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H.知的財産権の出願・登録状況

（予定を含む。）

1. 特許取得　　なし
2. 実用新案登録　　なし
3. その他　　なし

II. 研究成果の刊行に関する一覧表

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III. 研究成果の刊行物・別刷

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Bile Salt Export Pump (BSEP/ABCB11) Can Transport a Nonbile Acid Substrate, Pravastatin

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ABSTRACT

Pravastatin is a well known 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor. Cumulative studies have shown that pravastatin is taken up into hepatocytes by the organic anion transporting polypeptide family transporters and excreted into the bile as an intact form by multidrug resistance-associated protein 2 (MRP2). It is generally accepted that the bile salt export pump (BSEP/ABCB11) mainly transports bile acids and plays an indispensable role in their biliary excretion. Interestingly, we found that BSEP could accept pravastatin as a substrate. Significant ATP-dependent uptake of pravastatin by human BSEP (hBSEP)- and rat BSEP (rBsep)-expressing membrane vesicles was observed, and the ratio of the uptake activity of pravastatin to that of taurocholic acid (TCA) by hBSEP was 3.3-fold higher than that by rBsep. The K_m value of pravastatin for hBSEP was 124 μM. A mutual inhibition study between TCA and pravastatin revealed that they competitively interact with hBSEP. Several statins inhibited the hBSEP- and rBsep-mediated uptake of TCA; however, the specific uptake of other statins (cerivastatin, fluvastatin, and pitavastatin) by hBSEP and rBSEP was not detected. The inhibitory effects of hydrophilic statins (pravastatin and rosuvastatin) on the uptake of TCA by BSEP were relatively lower than those of lipophilic statins. These data suggest that BSEP may be partly involved in the biliary excretion of pravastatin in both rats and humans.

The liver plays an important role in the excretion of xenobiotics, including many kinds of drugs. It has been reported that several kinds of transporters are expressed on both the sinusoidal and canalicular membrane in the liver to excrete drugs efficiently into bile (Kullak-Ublick et al., 2004). ABC transporters, which are driven by ATP hydrolysis, are expressed on the canalicular membrane and are responsible for the export of endogenous and xenobiotic compounds from the intracellular compartment. For example, multidrug resistance-associated protein 2 (MRP2/ABCC2) recognizes organic anions, such as glutathione- and glucuronide-conjugates, as substrates, whereas multidrug resistance 1 (MDR1/P-glycoprotein/ABCB1) preferentially accepts neutral/cationic hydrophobic compounds, and breast cancer resistance protein (BCRP/ABCG2) can also accept several kinds of anionic compounds, such as sulfate conjugates (Kullak-Ublick et al., 2004). Bile salt export pump (BSEP/ABCB11) is also expressed in the canalicular membrane. BSEP shares a high degree of sequence homology with MDR1 and was originally called the sister of P-glycoprotein (SPGP) (Gerloff et al., 1998; Lecureur et al., 2000). However, it has been found that BSEP does not show a broad substrate specificity compared with MDR1, and mainly recognizes bile acids (Madon et al., 2000; Noe et al., 2001, 2002; Byrne et al., 2002). In addition, mutations in the BSEP gene are associated with progressive familial intrahepatic cholestasis type II, and therefore BSEP plays an essential role in the biliary excretion of bile acids (Strautnieks et al., 1998). On the other hand, only a few nonbile acid substrates (vinblastine and some fluorescent substrates) have been reported to date (Lecureur et al., 2000; Wang et al., 2003a).

Pravastatin cannot easily penetrate the cell membrane due to its hydrophilicity, but it is selectively distributed to the liver (Komai et al., 1992). Considering that pravastatin is mainly excreted into bile without extensive metabolism, several transporters are thought to be important regulators of the pharmacokinetics of pravastatin. Pravastatin is taken up into hepatocytes by organic anion transporting polypeptide

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ABBREVIATIONS: ABC, ATP-binding cassette; MRP/Mrp, multidrug resistance-associated protein; BSEP/bsep, bile salt export pump; OATP/Oatp, organic anion transporting polypeptide; EHBR, Eisai hyperbiliruminemic rat; SDR, Sprague-Dawley rat; CMV, canalicular membrane vesicle; E_1S, estrone-3-sulfate; TCA, taurocholic acid; MTX, methotrexate; hBSEP, human bile salt export pump; rBsep, rat bile salt export pump; GFP, green fluorescent protein; HEK, human embryonic kidney.

(OATP) 1B1 (OATP-C) in humans (Nakai et al., 2001) and several Oatp family transporters in rats (Yamazaki et al., 1993; Tokui et al., 1999), and it is excreted into bile in unchanged form, predominantly via Mrp2 in rats (Yamazaki et al., 1996). In particular, Yamazaki et al. (1997) reported that the biliary excretion clearance of pravastatin in Eisai hyperbilirubinemic rats (EHBR), which are Mrp2-deficient, was much lower than that in control Sprague-Dawley rats (SDR). Moreover, the ATP-dependent uptake of pravastatin in canalicular membrane vesicles (CMVs) prepared from EHBR was reduced compared with that in SDR (Yamazaki et al., 1997). From these results, it seems that Mrp2 is mainly responsible for the biliary excretion of pravastatin in rats. However, even in EHBR, the biliary excretion of pravastatin was partly maintained and the ATP-dependent uptake of pravastatin in CMVs prepared from EHBR was not abolished, suggesting that transporters other than MRP2 are also involved in the biliary excretion of pravastatin (Yamazaki et al., 1997), although they remain to be identified.

In a preliminary experiment, we found that pravastatin is a good substrate of human BSEP. In the present study, the transport properties of some statins were investigated using membrane vesicles expressing rat and human BSEP. We also observed the inhibitory effects of the statins on the uptake of TCA and clarified the relationship between the lipophilicity and inhibitory potency of the statins.

Materials and Methods

Materials. [^{3}H]Pravastatin (44.6 Ci/mmol) and [^{14}C]fluvastatin (45.7 mCi/mmol) were supplied by Sankyo Co., Ltd. (Tokyo, Japan) and Novartis (Basel, Switzerland), respectively. [^{3}H]Pitavastatin (16.0 Ci/mmol) and unlabeled 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors (atorvastatin, cerivastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, simvastatin acid, atorvastatin lactone, pitavastatin lactone, pravastatin lactone, and simvastatin) were donated by Kowa Co., Ltd. (Tokyo, Japan). [^{3}H]Estrone-3-sulfate (E_1S; 57.3 Ci/mmol) and [^{3}H]taurocholic acid (TCA; 3.50 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). [^{3}H]Methotrexate (MTX; 29.0 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). [^{14}C]Temocaprilat was prepared by hydrolysis of [^{14}C]temocapril (5 N NaOH for 5 h) (Schwab et al., 1992), which was supplied by Sankyo Co., Ltd. The radiochemical purity of [^{14}C]temocaprilat was checked by thin layer chromatography (*n*-butanol/acetic acid/distilled water; 4:1:1) and confirmed to be more than 95%. Unlabeled E_1S, TCA, and MTX were purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals were commercially available and of analytical grade.

Construction and Infection of Recombinant Adenovirus and Membrane Vesicle Preparation. The construction of recombinant adenovirus of human BSEP (hBSEP), rat BSEP (rBsep), and green fluorescent protein (GFP) has been described in detail previously (Hayashi et al., 2005). Membrane vesicles were prepared from hBSEP-, rBsep-, and GFP-transfected HEK293 cells according to the method described previously (Hayashi et al., 2005). HEK293 cells were cultured in Dulbecco's modified Eagle's medium (low glucose) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. For the preparation of the isolated membrane vesicles, HEK293 cells cultured in a 15-cm dish were infected by recombinant adenovirus of hBSEP or rBsep (25 multiplicity of infection). As a negative control, cells were infected with GFP (25 multiplicity of infection). Cells were harvested at 48 h after infection, and then the membrane vesicles were isolated from $1 \sim 2 \times 10^8$ cells using a standard method described in detail previously (Muller et al., 1994). Briefly, cells were diluted 40-fold with hypotonic buffer (1 mM Tris-HCl and 0.1 mM EDTA, pH 7.4, at 37°C) and stirred gently for 1 h on ice in the presence of 2 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, 1 μg/ml pepstatin, and 5 μg/ml aprotinin. The cell lysate was centrifuged at 100,000*g* for 30 min at 4°C, and the pellet was suspended in 10 ml of ice-cold isotonic TS buffer (10 mM Tris-HCl and 250 mM sucrose, pH 7.4) and then homogenized with a Dounce B homogenizer (glass/glass, tight pestle, 30 strokes). The crude membrane fraction was layered on top of a 38% (w/v) sucrose solution in 5 mM Tris-HEPES, pH 7.4, at 4°C, and centrifuged in a Beckman SW41 rotor at 280,000*g* for 60 min at 4°C. The turbid layer at the interface was collected, diluted to 23 ml with TS buffer, and centrifuged at 100,000*g* for 30 min at 4°C. The resulting pellet was suspended in 400 μl of TS buffer. Vesicles were formed by passing the suspension 30 times through a 27-gauge needle using a syringe. The membrane vesicles were finally frozen in liquid nitrogen and stored at −80°C until use. Protein concentrations were determined by the Lowry method, and bovine serum albumin was used as a standard.

Transport Studies with Membrane Vesicles. The transport studies were performed using a rapid filtration technique (Hirohashi et al., 1999, 2000). Briefly, 15 μl of transport medium (10 mM Tris-HCl, 250 mM sucrose, and 10 mM $MgCl_2$, pH 7.4) containing radiolabeled compounds, with or without unlabeled substrates, was preincubated at 37°C for 3 min and then rapidly mixed with 5 μl of membrane vesicle suspension (5 μg of protein). The reaction mixture contained 5 mM ATP or AMP, along with the ATP-regenerating system (10 mM creatine phosphate and 100 μg/μl creatine phosphokinase). The transport reaction was terminated by the addition of 1 ml of ice-cold stop solution containing 10 mM Tris-HCl, 250 mM sucrose, and 0.1 M NaCl, pH 7.4; the reaction mixture was filtered through a 0.45-μm HA filter (Millipore Corporation, Billerica, MA) and then washed twice with 5 ml of stop solution. Filters with trapped membrane vesicles were mixed with scintillation cocktail (Clear-sol I; Nacalai Tesque, Tokyo, Japan), and the radioactivity retained on the filter was determined in a liquid scintillation counter (LS6000SE; Beckman Coulter Inc., Fullerton, CA).

Estimation of the Inhibitory Effect of the Statins on the BSEP-Mediated Transport of Bile Acids in Humans. To estimate the maximum inhibitory effect of the statins on the BSEP-mediated transport of bile acids in humans, the maximum unbound concentration at the liver inlet ($I_{in,max,u}$) was calculated from the following equation as described previously (Ito et al., 1998).

$$I_{in,max,u} = \left(C_{max,blood} + \frac{k_a \cdot D \cdot F_a}{Q_h}\right) \times f_{u,blood} \quad (1)$$

where $C_{max,blood}$ and $f_{u,blood}$ are the reported values of the maximum blood concentration of drug after oral administration of the clinical dose (D) and the protein unbound fraction in humans. In addition, k_a is set to a theoretically maximum absorption rate constant (6 h^{-1}) to avoid the false negative prediction of a drug interaction. F_a is the estimated fraction of the dose absorbed into and through the gastrointestinal membranes and calculated by the following equation:

$$F_a = \frac{F}{1 - \frac{CL_h}{Q_h}} \quad (2)$$

where CL_h is the reported hepatic clearance of drug, F is bioavailability, and Q_h is the hepatic blood flow rate (96.6 l/h). We calculated the ratio of AUC in the presence of inhibitors to that without inhibitors (R) from the following equation, assuming that the unbound concentration in liver (I) was 20-fold higher than that in blood, because statins were taken up and accumulated into hepatocytes by active transporters:

$$R = 1 + \frac{I}{K_i} \quad (3)$$

Kinetic Analyses. Ligand uptake was expressed as an uptake volume (microliters per milligram of protein), given as the amount of radioactivity associated with the membrane vesicles (dpm per milligram of protein) divided by the substrate concentration in the incubation medium (dpm per microliter). The ATP-dependent uptake of ligands via hBSEP or rBsep was calculated by subtracting the ligand uptake in the presence of AMP from that in the presence of ATP. Kinetic parameters were obtained using the following equation:

$$v = \frac{V_{max} \times S}{K_m + S} \quad (4)$$

where v is the uptake velocity of the substrate (picomoles per minute per milligram of protein), S is the substrate concentration in the medium (micromolar), K_m is the Michaelis constant (micromolar), and V_{max} is the maximum uptake rate (picomoles per minute per milligram of protein). 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. Inhibition constants (K_i) of a series of compounds could be calculated by the following equation, if the substrate concentration was low enough compared with its K_m value:

$$CL(+I) = \frac{CL}{(1 + I/K_i)} \quad (5)$$

where CL represents the uptake clearance in the absence of inhibitor, $CL(+I)$ represents the uptake clearance in the presence of inhibitor, and I represents the concentration of inhibitor. When fitting the data to determine the K_i value, the input data were weighed as the reciprocal of the observed values.

Statistical Analysis. Statistical differences were determined using one-way analysis of variance followed by Fisher's least significant difference method. Significant differences were considered at $P < 0.05$.

Results

ATP-Dependent Uptake of [^{3}H]Taurocholic Acid and [^{3}H]Pravastatin into Membrane Vesicles. The time profiles for the uptake of [^{3}H]TCA and [^{3}H]pravastatin by membrane vesicles obtained from recombinant adenovirus-infected HEK293 cells are shown in Fig. 1. The uptake of [^{3}H]TCA into membrane vesicles from hBSEP- and rBsep-transfected HEK293 cells, but not into those from GFP-transfected control cells, was markedly stimulated by ATP (Fig. 1, A and B). Significant ATP-dependent uptake of [^{3}H]pravastatin into hBSEP- and rBsep-expressing membrane vesicles was observed compared with that into control vesicles (Figs. 1, C and D, and 2A). The relative ATP-dependent uptake clearance of pravastatin to TCA by hBSEP was 3.3-fold higher than that by rBsep.

Uptake of Other Statins into Membrane Vesicles. Uptake of other statins into BSEP-expressing membrane vesicles was determined as well (Fig. 2). We were unable to find any significant ATP-dependent uptake of [^{3}H]cerivastatin, [^{14}C]fluvastatin, and [^{3}H]pitavastatin by hBSEP- and rBsep-expressing membrane vesicles compared with that by GFP-transfected vesicles, whereas both rBsep and hBSEP significantly recognized pravastatin as a substrate (Figs. 1 and 2). [^{3}H]E_1S (0.1 μM), [^{3}H]MTX (0.1 μM), and [^{14}C]temocaplirat (10 μM), which are relatively hydrophilic drugs, were also not accepted as substrates of either hBSEP or rBsep (data not shown).

Inhibitory Effects of Pravastatin and TCA on the hBSEP-Mediated Uptake. The concentration dependence of the hBSEP-mediated ATP-dependent uptake of pravastatin and TCA is shown in Fig. 3. The K_m values of pravastatin and TCA were 124 ± 13 and 4.64 ± 0.19 μM, respectively (Table 1). To characterize the mode of inhibition of TCA and pravastatin, we also performed a mutual inhibition study with TCA and pravastatin. The K_m and V_{max} values of TCA and pravastatin in the presence and absence of unlabeled pravastatin and TCA by hBSEP-expressing membrane vesicles are shown in Table 1. Judging from the shape of the Eadie-Hofstee plots in Fig. 3, their inhibition was competitive.

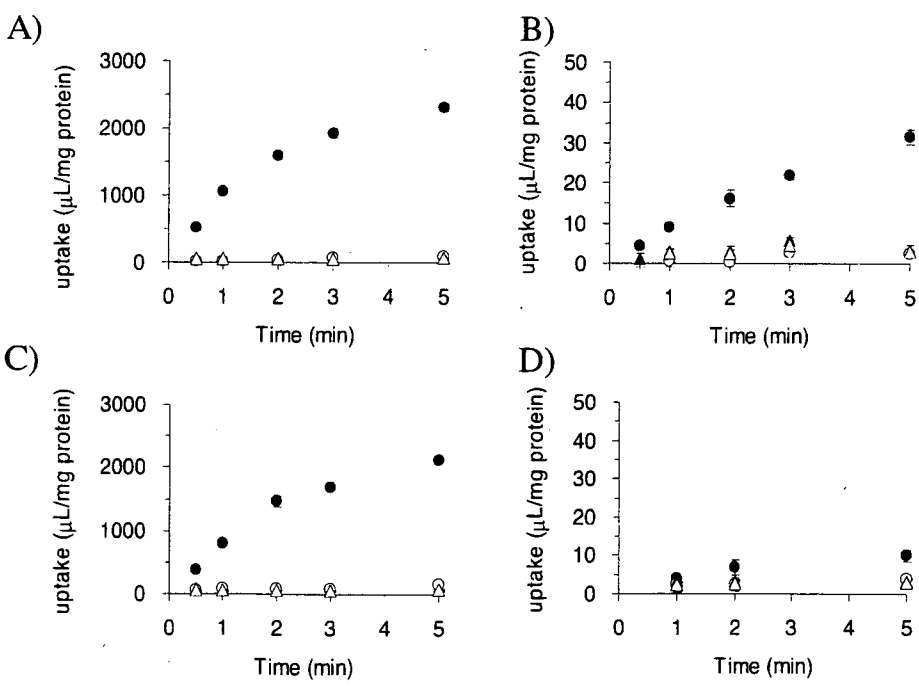
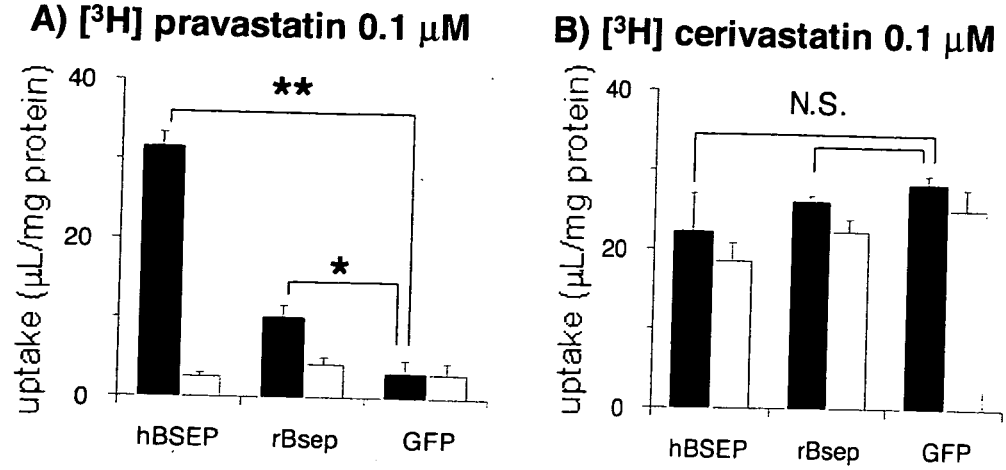


Fig. 1. Time profiles of the uptake of [^{3}H]TCA and [^{3}H]pravastatin by hBSEP and rBsep-expressing membrane vesicles. The uptake of 0.1 μM [^{3}H]TCA (A and B) and 0.1 μM [^{3}H]pravastatin (C and D) was observed at 37°C in the presence of 5 mM ATP (closed symbols) and 5 mM AMP (open symbols). The hBSEP- (A and C) and rBsep (B and D)-mediated uptake of [^{3}H]TCA or [^{3}H]pravastatin was determined. Circles and triangles represent the uptake in hBSEP- (or rBsep) and GFP-expressing membrane vesicles, respectively. Each point represents the mean ± S.E. (n = 3). Where vertical bars are not shown, the S.E. is within the limits of the symbols.



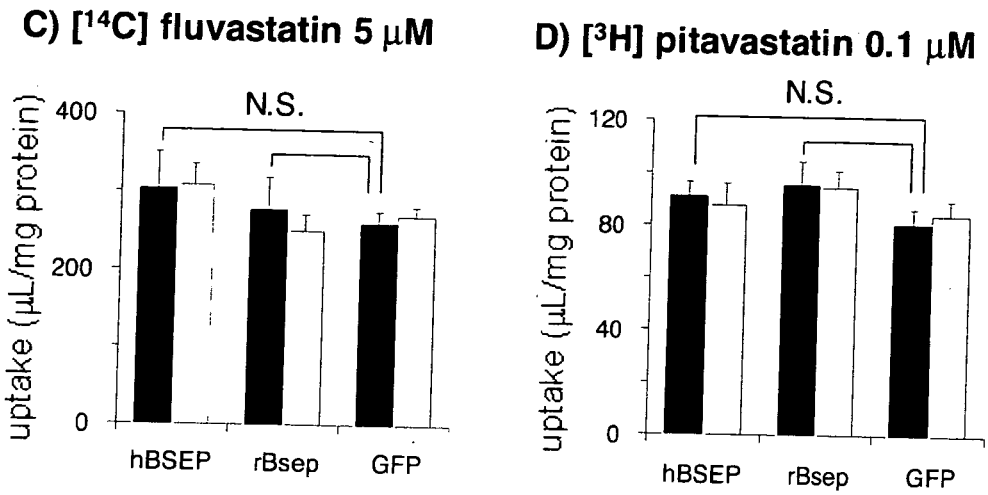


Fig. 2. Uptake of [^{3}H]pravastatin, [^{3}H]cerivastatin, [^{14}C]fluvastatin, and [^{3}H]pitavastatin by hBSEP- and rBsep-expressing membrane vesicles. The uptake of [^{3}H]pravastatin (A), [^{3}H]cerivastatin (B), [^{14}C]fluvastatin (C), and [^{3}H]pitavastatin (D) for 5 min was determined at 37°C in the presence of 5 mM ATP (closed symbols) and 5 mM AMP (open symbols). Each point represents the mean ± S.E. (n = 3). N.S., difference not significant compared with GFP-expressing control vesicles. *, $p < 0.05$; **, $p < 0.01$.

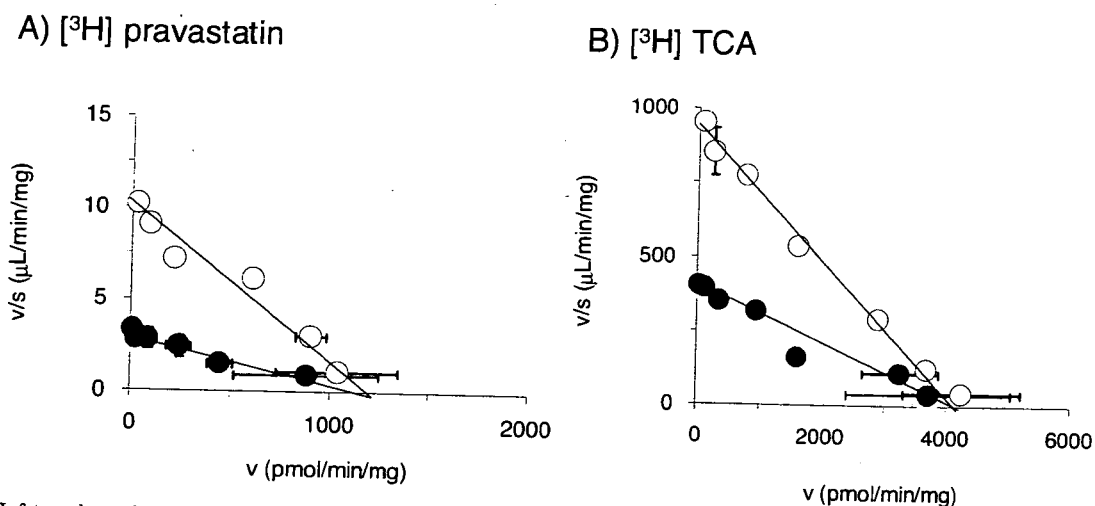


Fig. 3. Eadie-Hofstee plots of the ATP-dependent uptake of [^{3}H]pravastatin and [^{3}H]TCA in the presence and absence of unlabeled pravastatin and TCA by hBSEP-expressing membrane vesicles. The hBSEP-mediated uptake of [^{3}H]pravastatin (A) for 3 min and [^{3}H]TCA (B) for 2 min was measured in the presence (closed circles) and absence (open circles) of unlabeled TCA (3 μM) (A) and unlabeled pravastatin (250 μM) (B), respectively. Each point represents the mean ± S.E. (n = 3). The data were fitted to the Michaelis-Menten equation by nonlinear regression analysis as described under *Materials and Methods*, and each solid line represents the fitted curve.

Inhibitory Effects of the Statins on the ATP-Dependent Uptake of [^{3}H]TCA into hBSEP- and rBsep-Expressing Membrane Vesicles. We determined the inhibitory effects of the statins on the ATP-dependent uptake of [^{3}H]TCA by hBSEP- and rBsep-expressing membrane vesicles. All the statins (atorvastatin, cerivastatin, fluvastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin acid) that we tested were able to inhibit the ATP-dependent uptake of [^{3}H]TCA in a dose-dependent manner. The K_i values of the statins for hBSEP and rBsep are summarized in Table 2. On the other hand, the K_i value of TCA on the ATP-dependent uptake of pravastatin was 2.20 ± 0.52 μM (data not shown).

Comparison of K_i Values of the Statins for hBSEP with Those for rBsep. Comparing the K_i values of the

TABLE 1

K_m and V_{max} values of [^{3}H]pravastatin and [^{3}H]TCA in the presence and absence of unlabeled pravastatin and TCA by hBSEP-expressing membrane vesicles

The kinetic parameters were calculated from Fig. 3. The values are expressed as mean ± computer-calculated S.D.

Substrate	Inhibitor	K_m	V_{max}
		μM	*pmol/min/mg protein*
TCA		4.64 ± 0.19	4290 ± 120
	Pravastatin (250 μM)	9.91 ± 1.34	3880 ± 390
Pravastatin		124 ± 13	1220 ± 90
	TCA (3 μM)	366 ± 50	1110 ± 120

TABLE 2

Inhibitory effects of the statins on the ATP-dependent uptake of TCA by hBSEP- and rBsep-expressing membrane vesicles

The values are expressed as mean ± computer-calculated S.D. The log P values were estimated by Ishigami et al. (2001).

Statin	Log P	K_i Value for hBSEP	K_i Value for rBSEP
		μM	
Atorvastatin	1.5	9.12 ± 0.82	7.57 ± 1.17
Cerivastatin	2.3	11.4 ± 1.3	30.3 ± 4.1
Fluvastatin	1.8	30.2 ± 4.5	30.7 ± 3.1
Pitavastatin	1.5	25.6 ± 3.6	40.8 ± 4.8
Pravastatin	−0.47	163 ± 20	805 ± 97
Rosuvastatin	−0.30	120 ± 15	385 ± 59
Simvastatin acid	1.9	12.7 ± 1.4	30.0 ± 4.0
Atorvastatin lactone	4.2	13.7 ± 4.4	12.0 ± 5.3
Pitavastatin lactone	4.6[a]	59.9 ± 12.8	228 ± 48
Pravastatin lactone	2.4	37.6 ± 4.5	260 ± 35
Simvastatin (lactone)	4.4	23.1 ± 5.8	48.2 ± 10.4

[a] Calculated by ChemDraw version 7.0 (Chemical Drawing software; CambridgeSoft Corporation, Cambridge, MA).

statins for TCA transport by hBSEP with those by rBsep shown in Table 2, the inhibitory effect of the statins for hBSEP was slightly higher than that for rBsep. On the other hand, the rank order of the K_i values of the statins for hBSEP was almost the same as that for rBsep.

Discussion

In the present study, using membrane vesicles obtained from HEK293 cells infected with recombinant adenovirus of rat and human BSEP, we investigated the transport properties of pravastatin and other statins via BSEP in the biliary excretion process. We also examined the inhibitory effects of the statins on the uptake of TCA.

Significant ATP-dependent uptake of pravastatin was observed in both human and rat BSEP, but its relative uptake activity to TCA by hBSEP was 3.3 times higher than that by rBsep (Fig. 1). As shown in Fig. 3 and Table 1, the K_m values of pravastatin and TCA were 124 and 4.64 μM, which were almost consistent with the K_i values of pravastatin (163 μM; Table 2) and TCA (2.20 μM; data not shown), respectively. The mutual inhibition study between pravastatin and TCA was demonstrated that unlabeled TCA and pravastatin did not affect the V_{max} values in the ATP-dependent uptake of pravastatin and TCA, respectively (Fig. 3; Table 1). Judging from the shape of the Eadie-Hofstee plots, their inhibition might be competitive, suggesting that pravastatin and TCA might share the same binding site on hBSEP (Fig. 3). As shown in Fig. 2, the specific uptake of other statins, such as cerivastatin, fluvastatin and pitavastatin, was not detected in both hBSEP- and rBsep-expressing vesicles. In addition, at least, E_1S, temocaprilat and MTX are not accepted by BSEP (data not shown), implying that the substrate recognition of BSEP is relatively tightly controlled.

We previously demonstrated that the biliary excretion of pravastatin in EHBR was much lower than that in SDR and that ATP-dependent uptake of pravastatin by CMVs from EHBR was clearly reduced compared with that by CMVs from SDR. The K_m value (220 μM) of pravastatin uptake in CMVs from SDR was comparable with the K_i value (176 μM) for the uptake of 2,4-dinitrophenyl-*S*-glutathione, which is a typical substrate of Mrp2. These data suggest that Mrp2 is a major transporter in the biliary excretion of pravastatin in rats (Yamazaki et al., 1997). However, the biliary excretion as well as the ATP-dependent uptake in CMVs was slightly maintained even in EHBR, suggesting that transporters other than Mrp2 are also involved in the biliary excretion of pravastatin in rats (Adachi et al., 1996; Yamazaki et al., 1997). The K_m value of the ATP-dependent uptake of pravastatin in CMVs prepared from EHBR was 1050 μM (Adachi et al., 1996). In the present study, the ATP-dependent uptake of pravastatin into rBsep-expressing membrane vesicles was too low to determine the kinetic parameters. However, the K_i value of pravastatin for rBsep (805 μM) was comparable with the K_m value determined by CMVs from EHBR, and this does not conflict with our hypothesis that rBsep may be partly involved in the biliary excretion of pravastatin. In humans, the transcellular vectorial transport of pravastatin was clearly observed in MDCKII cells expressing OATP1B1 and MRP2 (Sasaki et al., 2002), whereas that of MDR1 and BCRP was relatively small (Matsushima et al., 2005). Although MRP2 is generally thought to be the main transporter for the biliary excretion of pravastatin, it is possible that BSEP is one of the candidates responsible for the remaining portion of its biliary excretion. Taking greater uptake activity of pravastatin by hBSEP into consideration, it is possible that the contribution of BSEP to the biliary excretion may become greater in humans than in rats. The relative contribution of BSEP to the biliary excretion of pravastatin will need to be clarified by using Bsep-knockout mice or by comparing the inhibitory effects of TCA on the biliary excretion of pravasta-

TABLE 3

Estimation of the inhibitory effect of the statins on BSEP-mediated transport of TCA in humans

The detailed method for calculation of these parameters is described under *Materials and Methods*. $C_{max,u,blood}$ ($=C_{max,blood} \times f_{u,blood}$) represents the reported values of the maximum unbound blood concentration, and $I_{in,max,u}$ represents the estimated maximum unbound concentration at the inlet to the liver. Simvastatin was administered as a lactone form. $C_{max,u,blood}$ was cited from other articles: simvastatin (Lilja et al., 1998); atorvastatin and pravastatin (Lilja et al., 1999); cerivastatin (Muck, 2000); pitavastatin (Fujino et al., 1998); and fluvastatin (Lennernas and Fager, 1997).

Statin	Atorvastatin	Cerivastatin	Fluvastatin	Pitavastatin	Pravastatin	Simvastatin
$C_{max,u,blood}$ (nM)	0.454	0.0696	4.18	1.67	58.6	0.444
$I_{in,max,u}$ (nM)	14.8	0.601	27.9	10.2	2294	104
Free concentration in liver (I) (μM)	0.295	0.0120	0.557	0.204	45.9	2.08
$1 + I/K_i$	1.03	1.00	1.02	1.01	1.28	1.16

tin between SDR and EHBR. In addition, when the expression or function of MRP2 is reduced by disease, genetic polymorphisms, and drug-drug interactions, BSEP may help excrete pravastatin into the bile, although the relative contribution of MRP2 and BSEP to the overall efflux of pravastatin remains to be determined in humans.

To investigate the affinities for BSEP among the statins, inhibitory effects of the statins were examined for the uptake of TCA by rBsep and hBSEP. The inhibition potency exhibited about a 100-fold difference among the statins. The rank order of the K_i values of the statins for hBSEP was almost the same as that for rBsep. In addition, the inhibitory effects of the acid forms of statins tended to be dependent on the lipophilicity of the drugs in both rBsep and hBSEP. The K_i values of hydrophilic acid forms of statins (pravastatin and rosuvastatin) for rBsep were larger than those for hBSEP, whereas those of lipophilic acid forms of statins were relatively similar (Table 2), which can partly explain why the relative uptake activity of pravastatin to TCA by rBsep was lower than that by hBSEP (Fig. 1). Regarding the lactone forms of statins, K_i value of the lactone forms except pravastatin for human and rat BSEP was slightly higher than that of the corresponding acid forms. Although the log P values of the lactone forms of statins are larger than those of acids forms, the K_i values of lactone forms were not different from those of lipophilic acid forms of statins, suggesting that the inhibition potency of acid and lactone forms of statins was not simply determined only by their lipophilicity.

Although only pravastatin was transported by BSEP, the K_i value of pravastatin was the highest among the statins. It is not surprising that the substrate for a transporter showed higher K_i value compared with nonsubstrate. For MRP2, the K_i values of vincristine and etoposide (802 and 756 μM, respectively) for the uptake of vinblastine were much higher than that of cyclosporine A (8.11 μM) (Tang et al., 2002). On the other hand, MRP2 could accept vincristine and etoposide (Chen et al., 1999; Guo et al., 2002), whereas there was no evidence that cyclosporine A was a substrate of MRP2.

In terms of inhibition of BSEP, a previous report has been indicated that many lipophilic drugs are inhibitors of BSEP such as tamoxifen, valinomycin, reserpine, rifamycin SV, cyclosporine A, troglitazone, and paclitaxel (Wang et al., 2003). Cyclosporine A and troglitazone, which are among the most potent known BSEP inhibitors, cause drug-induced cholestasis not only in rats but also in humans (Cadranel et al., 1992; Gitlin et al., 1998; Funk et al., 2001). It has been also reported that several kinds of the statins induce cholestasis in clinical situations (Ballare et al., 1991; Jimenez-Alonso et al., 1999; Batey and Harvey, 2002). So, to determine whether the inhibition of BSEP-mediated transport of bile acids by statins is clinically relevant, we calculated the maximum unbound concentration at the inlet to the liver using a method established in our laboratory to avoid the false negative prediction of drug-drug interactions (Ito et al., 1998). As shown in Table 3, even if the unbound concentration of the statins in the liver is 20-fold higher than that in plasma because uptake transporters can concentrate the statins efficiently into hepatocytes, the unbound concentration in liver is much lower than the K_i values for BSEP-mediated transport estimated in the present study. Therefore, although statins can inhibit BSEP-mediated transport in in vitro experiments, inhibition of BSEP-mediated transport by statins should not have a significant impact on drug interactions and statin-induced cholestasis in clinical situations.

So far, it has been reported that vinblastine and some fluorescent compounds (calcein-AM, bodipy, and dihydrofluoroscein) are accepted as substrates except for bile acids (Lecureur et al., 2000; Wang et al., 2003). In the present study, we also found that BSEP recognizes the nonbile acid pravastatin as a substrate and might be involved in the biliary excretion of pravastatin together with MRP2.

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Involvement of BCRP (ABCG2) in the Biliary Excretion of Pitavastatin

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ABSTRACT

Pitavastatin, a novel potent 3-hydroxymethylglutaryl coenzyme A reductase inhibitor, is distributed selectively to the liver and excreted into bile in unchanged form in rats. We reported previously that the hepatic uptake is mainly mediated by organic anion transporting polypeptide (OATP) 1B1, whereas the biliary excretion mechanism remains to be clarified. In the present study, we investigated the role of breast cancer resistance protein (BCRP) in the biliary excretion of pitavastatin. The ATP-dependent uptake of pitavastatin by human and mouse BCRP-expressing membrane vesicles was significantly higher compared with that by control vesicles with K_m values of 5.73 and 4.77 μM, respectively. The biliary excretion clearance of pitavastatin in Bcrp1(−/−) mice was decreased to one-tenth of that in control mice. The biliary excretion of pitavastatin was unchanged between control and Eisai hyperbilirubinemic rats, indicating a minor contribution of multidrug resistance-associated protein (Mrp) 2. This observation differs radically from that for a more hydrophilic statin, pravastatin, of which biliary excretion is largely mediated by Mrp2. These data suggest that the biliary clearance of pitavastatin can be largely accounted for by BCRP in mice. In the case of humans, transcellular transport of pitavastatin was determined in the Madin-Darby canine kidney II cells expressing OATP1B1 and human canalicular efflux transporters. A significant basal-to-apical transport of pitavastatin was observed in OATP1B1/MDR1 and OATP1B1/MRP2 double transfectants as well as OATP1B1/BCRP double transfectants, implying the involvement of multiple transporters in the biliary excretion of pitavastatin in humans. This is in contrast to a previous belief that the biliary excretion of statins is mediated mainly by MRP2.

The liver plays an important role in the excretion of xenobiotics, including many kinds of drugs. A number of reports have shown that several kinds of transporters are expressed on the canalicular membrane in the liver for the efficient elimination of drugs via the bile (Chandra and Brouwer, 2004). It has been generally accepted that transport of various organic anions across the canalicular membrane is mainly mediated by multidrug resistance-associated protein 2 (MRP2/ABCC2), whereas the bile salt export pump (BSEP/ABCB11) exclusively accepts bile acids, and multidrug resistance protein (P-glycoprotein, MDR1/ABCB1) can transport relatively hydrophobic neutral or cationic compounds (Chandra and Brouwer, 2004). However, several reports have shown that some anionic drugs can also be recognized by BSEP and MDR1 (Cvetkovic et al., 1999; Hirano et al., 2005), suggesting that multiple transport mechanisms are involved in the biliary excretion of organic anions.

Moreover, breast cancer resistance protein (BCRP/ABCG2) has been cloned recently and can accept various kinds of organic anions, especially sulfated conjugates of steroids and xenobiotics (Allikmets et al., 1998; Suzuki et al., 2003). Because BCRP is expressed on the bile canalicular membrane of hepatocytes as well as the brush-border membrane of enterocytes, trophoblast cells in placenta, and the apical membrane of lactiferous ducts in the mammary gland (Maliepaard et al., 2001), BCRP must also be considered as one of the routes for the biliary excretion of organic anions. Current evidence indicates that BCRP contributes to the membrane transport of some substrates, such as intestinal absorption and transfer to breast milk (Jonker et al., 2000, 2002; Adachi et al., 2004; Mizuno et al., 2004; Kondo et al., 2005; Merino et al.,

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ABBREVIATIONS: MRP (Mrp), multidrug resistance-associated protein; OATP (Oatp), organic anion transporting polypeptide; BCRP (Bcrp), breast cancer resistance protein; BSEP (Bsep), bile salt export pump; MDR (Mdr), multidrug resistance protein; HEK, human embryonic kidney; MDCK, Madin-Darby canine kidney; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; $E_2$17βG, 17β-estradiol-17β-D-glucuronide; EHBR, Eisai hyperbilirubinemic rat; LC/MS, high-performance liquid chromatography/mass spectrometry; GFP, green fluorescent protein; SNP, single nucleotide polymorphism; TS, Tris-HCl and sucrose; PE, polyethylene.