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## Short Communication

# *IN VIVO* EVIDENCE FOR ATP-DEPENDENT AND P-GLYCOPROTEIN-MEDIATED TRANSPORT OF CYCLOSPORIN A AT THE BLOOD–BRAIN BARRIER

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**Abstract**—To evaluate the significance of P-glycoprotein (P-gp)-mediated active efflux on the blood–brain barrier (BBB) permeability of cyclosporin A (CsA) *in vivo*, we investigated the effects of ATP depletion in the brain and of a multidrug-resistant (MDR) reversing agent on the transport of CsA across the BBB. Using transient brain ischemia obtained by 4-vessel occlusion of vertebral and common carotid arteries in rats to deplete ATP content in the brain, the estimated permeability surface area product (PS) value of [$^3$H]CsA was increased 2.7-fold compared with that in normal rats, whereas the PS value of [$^{14}$C]sucrose was not altered. Additionally, when quinidine hydrochloride (QND) was infused into the brain through a microdialysis probe implanted in the rat hippocampus, the extravascular extraction of CsA was increased to approximately 2.5-fold of the control, whereas no difference in the extravascular extraction between control and normal rats having no implanted dialysis probe was observed. Furthermore, the efflux rate from brain to blood of CsA was decreased remarkably to 5% of control at steady-state by co-administration of CsA with QND directly into the brain through the dialysis probe. The ATP-dependent and QND-sensitive efflux of CsA from the brain strongly indicates that P-gp in the brain capillary endothelial cells functions as an efflux pump under the physiological state, and that P-gp-mediated efflux of CsA is a major mechanism of the restricted transfer from blood into the brain.

*Key words:* active transport; blood–brain barrier; cyclosporin A; ischemia; multidrug resistance; P-glycoprotein

Although the permeability of drugs across the BBB† into the brain usually correlates with their lipophilicity [1, 2], that of highly lipophilic vincristine and CsA is unexpectedly low [2–5]. Accordingly, it is suggested that the BBB, made up of BCECs, regulates the movement of certain hydrophobic compounds as well as hydrophilic compounds, thereby maintaining brain homeostasis. Several mechanisms have been proposed to explain the unusually low BBB permeability of CsA: (i) that the lipid solubility of CsA transiently drops in the transmembrane transport process due to conformational changes [3], (ii) that CsA is present largely in bound form with plasma proteins or with red blood cells [6], (iii) that CsA is effectively trapped in the capillary endothelial cells of the brain [7], and (iv) that the molecular size of CsA is too large to cross the BBB, due to the presence of a threshold in the transport process [4, 8]. Contrary to these propositions, we have suggested that P-gp expression in the luminal membrane of the BBB as the mechanism responsible for reducing CsA permeability into the brain. This suggestion rests on results obtained using an *in vitro* BBB model to transport CsA [9], vincristine [10, 11] and rhodamine 123 [12] out of cells; these experiments demonstrated that CsA transport by BCECs is metabolic-energy dependent and is inhibited by various MDR-reversing agents [9]. Furthermore, CsA was shown to competitively inhibit the binding of vincristine to P-gp in MDR tumor cells [13]. However, the significance of P-gp in the disposition of these drugs at the BBB, under physiological conditions, is not clear.

In the present study, we evaluated the significance of P-gp-mediated efflux in the *in vivo* transfer of CsA from blood into the brain. P-gp-mediated transport shows two important features: ATP dependence and inhibition by MDR-reversing agents. In the first place, we studied the ATP dependence of the transfer of CsA into the brain by using forebrain ischemia, which can induce an ATP depletion state in the brain [14]. The effect of MDR-reversing agents on the transport of CsA at the BBB was investigated by administering MDR-reversing agents into the brain, using brain microdialysis. Implantation of a transcranial-type dialysis probe in the hippocampus causes no significant impairment of BBB function, as demonstrated by the permeation of $\alpha$-aminoisobutylate [15] and sucrose [16] into brain. According to the advantages described above, we infused MDR-reversing agents directly into the brains of living rats, thereby making it possible to evaluate the effect of MDR-reversing agents on the influx into brain and efflux from brain of CsA under the physiological state. QND was used as an MDR-reversing agent, because (i) it showed a significant effect on the steady-state uptake of

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† Abbreviations: $AcNH_2$, acetamide; BBB, blood–brain barrier; BCECs, brain capillary endothelial cells; BtOH, butanol; CsA, cyclosporin A; MDR, multidrug resistance; P-gp, P-glycoprotein; PS, permeability surface area product; QND, quinidine hydrochloride; and RHB, Ringer–HEPES buffer.

CsA by primary cultured BCECs [9], (ii) it has high water solubility and relatively low molecular size (324.4) among P-gp inhibitors, leading to an efficient permeability across microdialysis fiber, resulting in a high brain concentration, and (iii) it is expected to have relatively high affinity to P-gp [17].

*Materials and Methods*

*Animals.* Male Fischer rats (F344, Japan Charles River Co., Shiga, Japan) and male Wistar rats (Sankyo Laboratory Co., Toyama, Japan) were used for the brain ischemia studies and brain microdialysis studies, respectively. They had free access to food and water.

*Chemicals.* [Mebmt-$\beta$-$^3$H]CsA ([$^3$H]CsA, 10.1 Ci/mmol) was purchased from Amersham International plc (Buckinghamshire, U.K.), and [1-$^{14}$C]acetamide ([$^{14}$C]$AcNH_2$, 55 mCi/mmol) from American Radiolabeled Chemicals Inc. (St. Louis, MO, U.S.A.); *n*-[1-$^{14}$C]butanol ([$^{14}$C]-BtOH, 1.3 mCi/mmol), [$^{14}$C-U]sucrose (5.0 mCi/mmol) and Solvable (tissue solubilizer) were obtained from New England Nuclear (Boston, MA, U.S.A.). Unlabeled CsA was a gift from Sandoz AG (Basel, Switzerland). QND was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), BSA (Fraction V) from the Sigma Chemical Co. (St. Louis, MO, U.S.A.), and Clear-sol I (xylene based liquid scintillation fluid) from Nacalai Tesque Inc. (Kyoto, Japan). All other chemicals were of reagent grade and commercially available.

*Perfusion of* [$^3$H]*CsA or* [$^{14}$C]*sucrose into ATP-depleted brain.* Fisher rats weighing 200–250 g were used. BBB permeability of CsA under ATP depletion was measured by using the internal carotid artery perfusion method [18] in 4-vessel occluded forebrain ischemic rats [19, 20]. Briefly, at 48 hr after occlusion of both vertebral arteries, the rats were anesthetized with ketamine (120 mg/kg) and xylazine (1.2 mg/kg). After the right occipital and superior thyroid arteries were closed by electrocoagulation and the right pterygopalatine was ligated, the right external carotid artery was catheterized with polyethylene tubing (SP-10, Natsume Seisakusho Co., Tokyo, Japan) filled with sodium heparin (100 IU/mL). Perfusate (142 mM NaCl, 28 mM $NaHCO_3$, 4.2 mM $KH_2PO_4$, 1.0 mM $MgSO_4$, 1.7 mM $CaSO_4$, 6.0 mM D-glucose, 0.1% BSA, pH 7.4) was freshly prepared, oxygenated with 95% $O_2$–5% $CO_2$ and kept at 37°. Brain perfusion was initiated at 20 min after forebrain ischemia with clamping of both common carotid arteries, and both [$^3$H]CsA (2.0 $\mu$Ci/mL) and [$^{14}$C]sucrose (0.01 $\mu$Ci/mL) were perfused at the rate of 4.98 mL/min with a constant flow pump (Harvard Apparatus, South Natick, MA, U.S.A.) for 36 sec. Non-occluded rats were used as the control. Since it takes 6 sec for perfusate to reach the brain, the time for the transport assay was 30 sec. At the end of the perfusion, the rats were decapitated, and the hemisphere ipsilateral to the perfused side was solubilized in 3 mL of Solvable at 60° for 3 hr. Then, the sample was decolorized with 30% $H_2O_2$ and neutralized with 1 N HCl, and its radioactivity was measured with a liquid scintillation counter. Permeability of [$^3$H]CsA expressed as the cerebrovascular permeability surface area product (PS) was determined from equation 1 [18]:

$$PS = -F_{Pf} \cdot \ln[1 - q_B/(C_{Pf} \cdot T \cdot F_{Pf})] \quad (1)$$

where $F_{Pf}$ is the perfusion rate, $q_B$ is the amount of [$^3$H]-CsA in the cerebral hemisphere at the time of decapitation, $C_{Pf}$ is the concentration of [$^3$H]CsA or [$^{14}$C]sucrose in the perfusate, and T is the net perfusion time (30 sec). The intrinsic cerebrovascular permeability of [$^3$H]CsA was obtained after correction of the apparent vascular space estimated from [$^{14}$C]sucrose.

*Carotid artery injection with brain microdialysis.* A transcranial-type microdialysis probe was prepared as described previously [21]. The probe consists of a Cuprophan hollow-fiber (i.d., 0.2 mm; molecular cut off, 12,500; RENAK-E, Kawasumi Laboratories Inc., Tokyo, Japan) and stainless-steel tube (o.d., 0.2 mm; MT Giken, Tokyo, Japan). Fine stainless-steel tubes were inserted into a 22-mm long dialysis fiber to 7 mm depth in each end and attached with surgical adhesive (Aron Alpha A, Sankyo Co. Ltd., Tokyo, Japan). The effective dialysis part of the probe was 8 mm in length. To determine the relative recovery of CsA across the microdialysis probe, *in vitro* microdialysis was studied by a method reported previously [22]. Briefly, the dialysis probe was placed in 20 mL of RHB (141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 10 mM HEPES, 0.1% BSA, pH 7.2, 37°) containing [$^3$H]CsA (0.05 $\mu$Ci/mL) and unlabeled CsA (3 $\mu$M) in the absence and presence of 1.5 mM QND. Dialysis was carried out with RHB (pH 7.0) at a constant flow rate of 2.5 $\mu$L/min. *In vitro* relative recovery was estimated from the dialysate to reservoir concentration ratio.

The effect of QND on the permeability of [$^3$H]CsA into the brain was assessed *in vivo* using a combination of the carotid artery injection technique [23] with brain microdialysis. Briefly, the microdialysis probe was implanted horizontally in the hippocampus of the rats. The rats were allowed free access to food and water for 48 hr after implantation. QND in RHB (pH 7.0) at a concentration of 1.5 mM was infused into the brain through the dialysis probe for 60 min at a constant flow rate of 2.5 $\mu$L/min. Control rats were infused with RHB, and normal rats without implantation of the probes were also used to clarify the effect of the surgery. Two hundred microliters of [$^3$H]CsA (40 $\mu$Ci/mL) and [$^{14}$C]BtOH (0.2 $\mu$Ci/mL), which is an internal standard, in RHB (pH 7.4) was injected rapidly into the carotid artery. After 15 sec, the rats were decapitated, and then the radioactivity of the hemisphere ipsilateral to the injected side was measured. The extravascular extraction of [$^3$H]CsA was determined as described previously [24].

In vivo *steady-state efflux study with brain microdialysis.* Rats implanted with the microdialysis probe were anesthetized, and 3 $\mu$M [$^3$H]CsA in RHB (pH 7.0) was infused with or without 1.5 mM QND at a constant flow rate of 2.5 $\mu$L/min for 60 min into the brain via the implanted microdialysis probe. According to the reference method [22], QND concentration in the brain at steady state was estimated. At the end of an infusion, the inner space of the probe was washed out rapidly with RHB without radioisotope, the rats were decapitated, and the radioactivity of both hemispheres was determined. The steady-state efflux rate constant of [$^3$H]CsA in the absence and presence of QND was calculated as follows:

Table 1. Effect of brain ischemia on the transport of CsA into the brain

| | PS value ($\mu$L/min/g brain) | |
|---|---|---|
| | Control | Ischemia |
| [$^3$H]CsA* | 100 ± 21 | 315 ± 42† |
| [$^{14}$C]Sucrose | 29.9 ± 6.8 | 36.6 ± 5.4 |

Cerebrovascular permeability surface area products (PS) of [$^3$H]CsA and [$^{14}$C]sucrose were measured at 37° for 30 sec at the perfusion rate of 4.98 mL/min. Each value is the mean ± SEM of five experiments.

* PS value of CsA was corrected by the PS value of sucrose as the vascular space.

† Significantly different from control as determined by Student's *t*-test ($P < 0.05$).

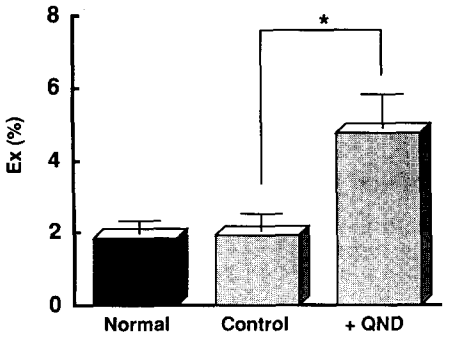


Fig. 1. Effect of QND on the extravascular extraction (Ex) of [$^3$H]CsA. To inhibit P-gp-mediated efflux, 1.5 mM QND in RHB (pH 7.0) was infused into the brain for 60 min through the dialysis probe. Rats infused with only RHB were represented by control and non-implanted rats by normal, respectively. Rats were decapitated at 15 sec after the bolus carotid artery injection with 200 $\mu$L of drug solution containing [$^3$H]CsA (40 $\mu$Ci/mL) and [$^{14}$C]BtOH (0.2 $\mu$Ci/mL). Each value is the mean ± SEM (N = 4–6). Key: (*) significantly different from control as determined by Student's $t$-test ($P < 0.05$).

*In vivo* dialysis clearance ($CL_D$) was estimated according to the method described in Ref. 25 and as given in equation 2:

$$CL_D = F_D \cdot [1 - \exp(-R_d \cdot PA_{vitro}/F_D)] \quad (2)$$

where $F_D$ is the dialysis flow rate, $PA_{vitro}$ is the *in vitro* permeability rate constant, and $R_d$ is the effective dialysis coefficient, which is the ratio of the *in vivo* to *in vitro* permeability rate constants ($PA_{vivo}/PA_{vitro}$) from the reference compound, antipyrine (0.389) [22, 25], by assuming that CsA has the same $R_d$ value as antipyrine.

Here, the mass balance of [$^3$H]CsA in the brain was obtained from the following equation 3:

$$V_B \cdot dC_B/dt = CL_D \cdot C_{in} - k_e \cdot V_B \cdot C_B \quad (3)$$

where $V_B$ is the apparent volume of distribution in the brain, $C_B$ and $C_{in}$ are the concentrations of [$^3$H]CsA in the brain and in the infused solution, respectively, and $k_e$ is the efflux rate constant. At steady state, $dC_B/dt = 0$; therefore, $k_e$ is determined as follows:

$$k_e = CL_D \cdot C_{in}/(V_B \cdot C_B) \quad (4)$$

*Results and Discussion*

To determine whether the transport of CsA into the brain is influenced by the ATP content in the brain *in vivo*, the effect of ATP depletion in the brain on the PS value of [$^3$H]CsA was examined. Using the 4-vessel occlusion technique in rats, ATP concentration in the brain is known to decrease markedly to a few percent of that of normal rats [14]. Accordingly, it was expected that P-gp could not function as an efflux pump, resulting in the apparently increased BBB transfer of CsA into the brain. As shown in Table 1, the PS value of [$^3$H]CsA increased markedly when ATP in the brain was depleted, whereas that of [$^{14}$C]-sucrose, which represents non-specific alterations in the BBB, such as vascular space and intercellular opening, was unchanged. This result strongly suggests that P-gp prevents the penetration of CsA into the brain in an ATP-dependent manner *in vivo*, a finding similar to that found in an *in vitro* BBB model reported previously from our laboratory [9].

To assess further the function of P-gp *in vivo*, brain microdialysis was employed as a tool for drug administration directly into the brain. This assay is based on the effect of QND on the efflux of CsA from brain mediated by P-gp. In the previous report, the steady-state uptake of [$^3$H]CsA by primary cultured BCECs was increased significantly to approximately 2.5-fold in the presence of 500 $\mu$M QND [9]. This increased uptake of [$^3$H]CsA was ascribed to the change of the process of efflux mediated by P-gp, but not to that of influx. When 1.5 mM QND solution was infused into the brain through the microdialysis probe at a rate of 2.5 $\mu$L/min, the unbound concentration of QND in the brain at steady state was calculated to be about 80 $\mu$M [22]. Since the $K_i$ value of QND obtained by the inhibition of vincristine binding to MDR tumor cell membrane was less than 1 $\mu$M [16], 80 $\mu$M is considered to be sufficient to inhibit the binding of CsA to P-gp. As shown in Fig. 1, the estimated extravascular extraction of [$^3$H]CsA by carotid artery injection was increased significantly to approximately 2.5-fold in the presence of QND. Since there were no differences in extravascular extraction between control rats and normal rats having no implanted dialysis probes (Fig. 1), it can be said that probe implantation did not cause a change in BBB permeability. Moreover, the extravascular extraction value of [$^3$H]CsA in both the normal and control rats was consistent with previous reports [3, 5, 7]. Therefore, the alteration of the extravascular extraction of CsA in the presence of QND was possibly due to a specific effect of QND on the efflux of CsA mediated by P-gp.

Table 2. Effect of QND on the steady-state efflux rate constants of CsA and $AcNH_2$*

| | $k_e$ (min$^{-1}$) | |
|---|---|---|
| | Control | +QND |
| [$^3$H]CsA | 0.777 ± 0.39 (0.89) | 0.0441 ± 0.013† (15.7) |
| [$^{14}$C]$AcNH_2$ | 0.197 ± 0.020 (3.52) | 0.127 ± 0.013 (5.46) |

Three micromolar [$^3$H]CsA or [$^{14}$C]$AcNH_2$ was infused into the brain through the microdialysis probe with or without 1.5 mM QND. The steady-state efflux rate constants ($k_e$) of each compound were calculated as described in the text. Each value is the mean ± SEM (N = 3–6).

* Mean half-lives (min) of the efflux process calculated by $0.693/k_e$ are shown in parentheses.

† Significantly different from control as determined by Student's $t$-test ($P < 0.05$).

The function of P-gp to exclude CsA from brain to blood was confirmed by the effect of QND on the efflux rate of CsA. First, to evaluate whether QND in the brain interferes only with the efflux by P-gp *in vivo*, the $k_e$ of the reference compound [$^{14}$C]$AcNH_2$ was examined, because (i) there is no report on specific carrier-mediated transport of $AcNH_2$ (it is supposed to transfer across the BBB by passive diffusion with the moderate PS value less than cerebral blood flow [1]), and (ii) no specific binding protein is present in tissue or cell. As shown in Table 2, the $k_e$ of [$^{14}$C]$AcNH_2$ was not affected significantly by QND. In contrast, the $k_e$ of [$^3$H]CsA in the brain was decreased remarkably to 5% of the control in the presence of QND (Table 2). Accordingly, P-gp in brain is confirmed to exclude CsA out of brain into blood under the physiological state. Very recently, it was demonstrated that a disruption of *mdr1a* gene in mouse leads to an increased accumulation of vinblastine and ivermectin in brain [26], that is, P-gp regulates entry of certain drugs into the brain. This result is comparable with ours, although it was not known whether

CsA transport at the BBB was also regulated by the *mdr1a* gene.

The BBB is constituted by BCECs connected with tight junctions [27]. Therefore, it has been believed that the permeability of drugs across the BBB can be predicted from their lipophilicity and molecular size [1, 2]. CsA is one compound where the BBB permeability is less than expected from its lipophilicity [3–5]. According to Levin's hypothesis [2], the estimated cerebrovascular permeability of CsA is $99 \times 10^{-6}$ cm/sec, whereas the observed value was $7.2 \times 10^{-6}$ cm/sec [4]. Hitherto, several mechanisms have been proposed to explain the unusual BBB permeability of CsA. They include a transient drop in its lipid solubility [3], binding to the blood component [6], trapping in BCECs [7], and threshold in the transport process [4, 8]. However, consistent with our previous finding in the *in vitro* BBB model [9], the results obtained in the present study indicate that P-gp in BCECs can pump out CsA from the brain into the bloodstream under physiological conditions. Since the $k_e$ of CsA from the brain at steady state markedly decreased to approximately 5% of the control in the presence of QND, P-gp-mediated efflux of CsA is thought to be a major mechanism of the restricted transport across the BBB. Consequently, P-gp expressed on the luminal surface of BCECs functions to restrict the permeation of cytotoxic compounds with high lipophilicity, that is, P-gp functions as the BBB against such compounds.

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