoles per minute per milligram of protein).

Inhibition constants (K_i values) of a series of compounds were obtained by examining their inhibitory effects on the Oat3- and PEPT2-mediated uptake. The substrate concentration was below the K_m values; thus, the inhibition constants can be obtained from the following equation, irrespective to the nature of the inhibition.

$$CL_{+I} = CL/(1 + I/K_i) \quad (2)$$

where CL is the uptake clearance, I is the concentration of inhibitor (in micromolar), and the subscript (+I) represents the value in the presence of inhibitor. Fitting was performed by the nonlinear least-squares method using a MULTI program (Yamaoka et al., 1981), and the Damping Gauss Newton Method algorithm was used for fitting.

Liquid Chromatography-Mass Spectrometry Analysis. The cellular accumulation of cefalexin and cefaclor by the cells and the medium concentration (45–55 μM) were quantified using liquid chromatography-mass spectrometry. Cefalexin and cefaclor in cell lysate were analyzed by liquid chromatography/mass spectrometry. The HPLC system consisted of a Separation Module (Waters 2695; Waters, Tokyo, Japan) connected to a Micromass ZQ with a ZSpray system (Waters). The system was controlled by version 3.5 of the Masslynx software. Chromatography was performed on a Capcell Pak C18 column (3 μm, 75 × 4.5 mm i.d.; Shiseido, Tokyo, Japan) protected by the same material. A gradient was applied using water (A) and acetonitrile (B), each containing 0.05% formic acid. The gradient conditions were as follows: initiate gradient with 100% A, ramp over 4 min to 30% A and 70% B, ramp over 1 min to 20% A and 80% B, hold for 0.5 min, and ramp over 0.1 min to 100% A to re-equilibrate the system. The total run time was 10 min. The compounds were eluted at a flow rate of 0.8 ml/min and allowed to pass into the electrospray source. The Micromass ZQ was operated in the atmospheric pressure chemical ionization-mass spectrometry positive ion mode with a corona current of 0.3 μA, cone voltage of 20 V, extractor voltage of 2.30 V, RF lens voltage of 0.3 V, source temperature of 100°C, cone temperature of 20°C, and desolvation temperature of 350°C. High-purity nitrogen gas was used as the desolvation gas at 300 ml/min, and the cone gas flow was 50 ml/min.

Intracerebroventricular Administration. The efflux of [^{3}H]GlySar after intracerebroventricular administration was studied using the method described previously in detail (Suzuki et al., 1989b; Ogawa et al., 1994). Rats were anesthetized with ketamine (60 mg/rat) and xylazine (0.984 mg/rat), and their heads were fixed in a stereotaxic apparatus. A hole was drilled in the skull, 1.5 mm to the left and 0.5 mm posterior to the bregma, into which a needle was fixed as a cannula for injection. An intracerebroventricular dose of [^{3}H]GlySar (20 nCi/rat) and [^{14}C]mannitol (0.4 nCi/rat), dissolved in 10 μl of artificial CSF, was administered to the left lateral ventricle. The prepared artificial CSF consisted of 122 mM NaCl, 25 mM $NaHCO_3$, 10 mM glucose, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, and 10 mM HEPES, adjusted to pH 7.3. For inhibition studies, unlabeled inhibitors of desired concentrations were administered simultaneously. At designated times, 50 to 100 μl of CSF was withdrawn by cisternal puncture and centrifuged to remove any contaminating blood. The remaining radioactivity in the CSF specimens was determined using a liquid scintillation spectrophotometer after the addition of scintillation fluid, and the elimination rate was calculated. Intracerebroventricular administration of cefaclor or cefalexin (1.25 μg/rat) and [^{14}C]mannitol (0.4 nCi/rat) was performed using the same method. The CSF specimens were subjected to HPLC analysis for quantification of cefalexin and cefaclor; and for [^{3}H]mannitol, the radioactivities associated with the CSF specimens were determined in a liquid scintillation spectrophotometer after addition of scintillation fluid. The elimination clearance from the CSF was determined by multiplying the elimination rate constant by the volume of CSF. In the present study, 250 μl was used as the volume of CSF (Cserr and Berman, 1978).

Transport Studies Using Isolated Rat Choroid Plexus. The uptake of [^{3}H]GlySar by rat choroid plexus was examined using the method described previously (Nagata et al., 2002). The choroid plexus was isolated from the lateral ventricles of SD rats and incubated at 37°C for 1 min in 500 μl of artificial CSF, consisting of 122 mM NaCl, 25 mM $NaHCO_3$, 10 mM glucose, 3 mM KCl, 1.4 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.4 mM K_2HPO_4, and 10 mM HEPES, equilibrated with 95% O_2/5% CO_2, and adjusted to pH 7.4. Incubation medium containing ligands, with or without inhibitors, was added to initiate uptake and at designated time points, the choroid plexus was washed three times in ice-cold artificial CSF to terminate the uptake. The tissue-to-medium concentration ratios of ligands were calculated with [^{14}C]urea as a cell water space marker. The ^{3}H and ^{14}C activity of the specimens was determined in a liquid scintillation spectrophotometer. The uptake of cefaclor by rat choroid plexus was examined as described above, and the samples were analyzed by HPLC. The uptake of cefaclor by the isolated rat choroid plexus was determined at different substrate concentrations ranging from 10 μM to 10 mM, and the effects of benzylpenicillin, GlySar, cimetidine, and *p*-aminohippurate were investigated at designated concentrations.

HPLC Assay. Concentrations of cefalexin and cefaclor in CSF and in the choroid plexus were analyzed by HPLC. The HPLC system included a pump (L-6200 intelligent pump; Hitachi, Ibaraki, Japan), a YMC-Pack Pro C18 column (5 μm, 250 × 4.6 mm; YMC, Tokyo, Japan) protected by the same material, a column oven (L-7300; Hitachi) set at 40°C, and a UV detector (L-4200 UV-VIS detector; Hitachi) operated at 260 nm. The system was controlled by an L-7200 autosampler (Hitachi). The mobile phase consisted of 0.01 M acetic acid, pH 4.7/methanol/2-propanol (80:20:1). The compounds were eluted at a flow rate of 1.0 ml/min.

Statistical Analysis. Statistical analysis of the effect of inhibitors on the uptake of cefaclor by the freshly isolated rat choroid plexus was performed by one-way analysis of variance followed by Fisher's t test to identify significant differences.

Results

Uptake of GlySar, Cefalexin, and Cefaclor by PEPT2-LLC. Transfection of rat PEPT2 cDNA increased the uptake of GlySar, a typical substrate of PEPT2 (Fig. 2A). The concentration dependence of the uptake of GlySar by PEPT2 was determined after a 3-min incubation. Nonlinear regression analysis yielded K_m and V_{max} values of 172 ± 21 μM and 2340 ± 240 pmol/min/mg protein, respectively (Fig. 2B).

Figure 2C shows the time profiles of the uptake of cefaclor and cefalexin by PEPT2. As for GlySar, the uptake of cefaclor and cefalexin was significantly greater in PEPT2-LLC than in vector-LLC. The K_i values of cefaclor for GlySar uptake by PEPT2 were determined to be 171 ± 27 μM (Fig. 2D).

Effect of Cefaclor, Cefadroxil, and Cefalexin on Benzylpenicillin Uptake by Oat3-LLC. The uptake of benzylpenicillin by Oat3- and vector-LLC for 5 min was 27.5 ± 2.1 and 5.32 ± 0.30 μl/mg protein, respectively. The uptake of cefaclor was determined in Oat3- and vector-LLC. It was found that they were comparable, and no specific uptake of cefaclor was detected by Oat3 (data not shown). The inhibitory effect of cefaclor, cephalexin, and cefadroxil on the uptake of benzylpenicillin by Oat3-LLC for 5 min was examined (Fig. 3). Cefaclor showed moderate potency, whereas cefalexin and cefadroxil showed weak potency. The K_i values of cefaclor, cephalexin, and cefadroxil were determined to be 105 ± 24, 677 ± 143, and 1060 ± 210 μM, respectively.

Elimination of GlySar, Cefalexin, and Cefaclor from CSF after Intracerebroventricular Administration. Figure 4 shows the CSF concentration of GlySar, cephalexin, or cefaclor and mannitol after intracerebroventricular ad-

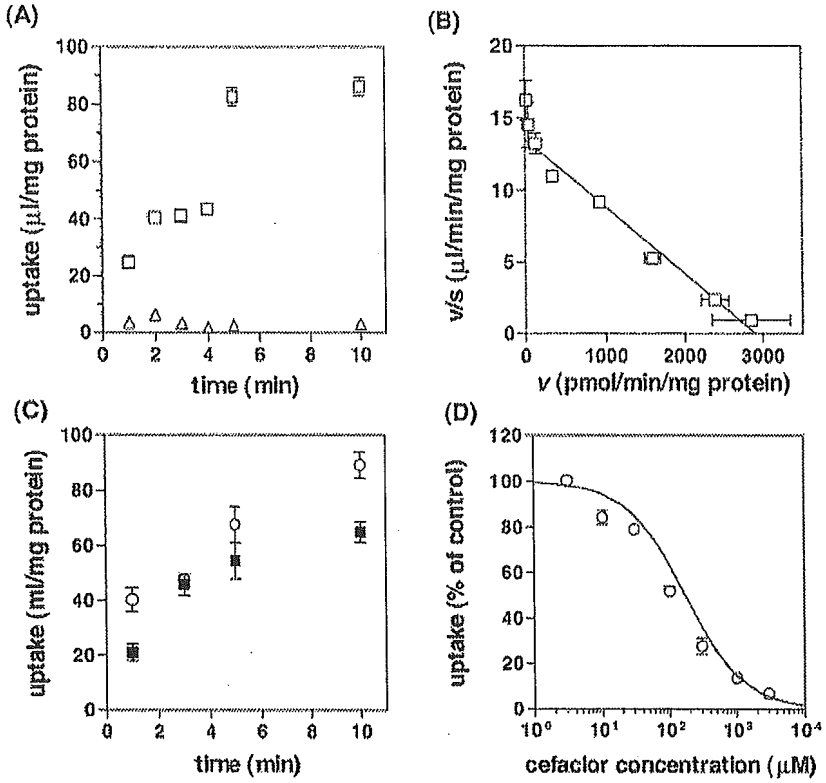

Fig. 2. Time-profile of the uptake of GlySar, cefalexin, and cefaclor by PEPT2-LLC, and the effect of cefaclor on PEPT2-mediated GlySar uptake. A, uptake of 1 μM GlySar by PEPT2-LLC (squares) and also by vector-LLC (triangles) was determined. B, concentration dependence of the uptake of GlySar by PEPT2 was determined after a 3-min incubation. The uptake was determined at different substrate concentrations (1 μM–3 mM). The fitted line was converted to the v/s versus v form (Eadie-Hofstee plot). C, uptake of 50 μM cefalexin (closed squares) and 54 μM cefaclor (open circles) by PEPT2-LLC was determined, and their uptake by vector-LLC was below the detection limit. D, uptake of [^{3}H]GlySar by PEPT2-LLC was determined after a 3-min incubation in the presence and absence of cefaclor at designated concentrations. Each point represents the mean ± S.E. (n = 3).

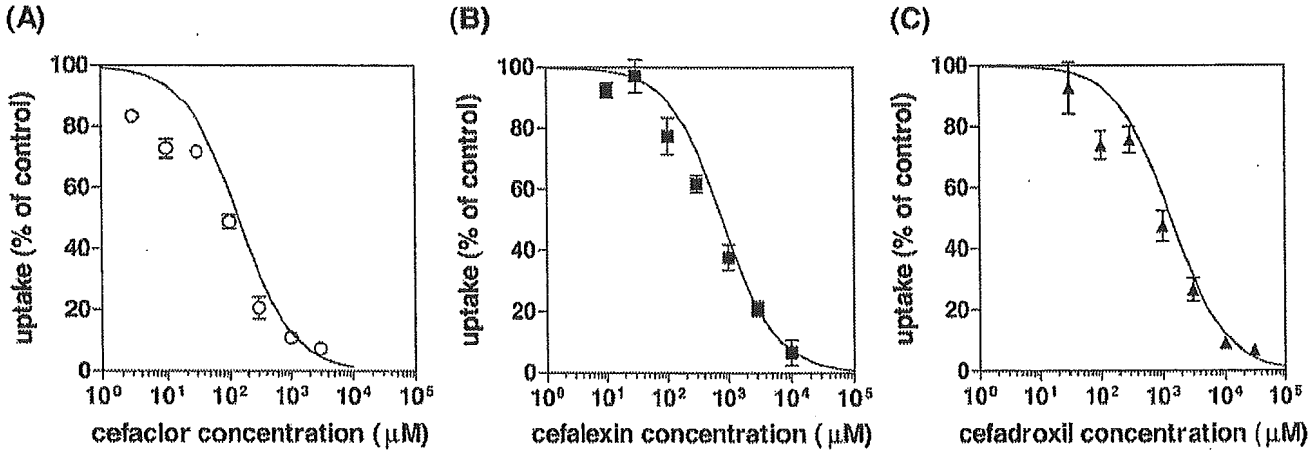

Fig. 3. Effect of cephalosporins on the uptake of [^{3}H]benzylpenicillin by Oat3-LLC. The uptake of [^{3}H]benzylpenicillin by Oat3-LLC was determined after a 5-min incubation in the presence and absence of the cephalosporins cefaclor (A), cefalexin (B), and cefadroxil (C) at the designated concentrations. Each point represents the mean ± S.E. (n = 3). The solid line represents the fitted line. Details of fitting are described under *Materials and Methods*.

ministration as a function of time. GlySar was eliminated from the CSF with a greater rate constant than mannitol, a reference compound for CSF turnover and diffusion into the brain interstitial space through the ependyma surface, with a rate constant of 0.073 min^{-1}. The elimination clearance of GlySar from the CSF (18 μl/min) was 2.5-fold greater than that of mannitol (7.23 ± 2.49 μl/min). The elimination of GlySar was saturated at the concentration examined (3 mM), whereas simultaneous injection of benzylpenicillin had no effect at a concentration sufficiently high to saturate its own elimination (Ogawa et al., 1994) (Fig. 5A).

Cefalexin was eliminated from the CSF with a rate constant of 0.0504 ± 0.0228 min^{-1} after intracerebroventricular administration (Fig. 4B), whereas cefaclor was more rapidly eliminated from the CSF with an elimination rate constant of 0.105 ± 0.002 min^{-1} (Fig. 4C). The elimination clearances of cefalexin and cefaclor were 13.0 ± 5.7 and 26.3 ± 0.5 μl/min, respectively. The elimination of both cephalosporins from the CSF was saturable, suggesting the involvement of a transporter (Fig. 5). Furthermore, the elimination of cefalexin was inhibited by GlySar, but it was not significantly affected by benzylpenicillin (Fig. 5B). Conversely, the elimination of cefaclor was inhibited by benzylpenicillin, but not by GlySar (Fig. 5C).

Uptake of Cefaclor by Isolated Rat Choroid Plexus. The uptake of cefaclor by freshly isolated rat choroid plexus was determined (Fig. 6A). The accumulation of cefaclor in the choroid plexus increased linearly for up to 5 min of incuba-

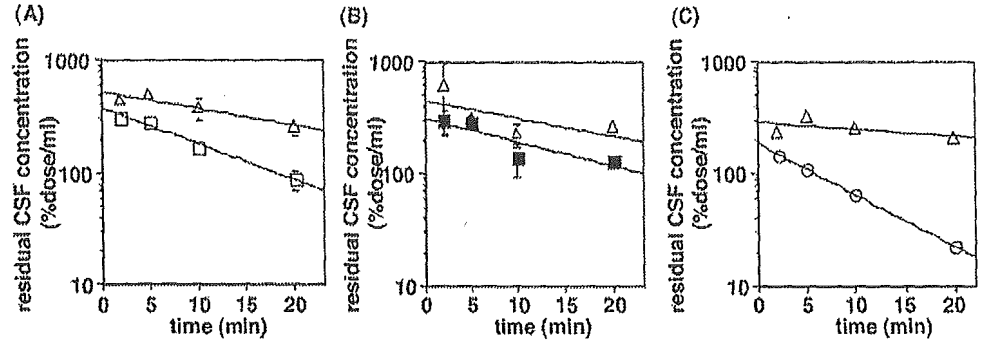

Fig. 4. CSF concentration versus time profiles of GlySar, cephalexin, and cefaclor after intracerebroventricular administration. [^{3}H]GlySar (A), cefalexin (B), and cefaclor (C) were administered into the lateral ventricle of rats. The initial concentration of [^{3}H]GlySar was 20 nM in the CSF, whereas that of cefalexin and cefaclor was 13 μM. The concentration of ligand remaining in the cisternal CSF was determined at the designated times. [^{14}C]Mannitol (open triangles) was coadministered as a reference for CSF turnover and passive diffusion into the brain parenchyma. The values are expressed as the percentage of the dose remaining per milliliter of CSF. Each point represents the mean ± S.E. of triplicate determinations using three rats. The solid lines represent the fitted elimination curve.

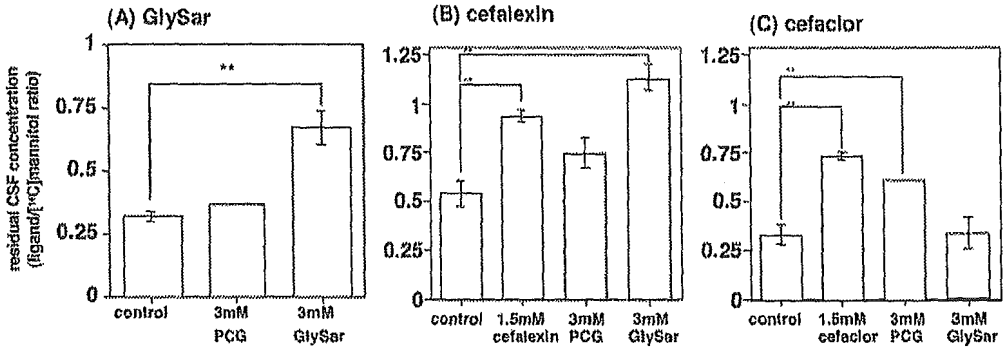

Fig. 5. Effects of benzylpenicillin and GlySar on the elimination of [^{3}H]GlySar, cephalexin, and cefaclor after intracerebroventricular administration. [^{3}H]GlySar (A), cefalexin (B), and cefaclor (C) were administered into the lateral ventricles of rats in the presence or absence of unlabeled benzylpenicillin (PCG) or GlySar. The designated concentrations represent their concentration in the CSF after injection. The concentration of ligand remaining in the cisternal CSF was determined at 20 min. [^{14}C]Mannitol was coadministered as a reference. The values are expressed as a ratio of the percentage of remaining ligand and [^{14}C]mannitol. Each point represents the mean ± S.E. of triplicate determinants using three rats. **, $p < 0.01$ (Student's t test).

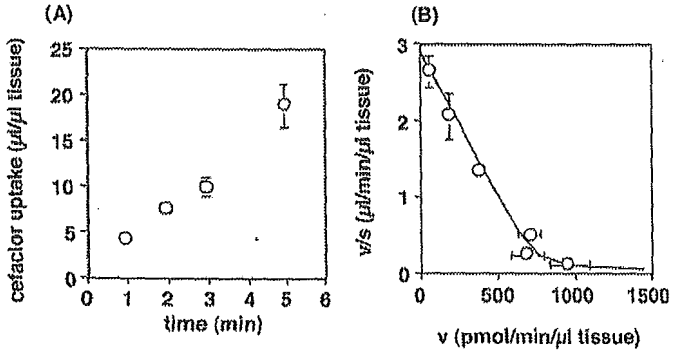

Fig. 6. Time profile and Eadie-Hofstee plot of cefaclor uptake by rat isolated choroid plexus. The uptake of 20 μM cefaclor was examined in the presence or absence of unlabeled cefaclor. The concentration dependence was determined after a 5-min incubation. The solid line represents the fitted line that was converted to the v/s versus v form (Eadie-Hofstee plot). The details of the fitting are described under *Materials and Methods*. Each point represents the mean ± S.E. of quadruple determinations.

tion; therefore, further studies were carried out at 5 min. Kinetic analyses based on Akaike's information criteria revealed that the K_m and V_{max} values for cefaclor uptake by rat isolated choroid plexus were 251 ± 31 μM and 722 ± 62 pmol/min/μl tissue, respectively, and the clearance corresponding to the nonsaturable component was 0.0242 ± 0.0099 μl/min/μl tissue (Fig. 6B). Inhibition studies were carried out to characterize the uptake of cefaclor by freshly isolated rat choroid plexus. Benzylpenicillin exhibited a significant inhibitory effect on the uptake in a concentration-dependent manner, and 3 mM benzylpenicillin had a significant effect (Fig. 7). In contrast, GlySar, cimetidine or p-aminohippurate had no significant inhibitory effect (Fig. 7).

Discussion

The efflux mechanisms in the choroid plexus have been shown to be an important factor governing the CSF concentration of β-lactam antibiotics and their therapeutic efficacy in bacterial meningitis. Regarding the efflux transport of β-lactam antibiotics from the CSF, Oat3 has been suggested to be responsible for the elimination of benzylpenicillin from the CSF and partly of the cephalosporin cefadroxil (Nagata et al., 2002; Ocheltree et al., 2004), whereas PEPT2 has been suggested to play a role in the elimination of cephalosporins with an α-amino group (Smith et al., 2004). In the present study, the elimination profiles of the analogs cefalexin and cefaclor from the CSF were compared after intracerebroventricular administration, and the uptake of cefaclor by the freshly isolated rat choroid plexus was characterized. Transport studies using the PEPT2-cDNA transfectant showed that both cefaclor and cefalexin are substrates of PEPT2 with

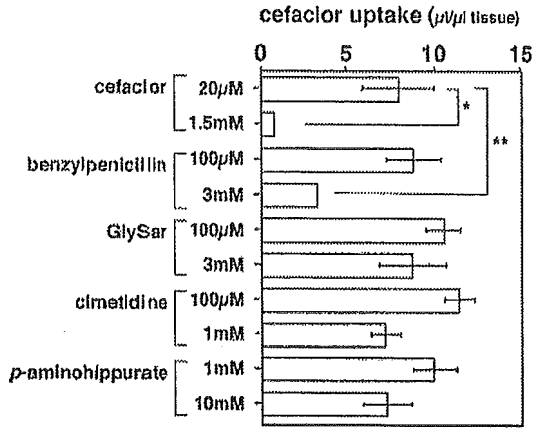

Fig. 7. Effects of benzylpenicillin, GlySar, cimetidine, and *p*-aminohippurate on the uptake of cefaclor by freshly isolated rat choroid plexus. The uptake of 20 μM cefaclor was determined at 5 min in the presence or absence of inhibitors at designated concentration. Each point represents the mean ± S.E. of quadruple determinations. **, $p < 0.01$; *, $p < 0.05$.

similar transport activities (Fig. 2). For Oat3, although the cephalosporins had a moderate or weak inhibitory effect (Fig. 3), no specific uptake of cefaclor by Oat3 could be detected (data not shown).

After intracerebroventricular administration, the elimination rates of cefalexin and cefaclor from the CSF were different. The elimination clearance of cefaclor from the CSF was 2-fold greater than that of cefalexin. Taking into consideration the clearance by nonspecific elimination pathways, such as a convective flow associated with CSF turnover and diffusion into the brain parenchyma across the ependyma surface, there was a 3-fold difference in the efflux clearance across the choroid plexus between cefaclor and cefalexin. To characterize the efflux transport, an in vivo inhibition study was carried out using benzylpenicillin and GlySar as inhibitors. It was found that there was a clear difference in the inhibition potencies of these inhibitors for the elimination of cefalexin and cefaclor from the CSF (Fig. 5), suggesting a difference in the contribution of the transporters responsible, despite their analogous chemical structures.

Furthermore, the uptake of cefaclor by isolated rat choroid plexus was characterized. Time-dependent and saturable uptake of cefaclor was observed in the freshly isolated rat choroid plexus (Fig. 6). Taking the water space of the rat choroid plexus (6 μl/rat) into consideration (Ogawa et al., 1994), it would be expected that the uptake clearance corresponding to the saturable fraction would account for the major part of the elimination clearance from the CSF (17 versus 26 μl/min/rat, respectively). Consistent with the in vivo observation (Fig. 5), benzylpenicillin had a significant effect on the uptake of cefaclor by the freshly isolated rat choroid plexus, whereas GlySar had no significant effect (Fig. 7). The uptake of benzylpenicillin by the isolated rat choroid plexus has been shown to be carrier-mediated (Suzuki et al., 1987; Nagata et al., 2002). However, its inhibition of the uptake of cefaclor was lower than expected from its own K_m value [58 μM (Suzuki et al., 1987), 111 μM (Nagata et al., 2002)] (Fig. 7). Furthermore, *p*-aminohippurate and cimetidine had no effect (Fig. 7), even at a concentration sufficient to inhibit benzylpenicillin uptake in the choroid plexus (Nagata et al., 2002). These kinetic results suggest that benzylpenicillin and cefaclor do not share the same uptake mechanism at the choroid plexus.

Considering the previous result obtained using another analog, cefadroxil (Ocheltree et al., 2004), PEPT2 is one of the candidate transporters responsible for the uptake of cefalexin by the choroid plexus. Inhibition of its elimination from the CSF by GlySar supports this hypothesis. In contrast to these analogs, the absence of inhibitory effect of GlySar on the elimination of cefaclor suggests a minimal contribution from PEPT2. Instead, a benzylpenicillin-sensitive transporter, distinct from the classical transporter responsible for benzylpenicillin uptake in the choroid plexus, accounts for the uptake of cefaclor by the choroid plexus and the rapid elimination of cefaclor from the CSF.

The present study demonstrates that even a minor modification of a single chemical group can dramatically alter the CSF retention time. Changing the methyl group of cefalexin to chloride alters the contribution of the responsible transporters, resulting in a marked increase in the elimination clearance across the choroid plexus. As discussed by Ocheltree et al. (2004), several cephalosporins without an α-amino group can achieve clinically adequate CSF concentrations for the treatment of bacterial meningitis. These clinical observations suggest that the α-amino group is also required for substrate recognition by the benzylpenicillin-sensitive transporter. This should be investigated further in future studies to establish a rational strategy for chemical modification to regulate the CSF concentrations of cephalosporins.

In addition to the CSF, it has been found that the brain parenchyma is the target of β-lactam antibiotics to prevent glutamate neurotoxicity through induction of glutamate transporters (Rothstein et al., 2005). The delivery of β-lactam antibiotics to the brain will become more important in future as far as increasing their therapeutic efficacy is concerned. There is another barrier, the blood-brain barrier, formed by a tight monolayer of brain capillary endothelial cells, in the central nervous system that prevents the passage of drugs into the central nervous system from the circulating blood. It has been suggested that the uptake of benzylpenicillin and cefodizime by the brain from the blood is mediated by a transporter (Suzuki et al., 1989a; Matsushita et al., 1991). However, benzylpenicillin has been suggested to undergo efflux across the blood-brain barrier, presumably by Oat3 (Kikuchi et al., 2003). Since the concentration of cefodizime in the extracellular fluid of the brain is less than the unbound plasma concentration (Matsushita et al., 1991), it could be that the efflux system for cefodizime is located in the blood-brain barrier. Further studies are necessary to investigate these transport mechanisms and the structure-activity relationships of cephalosporins governing their transport across the blood-brain barrier.

In conclusion, despite their very similar chemical structures, the elimination clearances of cefalexin and cefaclor from the CSF are markedly different. The results of the present study suggest that this distinction is accounted for by a difference in the contribution of the transporter responsible. A benzylpenicillin-sensitive transporter distinct from Oat3 accounts for the rapid elimination of cefaclor from the CSF, whereas a GlySar-sensitive transporter, presumably PEPT2, accounts for the elimination of cefalexin.

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Short Communication

Forced Exercise-Induced Flushing of Tail Skin in Ovariectomized Mice, as a New Experimental Model of Menopausal Hot Flushes

Hideki Shuto[1], Atsushi Yamauchi[1], Munehiko Ikeda[1], Yoshio Sohda[1], Ayako Koga[1], Kohji Tominaga[1], Takashi Egawa[1], and Yasufumi Kataoka[1,*]

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Forced Exercise-Induced Flushing of Tail Skin in Ovariectomized Mice, as a New Experimental Model of Menopausal Hot Flushes

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Abstract. Hot flushes are the most common complaint of menopausal women. In the present study, a new animal model of hot flushes was established. Tail skin temperature was measured with a thermo tracer after mice were subjected to a forced exercise task using a motor driven treadmill. In ovariectomized mice, forced exercise for 10 min was most effective in increasing tail skin temperature over that of sham-operated mice. This elevation was blocked by estradiol replacement (1 mg/kg per week for 3 weeks), suggesting that our model simulates menopausal hot flushes.

Keywords: ovariectomy, forced exercise, menopausal flushing

The menopausal hot flush is a bothersome symptom occurring in more than 75% of climacteric women (1). Manifesting as a transient increase in skin temperature and sweating hinders daily activity. Hot flushes have been linked to a transient disruption of the thermoregulatory mechanism that activates a heat-loss response including increased peripheral blood flow (2). Because hot flushes vary in duration, frequency, intensity, and duration of an individual flush, quantitative assessment of the disorder can be difficult (2). Flushing of the tail skin in ovariectomized (OVX) animals is known to be a good parameter of menopausal hot flushes, although the spontaneous appearance of flushing is irregular. Experimental manipulations including treatment with drugs trigger flushing in OVX-animals (3, 4). After morphine withdrawal with naloxone, a marked rise in tail skin temperature and an increase in heart rate appeared in OVX-rats (5). We previously demonstrated that nifedipine elevated tail skin temperature and that nifedipine-induced flushing was aggravated in mice with ovariectomy (6). This aggravation was blocked by estradiol replacement (6). In addition to drug-induced flushing, Rogers and Sheriff recently demonstrated that ovariectomy decreased hindlimb vascular conductance during graded mild-intensity treadmill locomotion in rats, this vascular modulation being reversed by estrogen replacement (7). Estrogen deficiency produces an abnormality of vascular tonus and/or insufficient autoregulation of the local vasculature. Therefore, we hypothesized that OVX-animals do not readily recover from forced exercise-increased peripheral vascular conductance. In the present study, we investigated changes in tail skin temperature before and after forced exercise on a motor-driven treadmill in OVX-mice as an experimental model of menopausal flushing.

Female ICR mice weighing 25 – 30 g were used (KyuDo Co., Ltd., Kumamoto). The mice were maintained on a 12-h light/dark schedule (lights on 7:00 a.m.) at a temperature of 24 ± 1°C with free access to food and water. All the procedures involving experimental animals adhered to the law (No. 105) and notification (No. 6) of the Japanese Government and were approved by the Laboratory Animal Care and Use Committee of Fukuoka University.

Mice underwent a bilateral ovariectomy or sham-operation under sodium pentobarbital anesthesia (50 mg /kg, i.p.) (6). Vehicle (sesame oil) and estradiol valerate (1.0 mg/kg) (Pelanin Depot; Mochida Pharmaceutical, Tokyo) were injected into the thigh muscle in a volume of 0.1 ml/100 g body weight once a week for 3 weeks starting 7 days after the operation. Twenty-eight days post-surgery, mice were subjected to the following

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experiment in a room maintained at a temperature of 25 ± 1°C. The body weight in sham-mice, OVX-mice, and estradiol-treated OVX-mice were 31.0 ± 0.2, 34.0 ± 0.4, and 33.8 ± 0.2 g, respectively.

Tail skin temperature was measured according to a procedure described previously (6). Mice were restrained in a holder in a conscious state and the tail skin temperature was measured at the dorsal surface of the tail about 1 cm from its base with a thermo tracer (TH5108ME; NEC San-ei, Tokyo) for 15 min. The data were stored in 1-min blocks and analyzed with the Thermal Image processing program (TH51-701, NEC San-ei) and Remote Control program (TH51-723, NEC San-ei). Two hours after the basal tail skin temperature was measured, mice were forced to run (15 m/min) on a motor driven treadmill (MK-680S; Muromachi Kikai, Tokyo) for a period of 5, 10, or 20 min and the running time was measured. After termination of the forced exercise, tail skin temperature was measured for 15 min. Changes in tail skin temperature were assessed using ΔTST. ΔTST = (tail skin temperature in each 1-min block after the forced exercise) – (average basal tail skin temperature for the period from 1 to 6 min).

Values are expressed as the means ± S.E.M. Statistical analysis was performed using the two-way analysis of variance (ANOVA) followed by the Tukey-Kramer test. A value of $P<0.05$ was considered to be statistically significant. The intraobserver or interobserver variation was <5% in each experiment.

As shown in Fig. 1A, OVX-mice subjected to the forced exercise (10 min) showed rapid and marked increases in tail skin temperature, with a return to the basal level within 7 – 8 min. Meanwhile, sham-operated (sham) mice showed only slight increases in the early stage after forced exercise. Based on these time-courses of ΔTST in sham- and OVX-mice (Fig. 1A), we evaluated the effect of forced exercise on tail skin temperature by accumulating ΔTST for the period from 1 to 6 min after the forced exercise (total ΔTST). Forced exercise for 10 and 20 min produced a marked increase in the total ΔTST of OVX-mice compared to the sham-mice (Fig. 1B). The total ΔTST of sham-mice increased with the amount of time the animals were forced to exercise. The difference in total ΔTST between OVX- and sham-mice was the greatest (9.73 ± 1.06°C) after a 10-min period of forced exercise. The total running time of OVX-mice in each period was the same as that of sham-mice (inset of Fig. 1B). When OVX-mice were treated with estradiol valerate (1.0 mg/kg) once a week for 3 weeks, forced exercise-induced increases in total ΔTST were markedly lowered by 81.3 ± 11.9% (Fig. 2A). In sham-mice, similar estradiol treatment did not influence the total ΔTST and the running time after

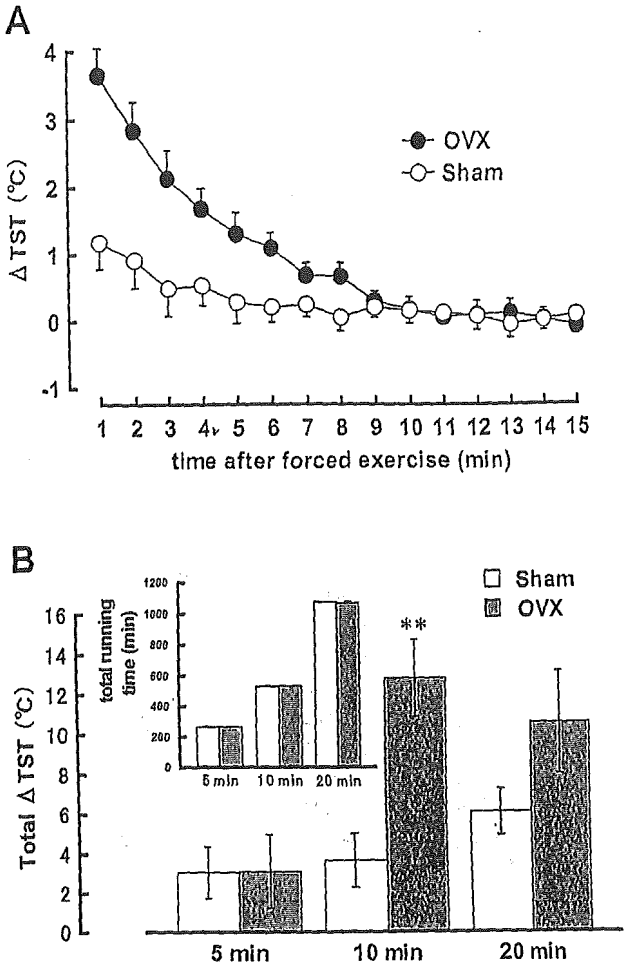

Fig. 1. Influence of treadmill locomotion (forced exercise) on tail skin temperature of sham-operated (sham) and ovariectomized (OVX) mice. A: Time course of changes in tail skin temperature (ΔTST) after forced exercise for 10 min. ΔTST was calculated as follows: ΔTST = (tail skin temperature in each 1-min block after the forced exercise) – (average basal tail skin temperature for the period from 1 to 6 min). B: Changes in accumulated ΔTST and running time (inset of panel B) in the period from 1 to 6 min (total ΔTST) after forced exercise for 5, 10, or 20 min in sham- and OVX-mice. Values represent the means ± S.E.M. for 5 to 7 animals. $^{**}P<0.01$, significant difference from sham-mice.

and during the forced exercise (10 or 20 min), respectively (data not shown). Representative thermograms show flushing of the tail skin in OVX-mice and estradiol-treated OVX-mice at 2 min after a 10-min period of forced exercise (Fig. 2B). There were no differences in running time between OVX-mice and estradiol-treated OVX-mice (inset of Fig. 2A).

In the present study, OVX-mice but not sham-mice showed a marked elevation in tail skin temperature at the early stage (1 to 6 min) after treadmill locomotion (15 m/min) for 10 min and this elevation was reversed by estrogen replacement. We propose that forced

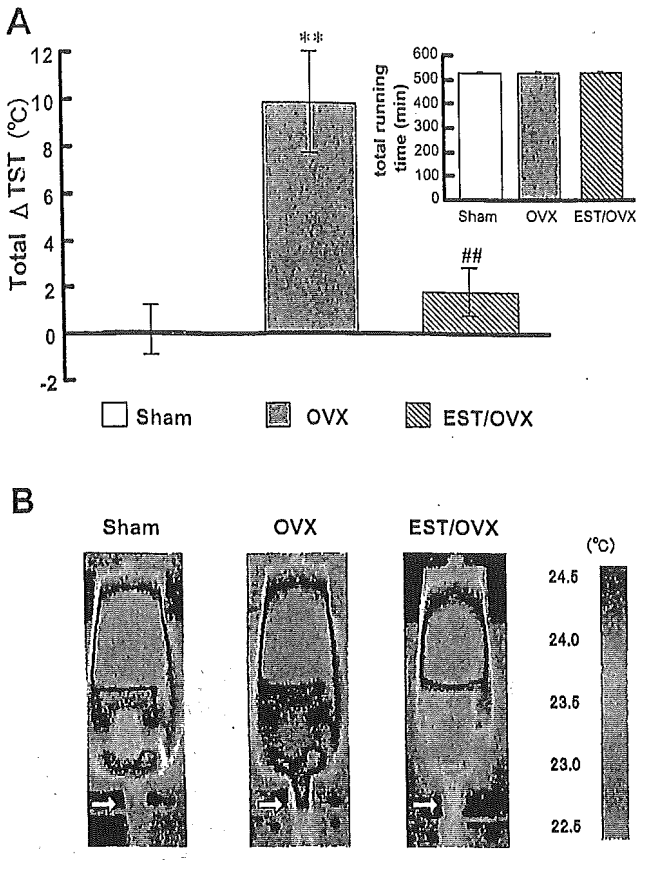

Fig. 2. Forced exercise-induced flushing of tail skin in mice with ovariectomy. A: Changes in tail skin temperature (total ΔTST) and total running time (inset of panel A) in the period from 1 to 6 min after forced exercise were induced by forced exercise for 10 min in sham-operated (sham) mice, ovariectomized (OVX) mice, and OVX-mice treated with estradiol (1 mg/kg) once a week for 3 weeks (EST/OVX). Values are the means ± S.E.M. for 10 to 13 animals. $^{**}P<0.01$ and $^{\#\#}P<0.01$, significant difference from sham- and OVX-mice, respectively. B: Representative thermograms showing flushing of the tail skin at 2 min after forced exercise (10 min) in sham-, OVX-, and EST/OVX-mice.

exercise-induced flushing of tail skin in OVX-mice is a conventional and useful experimental model of menopausal flushing. Changes in hindlimb vascular conductance during treadmill locomotion in OVX-rats were suggested to be positively associated with the production of endothelial nitric oxide (NO) (7). Chronic exercise increased the gene expression of endothelial NO synthase in canine aortic endothelial cells (8). Delp and Laughlin reported that levels of endothelial NO synthase protein in the aortas of rats increased after exercise training (9). Li et al. reported that basal NO generation is important in control of the cutaneous thermoregulatory microcirculation by ameliorating the arteriovenous anastomosis tone (10). These evidences together with the present findings suggest that the flushing of tail skin in OVX-rats is attributable to an insufficient vascular recovery from the vasodilatory response to forced exercise. Our previous findings demonstrated that ovariectomy significantly elevated the rise in tail skin temperature induced by nifedipine at a dose having no influence on blood pressure and that this event was blocked by estradiol replacement (6). Since estrogen promotes vascular relaxation and inhibits vascular contraction, the net result is a decrease in vascular resistance (11). It is, therefore, likely that estrogen deficiency leads to an abnormality of vascular tonus and/or insufficient autoregulation of the local vasculature. The protective effect of estrogen replacement in the present study may be related to an improvement of the abnormal local vascular tonus and autoregulation. On the other hand, calcitonin gene-related peptide (CGRP), a vasodilator neuropeptide is known to participate in the occurrence of menopausal hot flashes (12, 13). Recently, Noguchi et al. reported that ovariectomy not only potentiated CGRP-induced elevation of skin temperature and arterial vasorelaxation but also induced a lower concentration of endogenous CGRP in plasma and up-regulation of arterial CGRP receptors and that 17β-estradiol inhibited the CGRP-related responses in OVX-rats (14). Further studies are needed to clarify the CGRP-related mechanism in the present experimental model, forced exercise-induced flushing of tail skin in OVX-mice. Among sham-mice, OVX-mice, and estrogen-treated OVX-mice, there were no differences in the total running time during forced exercise (5, 10, and 20 min). This is consistent with the finding that changes in estrogen levels did not affect motor activity in rats (15). Therefore, the possibility that differences in forced exercise-induced flushing of the tail skin are due to changes in treadmill locomotion after estrogen withdrawal and/or estrogen replacement could be excluded.

In OVX-mice, flushing is detectable in the tail skin (3, 4), although experimental interventions are required for the induction of flushing. The present study demonstrated that forced exercise-induced flushing of tail skin in OVX-mice is a potentially useful experimental model of menopausal flushing.

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Short Communication

Cyclosporin A Aggravates Electroshock-Induced Convulsions in Mice with a Transient Middle Cerebral Artery Occlusion

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SUMMARY

1. To test whether an ischemic insult increases the susceptibility to cyclosporine A (CsA)-induced neurotoxicity, we examined the effect of CsA on the minimal electroshock-induced convulsions in mice treated with a transient middle cerebral artery occlusion (MCAO) for a short period (2 h).

2. This MCAO produced small to mid-sized infarcted regions in the cerebral hemisphere with increasing post-operative days. In MCAO mice, CsA (30 mg/kg, i.p.) elevated the incidence of minimal electroshock-induced convulsions to 90–100% over that in sham mice (20–30%) at 1–7 days but not 14 days post-surgery.

3. In light of these findings, the possibility that CsA increases the risk of convulsions in patients with cerebral infarction and/or at an early stage following focal cerebral ischemia would have to be considered.

KEY WORDS: cyclosporin A; electroshock-induced convulsions; middle cerebral artery occlusion; blood-brain barrier; mice.

INTRODUCTION

Cyclosporin A (CsA), an immunosuppressant, is widely used to prevent allograft rejection in solid organ transplantation and to treat various autoimmune diseases. CsA induces adverse events including renal, cardiovascular and gastrointestinal disorders. Neurological complications occur with a relatively high frequency (20–40%) (Gijitenbeek *et al.*, 1999; U. S. Group, 1994). The delivery of CsA into the brain is

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restricted by P-glycoprotein, a multi-drug efflux pump, and the tight junctions of brain capillary endothelial cells. CsA inhibited the function and expression of P-glycoprotein (Kochi *et al.*, 1999) and increased the permeability of brain endothelial cells (Dohgu *et al.*, 2000), suggesting that CsA could pass through the blood-brain barrier (BBB) due to the impaired function. In the present study, to test whether CsA induces neurotoxicity by passing through the damaged BBB and/or the reconstituted BBB with an incomplete function after an ischemic insult, we examined the effect of CsA on minimal electroshock-induced convulsions in mice treated with a transient middle cerebral artery occlusion (MCAO) for a short period (2 h).

MATERIALS AND METHODS

Male ddY mice weighing 25–35 g (Kyudo, Kumamoto, Japan) were housed in a room at a temperature of 22 ± 2°C under a 12-h light/dark schedule (lights on at 7:00 hours) and given water and food ad libitum. All the procedures involving experimental animals adhered to the law (No. 105) and notification (No. 6) of the Japanese Government, and were approved by the Laboratory Animal Care and Use Committee of Fukuoka University.

The pharmaceutical formulation of CsA (Sandimmun® injection, 250 mg/5 mL/ampule, Novartis Pharma, Tokyo, Japan) was used after a dilution with saline. The vehicle solution for CsA consisted of 13% polyoxyethylene castor oil (Cremophor EL®, Sigma, St. Louis, MO, USA), 7% ethanol, and 80% saline (the same mixture as the vehicle for Sandimmun® injection). A reagent for histological examination, 2,3,5-triphenyltetrazolium chloride (TTC), was purchased from Sigma.

Anesthesia was induced by 2% halothane (Flosen, Takeda, Osaka, Japan) and maintained with 1% halothane. Focal cerebral ischemia was induced by occlusion of the middle cerebral artery using the intraluminal filament technique (Mishima *et al.*, 2003). After a midline neck incision, the left common and external carotid arteries were isolated and ligated. An 8-0 nylon monofilament (Ethilon, Johinson & Johinson, Tokyo, Japan) coated with silicon resin (Xantopren, Heleus Dental Material, Osaka, Japan) was introduced through a small incision into the common carotid artery and was advanced to a position 9 mm distal from the carotid bifurcation for occlusion of the middle cerebral artery. Two hours after MCAO, animals were re-anesthetized with halothane, and reperfusion was established by withdrawal of the filament. The sham-operated mice were subjected to the procedure mentioned above without MCAO. At the end of the experiment, mice were decapitated under anesthesia with pentobarbital Na (40 mg/kg, i.p., Nembutal, Dainippon, Osaka, Japan) and brains were removed. Coronal sections were cut 2-mm thick and incubated in physiological saline containing 2% TTC at 37°C for 15 min for histological examination of the MCAO-induced brain damage.

Effect of CsA or vehicle on the minimal electroshock-induced convulsions was examined at 1, 3, 7 and 14 days after the sham-operation or MCAO. CsA (10 and 30 mg/kg) or vehicle was administered i.p. in a volume of 0.1 mL/10 g body weight. Sixty minutes after the injection, each mouse was subjected to the minimal electroshock (10 mA, at a frequency of 60 Hz, applied for 0.2 s) using

external corneal electrodes connected to an electroshock convulsive stimulator unit (MK-800, Muromachi Kikai, Tokyo, Japan) and placed individually in an acrylic cage (18 × 28 × 34 cm). The observations were made during a 2-min period with a camcorder (VL-DC1, Sharp, Tokyo, Japan) and stored on digital video tape. The durations of clonic and tonic-clonic convulsions were measured with a video player by two observers blinded to the pretreatment with CsA or vehicle. The minimal electroshock-induced convulsions were defined as positive when they lasted for more than 2 s. Only one of 12 normal mice showed convulsions for 1 s.

Statistical analysis was performed using Fisher's exact probability test. A value of $P < 0.05$ was considered significant.

RESULTS

Histological observations with TTC staining indicated that MCAO for 2 h produced a small infarcted region in the caudate putamen of the cerebral hemisphere with a relatively mild insult (an irregular pallor) at 1 day after the operation (Fig. 1). The size and intensity of infarction gradually increased in the cuadate putamen and cerebral cotex at 3–7 days, reaching a maximum at 14 days. Minimal electroshock did not induce convulsions in sham mice treated with vehicle. When CsA (10 or 30 mg/kg) was given, convulsions were induced by electroshock in 20–30% of sham mice at 1–14 days post-surgery (Fig. 2A). In MCAO mice treated with vehicle, electroshock-induced convulsion was observed in 1 of 8 mice at 1 day, but not at 3–14 days post-surgery (Fig. 2B). In MCAO mice, CsA (10 mg/kg) moderately increased the incidence of convulsions to 67% over the level in sham mice (20–30%) at 1 day but not at 3–14 days after operation (Fig. 2). CsA (30 mg/kg) markedly increased the incidence of convulsions to 90–100% at 1–7 days, but this increase was not observed at 14 days after the operation in MCAO mice (Fig. 2B).

DISCUSSION

Treatment with MCAO is employed to make an animal model of transient focal cerebral ischemia (Brown *et al.*, 1995; Sydserff *et al.*, 2002). In the present study, a short (2 h) MCAO produced small to mid-sized infarcted regions with increasing post-operative days in the caudate putamen and cerebral cortex, when compared with the usual MCAO method (4 h) (Mishima *et al.*, 2003) (Fig. 1).

A high dose of CsA (30 mg/kg) markedly aggravated the electroshock-induced convulsions in MCAO mice. This action appeared at an early stage (1–7 days) but not late stage (14 days) post-MCAO. An ischemic event has been known to cause disruption of the BBB (Cipolla *et al.*, 2004). Nishigaya *et al.* demonstrated using an immunohistological staining of endothelial barrier antigen that impairment and restoration of the brain endothelial barrier occurred in the post-ischemic period of 1–7 days and 14–28 days, respectively, in rats with MCAO for 2 h. Taken together with this evidence, the present findings suggest that CsA probably penetrates the brain through the impaired BBB at an early stage following ischemic insults and consequently has an adverse central action. The reconstituted BBB at a late-stage

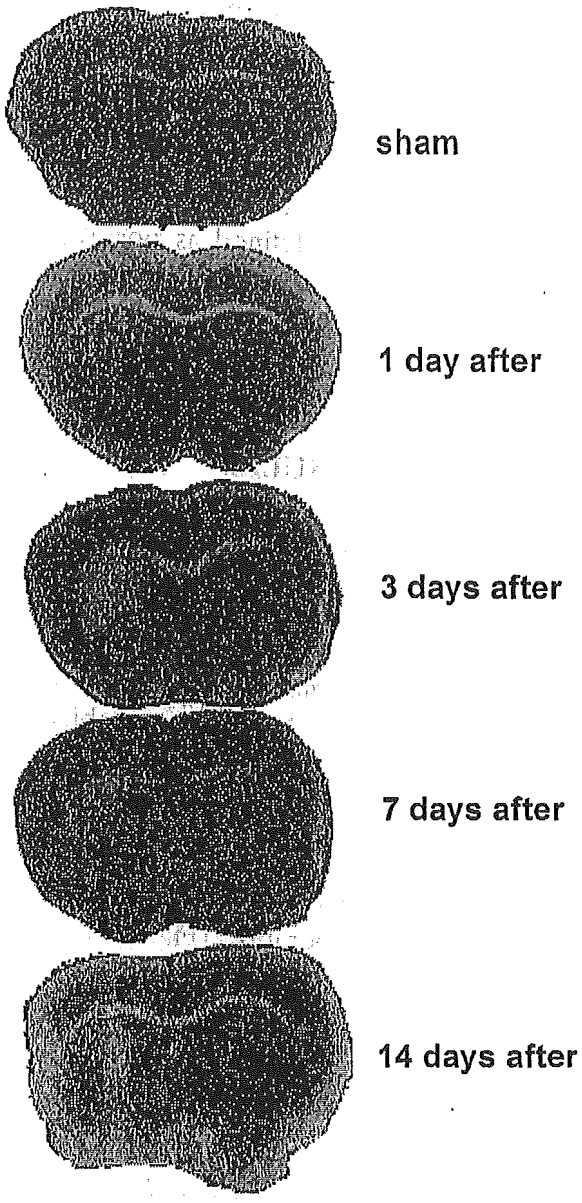

Fig. 1. Representative photographs showing coronal sections of the caudate putamen and cerebral cortex stained with 2% 2,3,5-triphenyltetrazolium chloride at 1, 3, 7 and 14 days after a transient middle cerebral artery occlusion for 2 h and at 7 days after sham operation (Sham).

post-MCAO may limit the delivery of CsA. Our previous findings *in vitro* demonstrated that CsA impairs brain endothelial barrier function by accelerating NO production in brain endothelial and astroglial cells (Ikesue *et al.*, 2000; Dohgu *et al.*, 2000; Yamauchi *et al.*, 2005). This action of CsA may be effective against the damaged BBB and/or the reconstituted BBB with an incomplete function at an early stage following the pathological insult. CsA is known to induce convulsions as a result of an

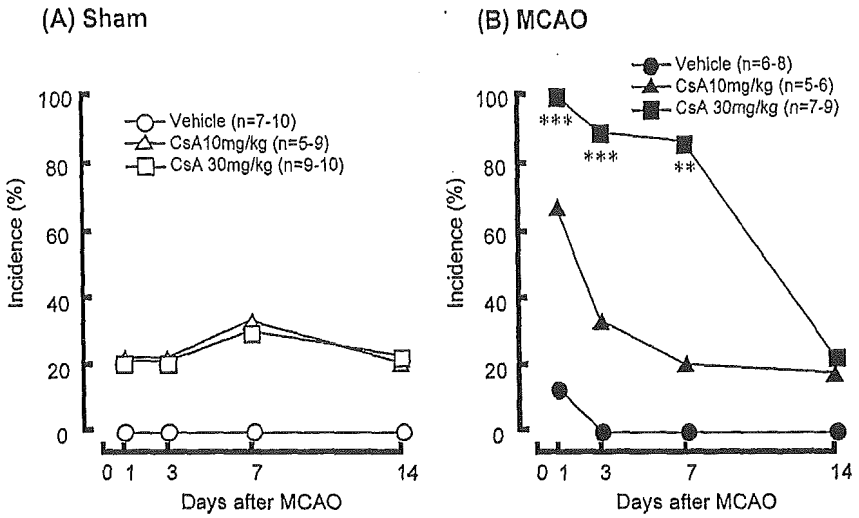

Fig. 2. Effect of cyclosporin A (CsA) on minimal electroshock-induced convulsions in sham-operated mice (Sham) (A) and in mice with a transient middle cerebral artery occlusion for 2 h (MCAO) (B). Values are expressed as the incidence (%) of convulsions in mice injected with vehicle or CsA 1 h before the test. The number of mice used in each treatment is indicated in parentheses. $^{**}P < 0.01$ and $^{***}P < 0.001$; significant difference from each corresponding group treated with vehicle.

interaction between NO and the γ-aminobutyric acid system in the hippocampus (Shuto *et al.*, 1999; Fujisaki *et al.*, 2002). This may be interpreted as a possible mechanism for the adverse central action of CsA after penetration of the brain. The possibility that MCAO influences these neuronal systems and increases the susceptibility to the deleterious effect of CsA on convulsions could not be excluded.

There is experimental evidence that CsA at low doses produces neuroprotective effects when given 30 min before or several minutes to 3 h after brain ischemia (Kuroda *et al.*, 1999; Yoshimoto *et al.*, 1999). In these studies, CsA prevented neuronal cell death including necrosis and apoptosis and decreased the infarct volumes in the brain. The different dosing schedule and doses of CsA are probably responsible for the discrepancy in the results.

In light of these findings, the possibility that CsA increases the risk of convulsions in patients with cerebral infarction and/or at an early stage following focal cerebral ischemia would have to be considered.

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