ABSTRACT

Systemically administered pravastatin effectively treats diabetic retinopathy without central nervous system side effects. The efflux transport mechanism of pravastatin from the brain has already been clarified. In this study, the influx of pravastatin across the blood-retinal and blood-brain barriers (BRB and BBB) and the efflux of pravastatin from the retina were investigated using rats. Pravastatin influx (blood-to-tissues) was assessed using the retinal and brain uptake index (RUI and BUI) methods, and microdialysis was performed to investigate the efflux (retina-to-blood) transport of pravastatin. The RUI and BUI values for [3H]pravastatin were lower than those expected based on its lipophilicity, suggesting that the influx transport across the BRB and BBB was less than the reverse-direction transport. The RUI and BUI values for [3H]pravastatin were significantly decreased by probenecid, digoxin, and ouabain, indicating that pravastatin undergoes carrier-mediated influx transport in the blood-to-tissues direction across the BRB and BBB. After intravitreal injection, [3H]pravastatin and the bulk flow marker [14C]D-mannitol were found to be eliminated biexponentially from the vitreous humor. The elimination rate constant of [3H]pravastatin during the terminal phase was 1.66-fold greater than that of [14C]D-mannitol. Efflux transport was reduced in the retinal presence of pravastatin, digoxin, and benzylpenicillin, suggesting that pravastatin is transported via efflux transporters. In conclusion, pravastatin is transported across the BRB via uptake and efflux transporters in both the blood-to-retina and retina-to-blood directions, and the retina-to-blood transporters are dominant, based on the lower values of the RUI compared with the values expected from the lipophilicity.

Introduction

Although some statins have pharmacologic effects on diabetic retinopathy, as well as central nervous system (CNS) side effects, systemically administered pravastatin reduces signs of diabetic retinopathy in diabetic patients and does not produce CNS side effects such as sleep disturbances (Gordon et al., 1991; Saheki et al., 1994).

Generally, the permeability of drugs into the retina from the blood is strictly regulated by the blood-retinal barrier (BRB), which is analogous to the blood-brain barrier (BBB). The BBB consists of both retinal capillary endothelial cells (inner BRB) and retinal pigment epithelial (RPE) cells (outer BRB), which together form the complex tight junctions that restrict paracellular solute transport. The control of the intraocular environment and the maintenance of neuroretinal homeostasis are mediated, in part, by the various transporters expressed at the BRB (Hosoya and Tomi, 2005).

Pravastatin is a substrate of the organic anion transporting polypeptides 1a4 (oatp2; slco1a4) and the organic anion transporter 3 (oat3; slc22a8) (Kikuchi et al., 2004). Oatp1a4 is found on both the luminal and abluminal membranes of the inner BRB and on the apical membrane of RPE cells of rats (Akanuma et al., 2013), whereas oat3 is expressed on only the abluminal side of the rat inner BRB and is not found on RPE cells (Hosoya et al., 2009). The expression pattern of these transporters at the BBB is similar to that at the BRB; specifically, oatp1a4 is found on both the luminal and abluminal sides of the rat brain capillary endothelium, whereas oat3 is found on only the abluminal side. Previously, transporters, including oatp1a4 and oat3, have been implicated in the brain-to-blood transport of pravastatin, and the efflux of pravastatin across the BBB has been found to be more than 3-fold greater than its uptake (Kikuchi et al., 2004). The transport mechanism of pravastatin across the BBB, however, remains unclear.

The aim of the present study was to clarify the characteristics of pravastatin transport across the BRB in both the blood-to-retina and retina-to-blood directions and to obtain further evidence to support the clinical effects of pravastatin on diabetic retinopathy. Pravastatin influx (blood-to-tissues) was assessed using the retinal uptake index (RUI) and compared with that of the BBB, and microdialysis was performed to investigate efflux (retina-to-blood) transport of pravastatin. In addition, estradiol 17β glucuronide (E17βG) influx was evaluated as a model substrate of oatp1a4 and oat3 because the efflux mechanism of E17βG has already been clarified; that is, E17βG is transported from the vitreous humor to the blood via at least oatp1a4 (Katayama et al., 2006) and from the brain to blood via the oat family (mainly oat3) and oatp1a4 (Sugiya, et al., 2001).

Materials and Methods

Animals and Reagents. Male Sprague-Dawley Mdr1a knockout rats (6–7 weeks old) and wild-type Sprague-Dawley rats (6–8 weeks old) were obtained from Sage Laboratories (St. Louis, MO) and Charles River Laboratories (Yokohama, Japan). All experiments were performed according to the Ethical Guidelines for Animal Experiments of Santen Pharmaceutical.
Company (Ikoma, Japan). Pravastatin [3H(G)] sodium salt ([3H] pravastatin, 5 Ci/mmol) and n-[1-14C]butanol ([14C]n-butanol, 2 mCi/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO), [Estradiol-6,7,8,9H(N)]-estradiol 17β-D-glucuronide ([3H]E17βG, 36.6 Ci/mmol) and n-[1-14C]mannitol ([14C]n-mannitol, 55 mCi/mmol) were purchased from PerkinElmer (Waltham, MA). All other chemicals were commercially available and of reagent grade.

**Uptake Index Method and Microdialysis Study.** The RUI and brain uptake index (BUI) were calculated according to previously reported uptake index methods. A microdialysis study was performed as previously described. Each experiment was performed once. The details are provided in the Supplemental Material.

**Data Analysis.** All data, which were obtained from independent biologic samples, are expressed as the mean ± S.E. (n = 3 or 4 rats). An unpaired, two-tailed Student’s t test was used to assess the significance of the differences between the means of the two groups. The statistical significance of the differences in the means of the groups was determined using Bartlett’s test followed by Dunnett’s multiple comparison test. *P < 0.05 and **P < 0.01, significantly different from control. Each column or symbol represents the mean ± S.E. (n = 3 or 4 rats).

**Results and Discussion.**

The RUI and BUI of [3H]pravastatin and [3H]E17βG in wild-type rats were almost the same as those in Mdr1a knockout rats and were lower than the lipophilicity trend line (Fig. 1, A and B). The results indicate that neither of these compounds is recognized by P-glycoprotein and that influx transport into tissues across the BRB and BBB is less than efflux transport. The RUI and BUI of [3H]pravastatin were significantly reduced by pravastatin (40 mM), probenecid (1 mM), which has a very high affinity for oatp1a4— and digoxin (0.01 mM)—which has a very high affinity for oatp1a4, and digoxin (0.01 mM)—which has a very high affinity for oatp1a4, and progesterone, which are expected to permeate by passive diffusion.

The line represents the linear regression curve according to the linear least-squares method for the three compounds in wild-type rats. RUI = 69.4 × log (0.493 × log (0.493 × log D7.4) (r² = 0.993) (A) and BUI = 16.9 × log (0.496 × log D7.4) (r² = 0.998) (B) (Fuji et al., 2014). (C) Time-profile of [3H]pravastatin and [14C]n-mannitol in the vitreous humor after their intravitreal injection into rats. Closed squares and open squares represent the concentrations of [3H]pravastatin and [14C]n-mannitol in the dialysate, respectively. (D) Inhibitory effect on the difference in the elimination rate constants (β) between [3H]pravastatin and [14C]n-mannitol during the terminal phase. Percentage of control was calculated as follows: β value of [3H]pravastatin – β value of [14C]n-mannitol in the presence of inhibitor/ β value of [3H]pravastatin – β value of [14C]n-mannitol in the absence of inhibitor × 100. The statistical significance of the differences in the means of the groups was determined using Bartlett’s test followed by Dunnett’s multiple comparison test. *P < 0.05 and **P < 0.01, significantly different from control. Each column or symbol represents the mean ± S.E. (n = 3 or 4 rats).

### Table 1

<table>
<thead>
<tr>
<th>Inhibitor Pravastatin</th>
<th>Uptake Index (%)</th>
<th>Percentage of Control (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retina Brain</td>
<td>Retina Brain</td>
</tr>
<tr>
<td>Control</td>
<td>2.58 ± 0.52</td>
<td>0.485 ± 0.031</td>
</tr>
<tr>
<td>40 mM Pravastatin</td>
<td>1.38 ± 0.22 *</td>
<td>0.263 ± 0.024 **</td>
</tr>
<tr>
<td>0.01 mM Digoxin</td>
<td>1.20 ± 0.24 *</td>
<td>0.224 ± 0.024 **</td>
</tr>
<tr>
<td>0.3 mM E17βG</td>
<td>1.21 ± 0.16 *</td>
<td>0.361 ± 0.079</td>
</tr>
<tr>
<td>1 mM Probenecid</td>
<td>1.36 ± 0.14 *</td>
<td>0.204 ± 0.025 **</td>
</tr>
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*P < 0.05 and **P < 0.01, significantly different from the control. E17βG, estradiol 17β glucuronide.
reduced by administration of digoxin and probenecid, although the BUI values were unchanged (Supplemental Fig. 1). These results suggested that oct1A4 plays a role in the blood-to-retina transport of organic anions and that oct1A4 is involved in both BRB- and BBB-mediated pravastatin influx. Further study is needed to elucidate the reason why no change was observed in the BUI for E17βG.

To investigate the elimination of pravastatin across the BRB, a microdialysis study was performed. The concentration of [3H]pravastatin and [14C]d-mannitol, co-injected as a bulk flow marker, in the dialysate [14C]d-mannitol coadministered with digoxin (0.01 mM) and benzylpenicillin (20 mM), an inhibitor of oat3, were both significantly lower than that of the control (Fig. 1D), whereas choline had no effect. These findings suggest that transporters, including oct1A4 and oat3, were involved in the retinal clearance of pravastatin, in addition to its elimination from the vitreous humor via bulk flow and passive diffusion. Thus, the efflux mechanism of pravastatin across the BRB is similar to that across the BBB (Kikuchi et al., 2004).

Moreover, pravastatin is a substrate of multidrug resistance–associated protein 2 (ABCC2/Mrp2), ABCC4 (Mrp4), and breast cancer resistance protein (ABCG2/Bcrp) (Yamazaki et al., 1997, Hirano et al., 2005; Uchida et al., 2007). Mrp2 protein is expressed in human retina and RPE (Pelis et al., 2009), but Mrp2 cannot be detected in the mouse inner BRB (Tachikawa et al., 2008) or in rat brain (Ohitsu et al., 2007) at the mRNA level. Mrp4 is found on the luminal side of the mouse inner BRB (Tajani et al., 2009) and both sides of the BBB (Kusuhara and Sugiyama, 2005). Bcrp is localized to the luminal side of the mouse inner BRB (Asahima et al., 2006) as well as the BBB. Taken together, the ATP-binding cassette (ABC) transporters may be involved in the transport of pravastatin across the BRB and BBB. Based on our results and previous reports, asymmetric pravastatin transport across both barriers would be expected (Supplemental Fig. 3). It is important to reveal whether the ABC transporters are involved in the efflux transport of pravastatin across the BRB and BBB in future experiments.

Recently, in a comparison between diabetic and normal rats, increased expression of oct1A4 mRNA and decreased expression of Mrp2 mRNA were found in the liver, as well as a markedly altered pravastatin disposition (Hasegawa et al., 2010). Decreases in renal oat3 function and expression have also been reported in diabetic rats (Phatchawhan et al., 2014). It is likely that there are changes in BRB transporters in diabetic rats. The functional and expressional differences in transporters between normal and pathologic conditions might be exploitable when treating diabetic retinopathy.

Oat1A4 falls into the same subfamily as human OATP1A2 (Hagenbuch and Meier, 2004). OATP1A2 and OAT3 are expressed in human retina/choroid at the mRNA level (Chan et al., 2015), as well as at the BBB (Cha et al., 2001; Gao et al., 2015). In addition, both transporters recognize pravastatin (Takeda et al., 2004; Shirasaka et al., 2010). Therefore, our results support the clinical effects of pravastatin on diabetic retinopathy without the CNS side effects. Pravastatin was transported into the target tissues, retinal endothelial cells, via uptake transporters and showed pharmacologic effects, whereas pravastatin taken into brain endothelial cells was transported via efflux transporters before reaching the CNS.

In conclusion, pravastatin is transported across the BRB and BBB via uptake and efflux transporters in both directions, and the efflux transporters are dominant, based on the lower values of the RUI and BUI than those expected from the lipophility. These findings provide useful information for the development of new approaches to the design of precision pharmaceuticals for the systemic delivery of drugs to retinal vessels without CNS side effects.

Acknowledgments

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Authorship Contributions

Participated in research design: Fujii, Kawazu, Hosoya. Conducted experiments: Fujii, Setoguchi. Contributed new reagents or analytic tools: Fujii. Performed data analysis: Fujii. Wrote or contributed to the writing of the manuscript: Fujii, Kawazu, Hosoya.

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