Involvement of Multiple Transporters in the Efflux of 3-Hydroxy-3-methylglutaryl-CoA Reductase Inhibitors across the Blood-Brain Barrier

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ABSTRACT

Statins, 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors, are frequently used for the treatment of hypercholesterolemia. The present study aimed to examine the involvement of organic anion transporters in the efflux transport of pravastatin and pitavastatin across the blood-brain barrier (BBB). Transport studies using cDNA-transfected cells revealed that these statins are substrates of multispecific organic anion transporters expressed at the BBB (rOat3:Slc22a8 and rOatp2:Slc10a4). The efflux of these statins across the BBB was characterized using the brain efflux index method. The efflux clearance of pitavastatin across the BBB, obtained from the elimination rate constant and the distribution volume in the brain, was greater than that of pravastatin (364 versus 59 μl/min/g brain). The efflux of pravastatin and pitavastatin was saturable (apparent K_m values: 18 and 5 μM, respectively) and inhibited by probenecid but unaffected by tetraethylammonium. Furthermore, an inhibitor of the efflux pathway for hydrophilic organic anions across the BBB (p-aminohippurate), and inhibitors of the efflux pathway for amphipathic organic anions (taurocholate and digoxin) inhibited the efflux of both statins. The degree of inhibition by p-aminohippurate was similar and partial for the efflux of pravastatin and pitavastatin. Taurocholate and digoxin completely inhibited the efflux of pitavastatin, whereas their effect was partial for the efflux of pravastatin. The results of the present study suggest the involvement of multiple transporters, including rOat3 and rOatp2, in the efflux transport of pravastatin and pitavastatin across the BBB, each making a different contribution.

The brain capillary endothelial cells are characterized by highly developed tight junctions and the expression of xenobiotic transporters (Kusuhara and Sugiyama, 2001a,b; Lee et al., 2001; Golden and Pollack, 2003; Sun et al., 2003). These transporters include the member(s) of the Oat family (Oat3) (Ohtsuki et al., 2002; Kikuchi et al., 2003), the Oatp family (Oatp2) (Asaba et al., 2000; Sugiyama et al., 2001), and the ATP-binding cassette transporters (Mdr1 and Mrp1) (Kusuhara et al., 1997; Sugiyama et al., 2003). Cumulative evidence suggests that these transporters facilitate the elimination of xenobiotics and endogenous compounds from the central nervous system (CNS) across the BBB, providing the barrier function between the blood and the brain.

Statins, HMG-CoA reductase inhibitors, have been used for the treatment of hypercholesterolemia. HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, is present in the liver and nonhepatic tissues, catalyzing the early conversion of HMG-CoA to mevalonic acid, and the enzyme inhibition in the liver by statins results in the lower serum level of total cholesterol (Reinoso et al., 2002). The adverse effects of statins include CNS side effects, such as sleep disturbance, as well as myopathy (Schaefer, 1988; Barth et al., 1990). Therefore, the liver selectivity of statins must be given priority in clinical situations to reduce the undesired toxicological effects on the body. On the other hand, several reports suggest that statins have a potentially neuroprotective effect (Cucchiara and Kasner, 2001). Thus, it is possible that statins could be used for the treatment of CNS diseases. Previously, Saheki et al. (1994) showed that the brain uptake clearance of pravastatin was quite low, almost comparable with that of sucrose using the in situ brain perfusion technique. However, taking into consideration the chronic administration of statins, it is necessary to...
investigate the efflux transport across the BBB since the steady-state brain concentration is governed by both the uptake and the efflux transport across the BBB.

Pitavastatin, one of the newly developed statins, contains a carboxylic acid group in its chemical structure like pravastatin. Although pitavastatin is more lipophilic than pravastatin (log D$_{7.4}$: 1.5 versus -0.47) (Ishigami et al., 2001), its brain-to-plasma concentration ratio has been reported to be lower than that of pravastatin (0.063 versus 0.48) (Komai et al., 1992; Kimata et al., 1998). The lower distribution of pitavastatin in the brain may be explained by the efflux transport across the BBB.

We have recently shown that rOat3 is expressed at the BBB (Kikuchi et al., 2003). rOat3 is a multispecific transporter, with substrates that include amphipathic organic anions as well as hydrophilic ones (Kusuhara et al., 1999). According to inhibition studies, it has been suggested that rOat3 is involved in the efflux of hydrophilic organic anions, but its contribution to the efflux transport of amphipathic organic anions, such as 17β-estradiol-3-17β-glucuronide, is limited (Sugiyama et al., 2001; Kikuchi et al., 2003). rOatp2, another multispecific organic anion transporter expressed at the BBB (Gao et al., 1999), has been suggested to account for the efflux of amphipathic organic anions across the BBB (Asaba et al., 2000; Hosoya et al., 2000; Sugiyama et al., 2001). Since pravastatin is a substrate of both rOat3 and rOatp2 (Tokui et al., 1999; Hasegawa et al., 2002), these transporters may be involved in the efflux of pravastatin and, possibly, of pitavastatin, from the brain across the BBB, accounting for the lower brain distribution of pitavastatin compared with that of pravastatin.

In the present study, we demonstrated that pravastatin and pitavastatin are substrates of both rOat3 and rOatp2 using cDNA-transfected cells. The efflux clearances of pravastatin and pitavastatin from the brain into the blood circulation across the BBB were calculated using the intracerebral microinjection technique (BEI method) and brain slice uptake experiments. In addition, the involvement of rOat3 and rOatp2 in the efflux processes was suggested in vivo by examining the inhibitory effect of several compounds.

### Materials and Methods

#### Chemicals.
[3H]Pravastatin (45.5 Ci/mmol) and unlabeled pravastatin sodium ([+]-3R,5R)-3,5-dihydroxy-7-[(1S,2S,6S,8S,8aR)-6-hydroxy-2-methyl-8-[(S)-2-methylbutyryloxy]-1,2,6,7,8,8a-hexahydro-1-naphthyl]heptanoate) were kindly donated by Sankyo (Tokyo, Japan), and [3H]pitavastatin (16 Ci/mmol) and unlabeled pitavastatin ([+]-monocalcium bis[(3R,5S,6E)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinoxinyl]-3,5-dihydroxy-6-heptanoate) were supplied by Kowa Company Ltd. (Tokyo, Japan). [14C]Carboxyl-inulin (2.5 mCi/g) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Unlabeled probenecid, PAH, and TCA were purchased from Sigma-Aldrich (St. Louis, MO), unlabeled digoxin was obtained from Aldrich Chemical Co. (Milwaukee, WI), and unlabeled tetraethylammonium was purchased from Wako Pure Chemicals (Osaka, Japan). Ketamine hydrochloride was purchased from Sankyo. Xylazine and ketamine hydrochloride were used as anesthetics. All other chemicals were commercially available, of reagent grade, and used without further purification.

#### Animals.
Sprague-Dawley male rats (supplied by Japan SLC, Shizuoka, Japan) weighing 220 to 250 g were used throughout this study and had free access to food and water. All experiments using animals in this study were carried out according to the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, The University of Tokyo).

#### Transport Study.
rOat3- and rOatp2-expressed LLC-PK1 cells were established and maintained as described previously (Sugiyama et al., 2001). Uptake was initiated by adding the radiolabeled ligands to the medium in the presence and absence of inhibitors after cells had been washed three times and preincubated with Kreb's-Henseleit buffer at 37°C for 15 min. The Krebs-Henseleit buffer consisted of 142 mM NaCl, 25.8 mM NaHCO$_3$, 4.93 mM KCl, 0.96 mM KH$_2$PO$_4$, 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 times by adding ice-cold Kreb's-Henseleit buffer, dissolved in 500 µl of 0.2 N NaOH and kept overnight. The radioactivity associated with the cells and medium was determined. The aliquots of cell lysate were used to determine the protein concentration by the method of Lowry (1951), with bovine serum albumin as a standard. Ligand uptake was given as the cell-to-medium concentration ratio determined as the amount of ligand associated with the cells divided by the medium concentration.

#### In Vivo Efflux Study.
The efflux of test compounds from the brain after microinjection into the cerebral cortex was investigated using the BEI method as described previously (Kakee et al., 1996). [3H]Pravastatin (15.6 nCi/rat) or [3H]pitavastatin (31.3 nCi/rat) with a nonpermeable reference compound ([14C]carboxyl-inulin (0.625 nCi/rat)) in 0.5 µl of ECF buffer (122 mM NaCl, 25 mM NaHCO$_3$, 10 mM d-glucose, 3 mM KCl, 1.4 mM CaCl$_2$, 1.2 mM MgSO$_4$, 0.4 mM K$_2$HPO$_4$, and 10 mM HEPES, pH 7.4) in the presence or the absence of different concentrations of various inhibitors was injected into the Par2 region (0.2 mm anterior and 5.5 mm lateral to the bregma, 4.5 mm in depth). After the microinjection, rats were decapitated, and the radioactivity that remained in the left and right cerebrum was determined. The 100-BEI (%), which represents the remaining percentage of the test compounds in the cerebrum, is described by eq. 1.

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100 - \text{BEI} (%) = \frac{\text{amount of test drug in the brain}}{\text{amount of reference in the brain}} \times 100 \quad (1)
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The elimination rate constant of the compounds from the brain ($k_e$) was obtained by fitting the 100-BEI (%) versus time data. A nonlinear least-squares regression program (MULTI) (Yamaoka et al., 1981) was used for the calculation.

The distribution volume of pravastatin and pitavastatin in the brain was determined by the intravitro brain slice uptake technique. Brain slices were prepared as reported previously with a minor modification (Kakee et al., 1997). A hypothalamic slice, 300-µm thick, was cut using a brain microslicer (DTK-2000; Dosaka, Kyoto, Japan), and kept in oxygenated ECF buffer equilibrated with 95% O$_2$/5% CO$_2$. After preincubation for 5 min at 37°C, the brain slice (15–25 mg) was transferred to 3 ml of oxygenated incubation medium containing [3H]pravastatin or [3H]pitavastatin (0.05 µCi/ml) and [14C]carboxyl-inulin (0.01 µCi/ml) at 37°C. At appropriate times, brain slices were collected, and the radioactivity was determined in a liquid scintillation counter. Ligand uptake was given as the amount of ligand associated with the slice divided by the medium concentration.

#### Results

#### Time Profiles of the Uptake of [3H]Pravastatin and [3H]Pitavastatin by cDNA-Transfected Cells.
The uptake of [3H]pravastatin and [3H]pitavastatin by rOat3- and rOatp2-transfected LLC-PK1 cells was significantly greater than that by vector-transfected cells (Fig. 1). The uptake of pravastatin by the cDNA-transfected cells increased linearly...
over 5 min, whereas that of pitavastatin increased over 2
min. Eadie-Hofstee plots of the specific uptake of pitavasta-
tin via rOat3 and rOatp2, obtained by subtracting the uptake
by vector-transfected cells from that by cDNA-transfected
cells, are shown in Fig. 2, A and B. Comparison of the
Akaike’s Information Criterion values (Yamaoka et al., 1981)
suggested that the specific uptake of pitavastatin by rOat3
consists of one saturable component, and the $K_m$ and $V_{\text{max}}$
values of pitavastatin for rOat3 were determined as 0.982 ±
0.176 μM and 4.76 ± 0.53 pmol/min/mg protein, respectively
(Fig. 2A). It was suggested that the specific uptake of pitavastatin
by rOatp2 consists of one saturable and one nonsat-
urable component. The $K_m$ and $V_{\text{max}}$ values of pitavastatin
for rOatp2 were 7.21 ± 0.96 μM and 80.9 ± 10.9 pmol/
min/mg protein, respectively, and the uptake clearance cor-
responding to the nonsaturable component was 1.24 ± 0.25
μl/min/mg protein (Fig. 2B).

**Time Profile of the Efflux of [3H]Pravastatin and [3H]Pitavastatin from the Brain across the BBB.** The time profiles of the efflux of pravastatin and pitavastatin from the brain after microinjection into the cerebral cortex are shown in Fig. 3. Both statins were effluxed from the brain into the systemic circulation following microinjection, and $k_{\text{el}}$ was calculated as 0.060 ± 0.002 min$^{-1}$ for pravastatin and 0.026 ± 0.004 min$^{-1}$ for pitavastatin.

**Uptake of Pravastatin and Pitavastatin by Brain Slices.** The distribution volume of pravastatin and pitavastatin in the brain, $V_d$brain, was determined in the in vitro brain slice uptake study. Figure 4, A and B, shows the time profiles of the uptake of [3H]pravastatin and [3H]pitavasta-

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**Fig. 1.** Time profiles of the uptake of [3H]pravastatin and [3H]pitavastatin by gene-transfected LLC-PK1 cells. The uptake of [3H]pravastatin (A and B) and [3H]pitavastatin (C and D) by rOat3- (A and C) and rOatp2- (B and D) transfected LLC-PK1 cells was examined at 37°C. Circles and squares represent the uptake by gene-
and vector-transfected cells, respectively. Each point represents the mean ± S.E. ($n = 3$).

**Fig. 2.** Concentration dependence of the uptake of [3H]pitavastatin by rOat3- and rOatp2-transfected LLC-PK1 cells. The uptake of [3H]pitavastatin by rOat3- (A) and rOatp2- (B) transfected LLC-PK1 cells in the presence of unlabeled pitavastatin was examined at 37°C. Specific up-
take was obtained by subtracting the uptake by vector-transfected cells from that by gene-trans-
ferred cells. The solid lines represent the fitted line obtained by nonlinear regression analysis. Each point represents the mean ± S.E. ($n = 3$).
Effect of Inhibitors on the Efflux of Statins across the BBB. The efflux of pravastatin and pitavastatin from the brain into the blood across the BBB was almost completely inhibited by 50 mM probenecid in the injectate whereas the effect of 50 mM tetraethylammonium was not significant for both statins (Fig. 6). The efflux of both statins was also inhibited by PAH, TCA, and digoxin in a concentration-dependent manner (Fig. 7).

Discussion

In the present study, the uptake of pravastatin and pitavastatin by rOat3- and rOatp2-transfected cells was determined, and the involvement of rOat3 and rOatp2 in the efflux transport across the BBB was examined.

Statins, except lovastatin and simvastatin which are administered in inactive lactone forms, are used in their active acid forms (Reinoso et al., 2002). Thus, it is possible that organic anion transporters are involved in regulating their brain concentrations. It has been shown that pravastatin is a substrate of both rOat3 and rOatp2 (Tokui et al., 1999; Hasegawa et al., 2002). Transport studies using cDNA-transfected cells demonstrated that pitavastatin is a substrate of both rOat3 and rOatp2 (Fig. 1). The transport activity of pravastatin and pitavastatin by rOat3 was comparable (Fig. 1, A and C), although the $K_m$ value of pitavastatin (0.98 $\mu$M) was more than 10-fold smaller than that of pravastatin reported previously (13 $\mu$M) (Hasegawa et al., 2002). The transport activity of pitavastatin by rOatp2 was much greater than that of pravastatin (Fig. 1, B and D), and the $K_m$ value of pitavastatin (7.2 $\mu$M) was approximately 5-fold smaller than that of pravastatin (38 $\mu$M) (Tokui et al., 1999). These results suggest the possibility that rOat3 and/or rOatp2 are involved in the efflux of statins from the brain across the BBB.

Pitavastatin was eliminated from the cerebral cortex more slowly than pravastatin after microinjection (Fig. 3). However, the intrinsic efflux clearance of pitavastatin from the brain across the BBB, calculated by multiplying the elimination rate constant by the distribution volume in the brain, was approximately 6-fold greater than that of pravastatin. The lower brain distribution of pitavastatin compared with that of pravastatin may be partly accounted for by the greater efflux clearance from the brain, although the difference in the uptake clearance from the blood circulation may
also be one of the reasons. The efflux clearance of pravastatin was more than 3-fold greater than the previously reported uptake clearance (59 versus 18 ml/min/g brain) (Saheki et al., 1994). These results led us to conclude that there is asymmetrical transport of pravastatin across the BBB. The in vivo uptake clearance of pitavastatin into the brain may be low because of its high plasma protein binding. Thus, it is possible that the transport of pitavastatin across the BBB is also asymmetrical.

The involvement of transporters in the efflux of pravastatin and pitavastatin was investigated in vivo using the BEI method. The efflux transport of statins across the BBB was determined at different substrate concentrations. The efflux of the two statins was saturable with the saturable fraction accounting for the majority of their total efflux (Fig. 5). To obtain some insight into the transporters involved, inhibition studies were carried out. The efflux of pravastatin and pitavastatin from the brain was almost completely inhibited by the simultaneous injection of probenecid, but tetraethylammonium had no effect (Fig. 6). Furthermore, PAH (Kakee et al., 1997; Kikuchi et al., 2003) or TCA and digoxin (Kitazawa et al., 1998; Sugiyama et al., 2001) have been used as selective inhibitors for the efflux transport of hydrophilic or amphipathic organic anions across the BBB, respectively. The efflux of both statins was inhibited by these inhibitors in a concentration-dependent manner (Fig. 7). PAH inhibited the efflux of pravastatin and pitavastatin, but the inhibitory effect was partial (60% and 50%, respectively) even at the concentration sufficient to saturate its own efflux (Kakee et al., 1997; Kikuchi et al., 2003). TCA and digoxin inhibited the efflux of both statins; however, their maximum inhibitory effect differed significantly between pravastatin and pitavastatin (Fig. 7). They completely inhibited the efflux of pitavastatin, whereas their effect on the efflux of pravastatin was partial, suggesting the different contribution of the transporters involved. These results suggest that the efflux of
Statins consists of PAH-, TCA- and digoxin-sensitive pathways. Since the efflux of 17βH9252estradiol-D-17βH9252glucuronide across the BBB after microinjection was completely inhibited by TCA, but partially by digoxin, the involvement of TCA-sensitive but digoxin-resistant transporters in the efflux of amphipathic organic anions has been suggested (Sugiyama et al., 2001). In the present study, TCA and digoxin inhibited the efflux of each statin to the same extent (Fig. 7). Therefore, it is likely that the TCA-sensitive but digoxin-resistant transporter(s) play a limited role in the efflux transport of statins across the BBB.

PAH has been used as an inhibitor of rOat3 (Kikuchi et al., 2003), whereas TCA has been used as an inhibitor of the amphipathic organic anion transport systems, including rOatp2, and digoxin is a specific inhibitor of rOatp2 (Sugiyama et al., 2001). The apparent Km values of the efflux of pravastatin and pitavastatin were not very different from their Km values for rOat3 and rOatp2: 13 and 38 μM for pravastatin (Tokui et al., 1999; Hasegawa et al., 2002), and 0.98 and 7.2 μM for pitavastatin (Fig. 2), respectively. The degree of inhibition of the efflux of pravastatin by PAH and TCA or digoxin was similar (Fig. 7, A–C) and accounted for the saturable fraction of the efflux transport. This result suggests the equal contribution of rOat3 and rOatp2 to the efflux transport of pravastatin across the BBB as high- and low-affinity sites, respectively. In the efflux transport of pitavastatin, the degree of inhibition by TCA or digoxin was greater than that by PAH (Fig. 7, D–F). However, the sum of the degree of inhibition by PAH and TCA or digoxin for the efflux of pitavastatin exceeded 100%. It is likely that these compounds inhibit other transporters at the BBB, including those expressed on the luminal membrane, since the net efflux across the BBB was evaluated by the BEI method. Assuming the PAH-sensitive fraction of the efflux of pravastatin represents the contribution of rOat3, the contribution of rOat3 to the efflux of pitavastatin across the BBB should be small since the transport activity of pitavastatin by rOat3 was similar to that of pravastatin (Fig. 1), and the intrinsic efflux clearance of pitavastatin was much greater than that of pravastatin. The difference between the transport activity of pravastatin and pitavastatin by rOatp2 may suggest a major contribution of rOatp2 to the efflux of pitavastatin across the BBB. Further studies are necessary to elucidate the transporters involved in the efflux of statins across the BBB.

Statins have been used for the drug treatment of hypercholesterolemia as inhibitors of HMG-CoA reductase. In addition to their lipid-lowering effects, increasing data suggest that these agents have properties that are potentially neuroprotective, i.e., endothelial protection via actions on the nitric oxide synthase system, as well as antioxidant, anti-inflammatory, and antiplatelet effects (Cucchiara and Kasner, 2001). Increasing the access of statins to the brain may improve their therapeutic effects in the CNS, although it may also increase the incidence of CNS side effects. The results of the present study indicate that increasing the lipophilicity is not necessarily followed by an improvement in the brain distribution, partly due to the difference in the efflux clearances from the brain. In vivo experiments such as in situ brain perfusion and the BEI method or in vitro ones using
BBB models, such as primary culture or immortalized cell lines and gene expression systems of the uptake and efflux transporters expressed at the BBB, will be required for the development of statins targeted to the CNS (Pardridge, 1998; Terasaki et al., 2003). Human OAT3 is expressed in the brain as shown by Northern blot analysis (Cha et al., 2001), and more recently, the expression of hOAT1 and hOAT3 at the choroid plexus, acting as a barrier between the blood and the cerebrospinal fluid, has been reported (Alebouyeh et al., 2003). Among the members of the human OATP family, hOATP-A has the highest homology to rOatp2 and is expressed at the BBB (Gao et al., 2000). It is possible that these human organic anion transporters play an important role in the efflux transport of organic anions across the barriers of the CNS. Their cDNA-transfected cells will provide screening systems for statins and other candidate drugs with anionic moieties.

In conclusion, both pravastatin and pitavastatin undergo efflux from the brain into the blood across the BBB, and at least two transporters, rOatp3 and rOatp2, are involved in the efflux processes, each making a different contribution. It is likely that one of the underlying mechanisms of the lower brain distribution of pitavastatin compared with pravastatin despite its higher lipophilicity is the difference in the efflux transport clearance, i.e., in the transport activity by rOatp2.

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References


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