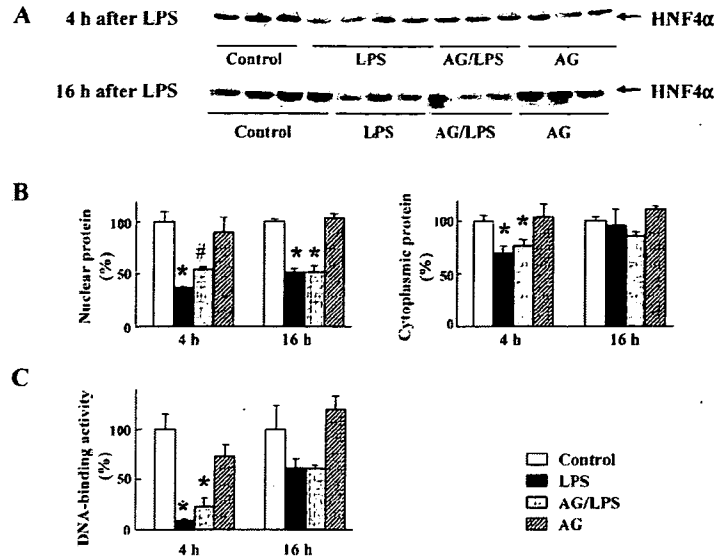

Fig. 7. Effect of AG on LPS-Induced Decrease in HNF4α Protein Levels and HNF4α DNA-Binding Activities

Rats were treated with saline or AG (400 mg/kg) 0.5 h before saline or LPS (1 mg/kg). Western blot analysis was performed with nuclear and cytoplasmic protein of liver isolated 4 h or 16 h after LPS. (A) Representative immunoblots of nuclear protein. (B) Relative nuclear and cytosolic HNF4α protein levels. The protein data from nuclear and cytoplasmic fractions after densitometric analysis are expressed as a percentage of the controls. (C) Relative HNF4α DNA-binding activity. DNA-binding activities measured with TransAM™ are expressed as a percentage of the controls. Each bar represents mean±S.E.M. (n=3—4). *p<0.05 compared with control groups. #p<0.05 compared with LPS groups.

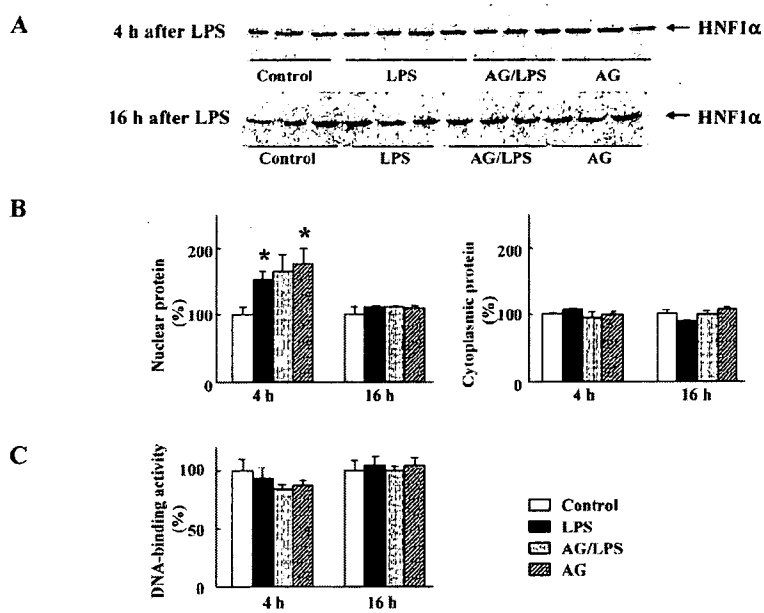

Fig. 8. Effect of AG on LPS-Induced Changes in HNF1α Protein Levels and HNF1 DNA-Binding Activities

Rats were treated with saline or AG (400 mg/kg) 0.5 h before saline or LPS (1 mg/kg). Western blot analysis was performed with nuclear and cytoplasmic protein of liver isolated 4 h and 16 h after LPS. (A) Representative immunoblots of nuclear protein. (B) Relative nuclear and cytosolic HNF1α protein levels. The protein data from nuclear and cytoplasmic fractions after densitometric analysis are expressed as a percentage of the controls. (C) Relative HNF1 DNA-binding activity. DNA-binding activities measured with TransAM™ are expressed as a percentage of the controls. HNF1 DNA-binding activities are expressed as a percentage of the controls. Each bar represents mean±S.E.M. (n=3—4). *p<0.05 compared with LPS groups.

tion. Antioxidant drugs such as *N*-acetylcysteine, α-tocopherol and selenium are known to attenuate LPS-induced NF-κB activation and thereby reduce mRNA levels for TNF-α, IL-1β and iNOS.[37—39] Further, *N*-acetylcysteine attenuates IL-1β release by inhibiting LPS-induced ICE activity.[36]

Protein kinase inhibitors also inhibit LPS-induced NF-κB-dependent gene transcription. Of three major MAPK subfamilies (p38MAPK, extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun-N-terminal kinase (JNK)), p38MAPK and ERK1/2 are thought to play crucial roles in LPS-induced cytokine production. p38MAPK and ERK1/2 inhibitors decrease the transcription of IL-1β, IL-6 and iNOS[40] and release of TNF-α and IL-1β.[41] AG has been reported to exert antioxidant activity by preventing ROS formation *in vivo*[42] and by directly scavenging H_2O_2, hydroxyl radical and peroxynitrite (a ROS formed from NO and superoxide) *in vitro*.[43]

Taken together, these findings indicate that the suppressive effects of AG on LPS-induced NF-κB activation result from several of its antioxidant properties, namely ROS quenching, inhibition of regulatory pathways distal to NF-κB activation such as p38MAPK and ERK1/2, or both.

Here, LPS induced a number of changes in mRNA expression of transporters in hepatocytes, namely the down-regulation of Ntcp, Oatp1, Oatp2, Oatp4, Oct1, Mrp2 and Mdr1a and up-regulation of Mdr1b. In addition to these changes, Cherrington *et al.*[2] reported that LPS also produced a significant decrease in mRNA levels for Oat3 and Bsep and an increase for Mrp3. These differences may have resulted from their use of a four-fold-higher dose of LPS than the 1 mg/kg used here.

Of interst, AG attenuated the up-regulated and constitutive Mdr1b transcription. Mdr1b is mainly regulated through NF-κB signaling (Table 5). The increase in Mdr1b mRNA expression by ROS generated in response to various stimuli, including LPS as well as extracellulary applied H_2O_2, is attenuated by antioxidant drugs.[44] Since cytokines produced in KCs stimulate hepatocytes, thereby leading to NF-κB activation, this finding indicates that AG reduces the transcription of NF-κB-dependent genes in hepatocytes *via* a decrease in LPS-induced KCs activation.

Transcription factors (RXRα, HNF1α, HNF4α) are known to be involved in the down-regulation of Ntcp, Oatp1, Oatp2, Oatp4, Mrp2 and Mdr1a transcription in response to LPS (Table 5). In the present study, LPS resulted in a decrease in nuclear RXRα protein levels with an associated decrease in mRNA levels, but also in a decrease in HNF4α DNA-binding activities and nuclear protein levels without any change in mRNA levels. These results are consistent with those of Beigneux *et al.*[45] and Wang *et al.*[46] However, our finding that HNF1α DNA-binding activities were not reduced by LPS is inconsistent with previous reports.[46—49] Despite the presence of an HNF4α binding site on the HNF1α promoter, the decrease in HNF4α DNA-binding activities did not decrease HNF1α mRNA levels or protein levels. The rapid decrease in DNA-binding activities of HNF1α and HNF4α within 1 h after LPS[48] may suggest that HNF1α DNA-binding activities are independent of HNF4α. However, further experiments are needed to examine whether LPS reduces HNF1α DNA-binding activities at an earlier time than the 4 h in our rat model, and also to clarify the meaning of the increase in HNF1α and HNF4α mRNA expression by AG alone.

RXRα and HNF4α protein are reported to be degraded *via* the ubiquitin/proteasome pathway.[46,50] In the case of RXRα, a decrease in mRNA also participates in the decrease in RXRα protein levels but, given the lack of the difference in reduced RXRα protein levels at 16 h between the LPS and AG/LPS groups in spite of the attenuation of LPS-reduced RXRα mRNA levels by AG, presumably to a lesser extent than the protein degradation. IL-1β, produced in KCs in response to LPS, is reported to be the main regulator of RXRα and HNF4α. In IL-1β-induced cell signaling, the JNK pathway produces a decrease in nuclear RXRα levels by inducing the export of phosphorylated RXRα from the nucleus and the degradation of RXRα protein in cytosol,[51] and a decrease in RXRα DNA-binding activity.[52] IL-1β also reduces HNF4α DNA-binding activity by decreasing nuclear protein levels[46]; and HNF4α phosphorylated by various protein kinases, including JNK, loses its DNA-binding activity.[53] Our results indicate that HNF4α DNA-binding activities were decreased to a greater degree than would be expected from the decreased nuclear protein levels at 4 h after LPS as reported by Cheng *et al.*[48] The transcriptional activity of RXRα and HNF4α is presumed to be regulated by IL-1β-induced cell signaling pathways, which lead to a decrease in nuclear protein levels *via* the proteasome pathway and to a change in phosphorylation states.

Hepatocytes are known to be stimulated by mediators from KCs activated by LPS. AG inhibited IL-1β release from KCs, but had little effect on the prolonged LPS-induced decrease in RXRα and HNF4α transcriptional activity. We wondered why the transcriptional activities of HNF4α, mainly in hepatocytes,[46] and RXRα, in both hepatocytes and nonparenchymal cells,[54] were down-regulated in the AG/LPS and LPS groups to almost the same extent. As a possible answer, it was recently demonstrated that hepatocytes express the components needed to respond to LPS, such as TLR4 and CD14, and can therefore respond to LPS by activating NF-κB and MAPKs in the absence of cytokines from KCs.[55] Our results also suggested that hepatocytes were stimulated directly by LPS, which lead to the activation of NF-κB and the reduction of RXRα and HNF4α transcriptional activities as rapid acute-phase responses. These rapid responses are thought to be mediated by cell signaling pathways common to LPS and IL-1β, but independent of IL-1β and NO released from KCs by LPS, the production of which requires time. This suggestion is supported by the results of Geier *et al.*, who found that even complete IL-1β inactivation cannot attenuate an LPS-induced decrease in RXRα protein levels.[49]

Our findings show that LPS stimulates hepatocytes directly to reduce either or both RXRα- and HNF4α-dependent gene transcription, such as Ntcp, Oatp1, Oatp2, Oatp4, Mrp2 and Mdr1a, and to induce NF-κB-dependent gene transcription such as Mdr1b, as early acute responses. As a longer-term response, cytokines and NO produced in KCs *via* LPS-induced activation of NF-κB enhanced the changes in Ntcp, Oatp1, Oatp4 and Mdr1b transcription in hepatocytes. Therefore, AG suppressed LPS-induced decreases in Ntcp, Oatp1 and Oatp4 transcription and increase in Mdr1b transcription by decreasing NF-κB activation in both hepatocytes and KCs, as well as cytokines and NO release from NF-κB-activated KCs.

In conclusion, NF-κB, cytokines and NO produced *via* NF-κB activation play a role in the regulation of Ntcp, Oatp1, Oatp4 and Mdr1b transcription. In contrast, RXRα and HNF4α, which participate in the down-regulation of transporters, are independent of cytokines and NO. It was impossible to discriminate the roles of cytokines and NO in this *in vivo* study using AG. The possible role of NO in the regulation of liver transporters deserves further *in vitro* investigation.

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Establishment of a Set of Double Transfectants Coexpressing Organic Anion Transporting Polypeptide 1B3 and Hepatic Efflux Transporters for the Characterization of the Hepatobiliary Transport of Telmisartan Acylglucuronide

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ABSTRACT:

In the hepatic uptake of organic anions, organic anion transporting polypeptide (OATP) 1B1 is believed to be mainly involved. We have constructed a set of double-transfected cells coexpressing OATP1B1 and hepatic efflux transporters and characterized the transcellular transport of several anions. Recent reports have also suggested the importance of OATP1B3 in the hepatic uptake of some compounds. However, there is little information about OATP1B3-selective substrate and no good tool for the evaluation of efflux transporters of OATP1B3 substrates. In the present study, we found an OATP1B3-selective substrate and established a novel set of double transfectants expressing OATP1B3. Telmisartan acylglucuronide (tel-glu) is a main metabolite of telmisartan, an angiotensin II receptor antagonist. Tel-glu is recognized by hepatobiliary transport systems and efficiently distributed to liver. Several studies using rat and human hepatocytes and transporter-expressing cells revealed that OATP1B3 was responsible for the hepatic uptake of tel-glu in humans. By using double transfectants expressing OATP1B3, we investigated the transcellular transport of tel-glu as well as estradiol 17β-D-glucuronide ($E_2$17βG) and cholecystokinin octapeptide (CCK-8) to identify the responsible efflux transporters in their biliary excretion. Vectorial basal-to-apical transport of tel-glu was observed in all kinds of double transfectants expressing OATP1B3. In contrast, basal-to-apical transport of $E_2$17βG and CCK-8 was seen only in the OATP1B3/MRP2 double transfectant compared with OATP1B3-expressing cells. Therefore, the newly established set of double transfectants expressing OATP1B3 combined with OATP1B1-expressing double transfectants can be used as a powerful tool for the rapid identification of hepatic uptake and efflux transporters of organic anions.

The hepatobiliary transport of xenobiotics is coordinated by uptake and efflux processes. Accumulating evidence has shown that OATP1B1 (Abe et al., 1999; Hsiang et al., 1999; König et al., 2000a) and OATP1B3 (König et al., 2000b) are responsible for the hepatocellular uptake of organic anions and that several kinds of ATP-binding cassette (ABC) transporters such as multidrug resistance 1 (MDR1), multidrug resistance-associated protein 2 (MRP2), and breast cancer resistance protein (BCRP) play an important role in the biliary excretion of many compounds including clinically important drugs.

The hepatic export of organic anions is generally believed to be mediated predominantly by MRP2 (Evers et al., 1998; Cui et al., 1999; König et al., 1999). However, recent reports suggested that some anionic compounds such as morphine 6-glucuronide (Huwyler et al., 1996) and fexofenadine (Cvetkovic et al., 1999) could be transported by MDR1. Hirano et al. (2005) showed that biliary excretion of pitavastatin is accounted for largely by BCRP in mice. Matsushima et al. (2005) established a set of double-transfected MDCKII cells coexpressing OATP1B1 and MRP2, MDR1, or BCRP and demonstrated that not only MRP2 but also BCRP and MDR1 are involved in the biliary excretion of several organic anions.

In the uptake process of anions, the broad substrate specificity of OATP1B3 commonly overlaps that of OATP1B1, so several compounds can be bisubstrates of both OATP1B1 and OATP1B3 (Vavricka

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ABBREVIATIONS: OATP/Oatp, organic anion transporting polypeptide; ABC, ATP-binding cassette; MDR1, multidrug resistance 1; MRP2, multidrug resistance-associated protein 2; BCRP, breast cancer resistance protein; CCK-8, cholecystokinin octapeptide; tel-glu, telmisartan acylglucuronide; $E_2$17βG, estradiol 17β-D-glucuronide; E-sul, estrone-3-sulfate; TCA, taurocholate; TEA, tetraethylammonium; MDCKII, Madin-Darby canine kidney strain II; HPLC, high-performance liquid chromatography; HSA, human serum albumin; PBS, phosphate-buffered saline.

et al., 2002; Hirano et al., 2004). It has recently been reported that some anions such as cholecystokinin octapeptide (CCK-8), telmisartan, and fexofenadine are taken up into hepatocytes mainly by OATP1B3 rather than OATP1B1 (Ismair et al., 2001; Shimizu et al., 2005; Ishiguro et al., 2006). Thus, OATP1B3 and OATP1B1 could be important transporters for the hepatocellular uptake of anionic compounds, and experimental methods to distinguish their contributions have been established (Hirano et al., 2004; Ishiguro et al., 2006).

For the characterization of efflux transport, there are several methods such as transcellular transport study using single ABC transporter-expressing cells and double transfectants in which uptake and efflux transporters are expressed and uptake study into membrane vesicles prepared from transporter-expressing cells. Using single ABC transporter-expressing cells, we can evaluate the transcellular transport of lipophilic compounds with high membrane permeability but not that of hydrophilic compounds including several organic anions such as pravastatin because of their limited access to efflux transporters from intracellular compartments (Sasaki et al., 2002, 2004). For the low membrane-permeable compounds, a membrane vesicle study may be a useful alternative. However, this experiment cannot be applied to compounds that easily adsorb to the membrane filter because it is difficult to distinguish between transporter-mediated uptake into membrane vesicles and nonspecific adsorption. In the transcellular transport assay using double transfectants, we measure the drug concentration in the buffer, so the effect of nonspecific adsorption to the cells and labware is basically negligible in the quantification of ligand concentration. We can also detect the efflux more sensitively because of the intracellular accumulation of ligands by a basolateral uptake transporter. To date, several kinds of double transfectants have been constructed (Cui et al., 2001; Sasaki et al., 2002, 2004; Letschert et al., 2004, 2005; Kopplow et al., 2005; Matsushima et al., 2005). However, there was no good tool for the identification of transporters involved in the biliary excretion of OATP1B3-specific substrates. Thus, this study was performed to demonstrate the usefulness of double transfectants expressing OATP1B3 for the investigation of the transport of tel-glu.

After oral administration of telmisartan, a part of the oral dose is conjugated with glucuronate in intestine and accumulated selectively in liver. Telmisartan is extensively glucuronidated in liver and excreted into bile as tel-glu. (Wienen et al., 2000; Stangier et al., 2000a,b). Thus, the identification of transporters to the hepatobiliary transport of telmisartan and tel-glu is important for predicting the pharmacokinetics and subsequent pharmacological effect of telmisartan. We have reported that telmisartan is taken up into liver mainly via OATP1B3, and the efflux of tel-glu into bile in rats is mediated by MRP2 and another unknown transporter(s) (Nishino et al., 2000; Ishiguro et al., 2006).

In the present study, we show that tel-glu is recognized mainly by OATP1B3 rather than by OATP1B1 using transporter expression systems and human hepatocytes. We also establish a novel set of double transfectants coexpressing OATP1B3 and MDR1, MRP2, or BCRP and characterize the transcellular transport of estradiol 17β-D-glucuronide ($E_2$17βG), CCK-8, and tel-glu, which are substrates of OATP1B3, to identify the efflux transporters responsible for their biliary excretion.

Materials and Methods

Chemicals. [^{3}H]Telmisartan (762 GBq/mmol, radiochemical purity >98%), 4′-[[4-methyl-6-(1-methyl-2-benzimidazolyl)-2-propyl-1-benzimidazolyl]methyl]-2-biphenyl carboxylic acid, and unlabeled telmisartan were synthesized by Boehringer Ingelheim Pharma KG (Biberach, Germany). Unlabeled telmisartan 1-*O*-acylglucuronide (purity >98%) was isolated from rat bile after i.v. dosing of telmisartan by Charles River (Tranent, Scotland, UK). [^{3}H]$E_2$17βG and [^{3}H]estrone-3-sulfate (E-sul), and [^{3}H]taurocholate (TCA) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA), and [^{3}H]CCK-8 was purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Unlabeled $E_2$17βG, E-sul, TCA, CCK-8, tetraethylammonium (TEA), and digoxin were purchased from Sigma-Aldrich (St. Louis, MO). Parental MDCKII cells and MDCKII cells expressing human MRP2 (Evers et al., 1998) and MDR1 were kindly provided by Dr. Piet Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). All other chemicals and reagents were commercial products of reagent grade.

Cell Culture of Transporter-Expressing HEK293 Cells. OATP1B1-, OATP1B3-, and OATP2B1-expressing or vector-transfected HEK293 cells were established previously (Hirano et al., 2004; Shimizu et al., 2005). HEK293 cells were grown in Dulbecco's modified Eagle's medium (low glucose) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen) and 1% antibiotic-antimycotic solution (Invitrogen) at 37°C with 5% CO_2 and 95% humidity. Cells were then seeded in 12-well plates at a density of 1.5×10^5 cells/well. For the transport study, the cell culture medium was replaced with culture medium supplemented with 5 mM sodium butyrate for 24 h before the transport assay to induce the expression of OATP1B1, 1B3, and 2B1.

Transport Study Using Transporter-Expressing HEK293 Cells. The transport study was carried out as described previously (Hirano et al., 2004). Uptake was initiated by adding Krebs-Henseleit buffer containing radiolabeled and unlabeled compounds after cells had been washed twice and preincubated with Krebs-Henseleit buffer at 37°C for 15 min. The uptake was terminated at a designated time by adding ice-cold Krebs-Henseleit buffer after removal of the incubation buffer. Then cells were washed twice with 1 ml of ice-cold Krebs-Henseleit buffer, solubilized in 1 N NaOH, and kept for 1 h at 37°C. During this incubation period, tel-glu was completely converted to telmisartan. Aliquots were transferred to scintillation vials or sample tubes after adding ½ volume of 2 N HCl. The radioactivity associated with the cells and incubation buffer was measured by a liquid scintillation counter (Tri-Carb 2500TR, PerkinElmer Life and Analytical Sciences) after transfer to 2-ml scintillation vials. The aliquots for tel-glu were stored at −20°C until HPLC analysis. The remaining 50 μl of cell lysate was used to determine the protein concentration by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Preparation of Rat and Human Hepatocytes before Transport Assay. Isolated rat hepatocytes were prepared from Sprague-Dawley rats weighing 200 to 300 g by the collagenase perfusion method described previously (Yamazaki et al., 1993). Isolated hepatocytes (viability >80%) were suspended in Krebs-Henseleit buffer, adjusted to 2.0×10^6 cells/ml, and stored on ice before the uptake experiment. Cryopreserved human hepatocytes (lots HH-OCF, HH-094, and HH-TDH) were purchased from Celsis In Vitro Technologies (Baltimore, MD). The hepatocytes were treated as described previously (Shitara et al., 2003). The cryopreserved human hepatocytes were resuspended in Krebs-Henseleit buffer to give a final cell density of 1.0×10^6 viable cells/ml for the uptake study. The number of viable cells was determined by trypan blue staining. To measure the uptake in the absence of Na^+, sodium chloride and sodium bicarbonate in Krebs-Henseleit buffer were replaced with choline chloride and choline bicarbonate, respectively.

Transport Study Using Isolated Hepatocytes. Before the uptake studies, cell suspensions were prewarmed in an incubator at 37°C for 3 min. The uptake studies were initiated by adding an equal volume of buffer containing radiolabeled and unlabeled compounds to the cell suspension. After incubation at 37°C for 0.5, 2, or 5 min, the reaction was terminated by separating the cells from the substrate solution. For this purpose, an aliquot of 80 μl of incubation mixture was collected and placed in a centrifuge tube (450 μl) containing 50 μl of 2 N NaOH under a layer of 100 μl of oil (density of 1.015, a mixture of silicone oil and mineral oil; Sigma-Aldrich), and subsequently the sample tube was centrifuged for 15 s in a centrifuge (15,000 rpm, MX-100; Tomy Seiko, Tokyo, Japan). During this process, hepatocytes passed through the oil layer into the alkaline solution. After an overnight incubation in alkali to dissolve the hepatocytes and to allow the conversion of tel-glu into telmisartan, the centrifuge tube was cut, and each compartment was transferred to a scintillation vial or experimental tube. The compartment containing the dissolved cells was neutralized with 50 μl of 2 N HCl. The aliquots for the radioactivity determination were mixed with scintillation cocktail, and the radioactivity was

measured by a liquid scintillation counter. The aliquots for tel-glu were stored at −20°C until HPLC analysis.

Estimation of Protein Unbound Concentration of Tel-Glu in the Presence of Human Serum Albumin. The protein unbound concentration of tel-glu in the presence of human serum albumin (0, 0.1, 0.3, 1, 3, and 5%) was determined after a 2-h incubation at 37°C by equilibrium dialysis (DIANORM; Dainippon Pharmaceutical Co. Ltd., Osaka, Japan).

Determination of Tel-Glu Concentration by HPLC. Telmisartan and tel-glu concentrations were determined by HPLC. A Waters alliance HPLC system combined with a fluorescence detector (Waters 474; Waters, Milford, MA) was used. After adding ¼ volume of acetonitrile, 10-μl aliquots were separated on an XTerra RP18 column (3.5 μm, 4.6 × 150 mm; Waters) using a mobile phase (acetonitrile-water-pyridine, 234:800:0.16) at a flow rate of 1.0 ml/min. The analyte peaks (telmisartan 9 min and tel-glu 15 min) were detected by fluorescence (excitation 300 nm and emission 385 nm). The stability of telmisartan and tel-glu in the Krebs-Henseleit buffer supplemented with 0.3% HSA was confirmed up to 1 h of incubation by this HPLC analysis.

Construction of Stably Transporter-Transfected MDCKII Cell Lines. MDCKII cells expressing MDR1 and MRP2 and parental MDCKII cells were kindly provided by Dr. Piet Borst (The Netherlands Cancer Institute) (Evers et al., 1998; Tang et al., 2002). MDCKII cells were cultured at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (low glucose) supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic (Invitrogen). For construction of the BCRP-expressing MDCKII cells, parental MDCKII cells were transfected with the expression vector pcDNA3.1(+) containing human BCRP cDNA (Kondo et al., 2004) by using FuGENE6 reagent (Roche Diagnostics, Indianapolis, IN). At 50% confluence, cells on six-well plates were exposed to serum-free Opti-MEM I (Invitrogen) containing plasmid and FuGENE6 according to the manufacturer's instructions. At 6 h after the initiation of transfection, the plasmid-FuGENE6 solution was replaced with the normal culture medium. The transfected MDCKII cells were selected with neomycin (500 μg/ml; Invitrogen). Expression of BCRP was screened by the detection of BCRP mRNA in cells of each clone. For construction of double-transfected MDCKII cells, MRP2-, BCRP-, and MDR1-expressing MDCKII cells were transfected with the expression vector pcDNA3.1/Zeo(+) containing SLCO1B3 cDNA (Hirano et al., 2004; Iwai et al., 2004), and selection was performed with Zeocin (700 μg/ml; Invitrogen).

Western Blot Analysis. For Western blot analysis, a crude membrane was prepared from MDCKII cells according to the method described in a previous report (Gant et al., 1991). After the crude membrane was suspended in PBS, it was frozen in liquid N_2 and stored at −80°C until use. The protein concentrations in the crude membranes prepared from MDCKII cells were determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard. The membrane fraction was dissolved in 3× SDS sample buffer (New England Biolabs, Beverly, MA) with 0.125 M dithiothreitol and loaded onto a 7.5% SDS-polyacrylamide electrophoresis gel (Daiichi Pure Chemical Co. Ltd., Tokyo, Japan). The molecular weight was determined using a prestained protein marker (New England Biolabs). Proteins were transferred electrophoretically to a polyvinylidene difluoride membrane (Pall, East Hills, NY) using a blotter (Trans-blot; Bio-Rad, Hercules, CA) at 15 V for 1 h. The membrane was blocked with Tris-buffered saline with 0.05% Tween 20 (TTBS) and 5% skimmed milk overnight at 4°C. After washing with TTBS, the membrane was incubated at room temperature in TTBS with 1000-fold diluted anti-OATP1B3 polyclonal antiserum, which was raised in rabbits against the 21 amino acids at the carboxyl terminus of the deduced OATP1B3 sequence (Hirano et al., 2004), for 1 h, 125-fold diluted monoclonal antibody against MRP2 (M_2III-6; Biochemicals, Gruenberg, Germany) for 2 h, 100-fold diluted monoclonal antibody against MDR1 (C219; Signet Laboratories, Inc., Dedhem, MA) for 1 h, or 200-fold diluted monoclonal antibody against BCRP (BXP-21; Kamiya Biomedical Company, Seattle, WA) for 2 h. For the detection of each transporter, the membrane was placed in contact with 2500-fold diluted donkey anti-rabbit (OATP1B3) or anti-mouse IgG (MRP2, MDR1, and BCRP) conjugated with horseradish peroxidase (GE Healthcare) for 1 h in TTBS. The band was detected using an ECL Plus Western blotting starter kit (GE Healthcare).

Immunocytochemical Staining. For immunocytochemical staining, transfectants were plated at a density of 5.4 × 10^5 cells in 12-well plates 96 h before the experiments. Sodium butyrate (5 mM) was added to the culture medium 1 day before the experiments. After fixation with methanol at −20°C for 10 min and permeabilization with 1% Triton X-100 in PBS at room temperature for 5 min, cells were incubated for 1 h at room temperature with 50-fold diluted anti-OATP1B3 antiserum, 40-fold diluted monoclonal antibody against MRP2 (M_2III-6), 40-fold diluted monoclonal antibody against MDR1 (C219), or 40-fold diluted monoclonal antibody against BCRP (BXP-21). Then the cells were washed with PBS three times and incubated for 1 h at room temperature with 250-fold diluted goat anti-rabbit IgG Alexa 488 (Invitrogen) for OATP1B3 or 250-fold diluted goat anti-mouse IgG Alexa 568 (Invitrogen) for MRP2, MDR1, and BCRP. Nuclei were stained with 250-fold-diluted TO-PRO-3 iodide (Invitrogen). The localization was visualized by confocal laser microscopy (Zeiss LSM-510; Carl Zeiss Inc., Thornwood, NY).

Transcelluar Transport Study Using Double-Transfected MDCKII Cells. MDCKII cells coexpressing OATP1B1 and each hepatic efflux transporter (MDR1, MRP2, and BCRP) (Evers et al., 1998; Tang et al., 2002; Matsushima et al., 2005) and coexpressing OATP1B3 and each hepatic efflux transporter (MDR1, MRP2, and BCRP) were used for transcellular transport studies. The transcellular transport study was performed as reported previously (Sasaki et al., 2002). Briefly, MDCKII cells were seeded on the Transwell (6.5-mm diameter, 0.4 μm pore size; Corning Coster, Bodenheim, Germany) at a cell density of 1.4 × 10^5 cells/well and grown on Transwell membrane inserts at confluence for 7 days, and the expression of transporters was induced by replacing the culture medium with medium containing 5 mM sodium butyrate for 48 h before the transport study. Cells were first washed with Krebs-Henseleit buffer (118 mM NaCl, 23.8 mM $NaHCO_3$, 4.8 mM KCl, 1.0 mM KH_2PO_4, 1.2 mM $MgSO_4$, 12.5 mM HEPES, 5.0 mM glucose, and 1.5 mM $CaCl_2$ adjusted to pH 7.4) and preincubated with Krebs-Henseleit buffer at 37°C for 20 min. Subsequently, the transport study was initiated by adding Krebs-Henseleit buffer containing test compounds to either the apical compartments (250 μl) or the basolateral compartments (1 ml) after removal of Krebs-Henseleit buffer used for preincubation. In the case of tel-glu, the incubation volume was changed to 100 μl for the apical compartments and to 600 μl for the basolateral compartments, and 0.3% HSA was supplemented in Krebs-Henseleit buffer. After a designated period, for the radioactive compounds, the radioactivity in 100 μl of medium in the opposite compartments was measured by a liquid scintillation counter (Tri-Carb 2500TR; PerkinElmer Life and Analytical Sciences). For the measurement of tel-glu, a sample of 50 μl was removed from the opposite compartments, and 10-μl aliquots were mixed with an equal volume of 1 N NaOH and stored for 1 h at 37°C for the complete conversion from tel-glu to telmisartan. The mixtures were neutralized with 5 μl of 2 N HCl, and then the tel-glu concentration in the medium was measured by HPLC fluorescence as described above after addition of 6.25 μl of acetonitrile. Additionally, the tel-glu concentration in a 20-μl sample was measured after adding 30 μl of distilled water and 12.5 μl of acetonitrile to assess the decomposition from tel-glu to telmisartan during the incubation period. At the end of the experiments, the cells were washed three times with 1.5 ml of ice-cold Krebs-Henseleit buffer and solubilized in 150 μl of 1N NaOH. After addition of 75 μl of 2 N HCl and 56 μl of acetonitrile, the tel-glu concentration in the cells was determined by HPLC fluorescence. Twenty-microliter aliquots of cell lysate were used to determine protein concentrations by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Kinetic Analysis. Ligand uptake was expressed as the uptake volume (microliters per milligram of protein), given as the radioactivity associated with the cells (disintegrations per minute per milligram of protein) divided by its initial concentration in the incubation medium (disintegrations per minute per microliter). Specific uptake was obtained by subtracting the uptake into vector-transfected cells from the uptake into cDNA-transfected cells. Kinetic parameters were obtained using eq. 1:

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

where v is the uptake velocity of the substrate (picomoles per minute per milligram of protein), S is the substrate concentration in the medium (micromolar), K_m is the Michaelis constant (micromolar concentration), 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). The half-inhibitory concentration (IC_{50}) of

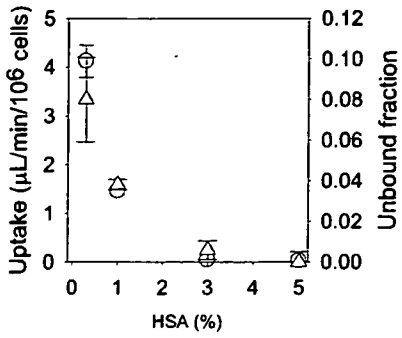

FIG. 1. Effect of various concentrations of human serum albumin on the uptake of tel-glu in isolated rat hepatocytes and the protein unbound fraction of tel-glu. Δ and O represent the uptake of tel-glu into isolated rat hepatocytes (microliters per minute per 10^6 cells) and protein unbound fraction of tel-glu in the incubation media, respectively. Uptake of tel-glu was measured by incubating cells with 5 μM or 200 μM tel-glu, and the saturable uptake of tel-glu by isolated rat hepatocytes was determined by using eqs. 3 and 4. HSA concentrations used were 0.3, 1, 3, and 5%. Each point with vertical bar shows the mean ± S.E. of three separate determinations.

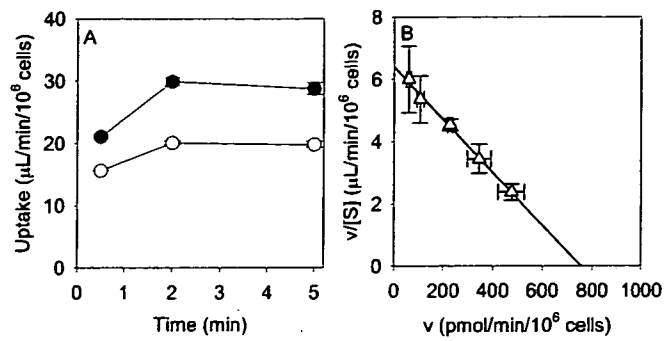

FIG. 2. Time profiles (A) and Eadie-Hofstee plot (B) of the uptake of tel-glu by isolated rat hepatocytes in the presence of 1% HSA. A, concentrations of tel-glu used were 2 (●) and 200 μM (○). B, the uptake of tel-glu in isolated rat hepatocytes as a function of a range of tel-glu concentrations (10–200 μM) was measured at a concentration between 10 and 200 μM tel-glu. The initial uptake rate of tel-glu in isolated rat hepatocytes was determined using (eq. 3). The solid line represents the fitted curve. Each point with bar represents the mean ± S.E. of three separate determinations.

inhibitors was obtained by examining their inhibitory effects on the uptake of CCK-8, $E_2$17βG, and tel-glu based on eq. 2:

$$CL_{+I} = CL \Big/ \left(1 + \frac{I}{IC_{50}}\right) \quad (2)$$

where CL and CL_{+I} represent the uptake clearance in the absence and presence of inhibitor, respectively, and I is the concentration of inhibitor. IC_{50} values were estimated by the nonlinear least-squares method using a MULTI program (Yamaoka et al., 1981). To determine the saturable hepatic uptake clearance in human hepatocytes, we first determined the hepatic uptake clearance [$CL_{(2\ min-0.5\ min)}$] (microliters per minute per 10^6 cells) by calculating the slope of the uptake volume (V_d) (microliters per 10^6 cells) between 0.5 and 2 min (eq. 3). The saturable hepatic uptake clearance (CL_{hep}) was determined by subtracting $CL_{(2\ min-0.5\ min)}$ in the presence of an excess of cold substrate (excess) from that in the presence of tracer amount of substrate (tracer) (eq. 4).

$$CL_{(2\ min-0.5\ min)} = \frac{(V_{d,\,2\ min} - V_{d,\,0.5\ min})}{2-0.5} \quad (3)$$

$$CL_{hep} = CL_{(2\ min-0.5\ min),\,tracer} - CL_{(2\ min-0.5\ min),\,excess} \quad (4)$$

Results

Uptake of Tel-Glu into Isolated Rat Hepatocytes. First, we evaluated the involvement of transporters in the uptake of tel-glu by isolated rat hepatocytes. Because tel-glu is also anticipated to exhibit very high adsorption to the cell surface and/or culture materials as well as telmisartan (Ishiguro et al., 2006), we checked the effect of HSA on tel-glu uptake and measured the protein unbound fraction of tel-glu in incubation media in each condition before the uptake experiment with tel-glu. Tel-glu exhibited a very high apparent uptake, which may include nonspecific adsorption to cells, transporter-mediated uptake, and passive diffusion in the absence of HSA (tracer, 5 μM: 268 ± 5 μl/2 min/10^6 cells; excess, 200 μM: 200 ± 3 μl/2 min/10^6 cells). The apparent uptake was reduced with an increase in HSA concentration (Fig. 1). The protein unbound fractions of tel-glu in the incubation media with 0.3, 1, 3, and 5% HSA were reduced to 9.9, 3.5, 1.1, and 0.7%, respectively (Fig. 1). Both the apparent uptake into hepatocytes and the unbound fraction of tel-glu were reduced in parallel with increasing concentrations of HSA. This observation suggested that the saturable uptake of tel-glu followed the "free" hypothesis according to which only unbound ligand can be recognized by transporters (Fig. 1). Thus, the tel-glu uptake was assessed with at least 0.3% HSA in the incubation media to prevent the extensive adsorption of tel-glu to the cells and/or culture materials and to use the same experimental conditions as in the telmisartan study (Ishiguro et al., 2006). In the presence of 1% HSA, tel-glu was taken up into isolated rat hepatocytes in a linear fashion for up to 2 min. The uptake was reduced by the presence of an excess of tel-glu (200 μM), indicating that transporter(s) are involved in the tel-glu uptake by isolated rat hepatocytes (Fig. 2A). The concentration dependence of the uptake of tel-glu was studied over the concentration range of 10 to 200 μM in the presence of 1% HSA. An Eadie-Hofstee plot showed one saturable component (Fig. 2B), and the apparent K_m and V_{max} values for tel-glu uptake in the presence of 1% HSA were 118 ± 4 μM and 754 ± 12 pmol/min/10^6 cells, respectively. To evaluate the nonsaturable uptake of tel-glu, a tel-glu concentration of 200 μM was used, which is not high enough to saturate the transporter-mediated transport completely, but represents the maximum solubility of tel-glu in the incubation media. Depletion of Na^+ in the incubation media did not reduce the uptake of tel-glu (Fig. 3A). Although 10 μM pravastatin slightly stimulated the uptake of tel-glu, the uptake was inhibited by pravastatin, digoxin, and TCA, which are substrates and inhibitors of Oatp isoforms (Noe et al., 1997; Kouzuki et al., 1999; Tokui et al., 1999; Cattori et al., 2000; Sasaki et al., 2002, 2004), with IC_{50} values of 58.9 ± 26.7, 22.9 ± 9.8, and 7.12 ± 3.18 μM, respectively. An excess amount of TEA (1 mM), a typical substrate of organic cation transporters, caused at most a 30% reduction in the uptake of tel-glu (Fig. 3B). On the basis of these results, Oatp isoforms are considered to be involved in the tel-glu transport by isolated rat hepatocytes.

Uptake of Tel-Glu in Transporter-Expressing HEK293 Cells. To identify which transporters are important for the hepatic uptake of tel-glu in humans, uptake assays were performed using OATP1B1-, OATP1B3- and OATP2B1-expressing HEK293 cells. For these studies the HSA concentration was reduced from 1 to 0.3% in the incubation media. This was done because only minimal transport activity of $E_2$17βG, which is a typical ligand for OATP1B1, was detected in OATP1B1-expressing cells in the presence of 1% HSA owing to the significant reduction in its unbound concentration by binding to HSA (data not shown). Tel-glu was transported by OATP1B3 and OATP2B1 but not by OATP1B1 (Fig. 4A). The concentration dependence of the initial uptake of tel-glu by OATP1B3 and OATP2B1 was studied over the concentration ranges of 0.2 to 20 μM and 0.2 to 50 μM, respectively (Fig. 4B). The K_m and V_{max} values of OATP1B3- and OATP2B1-mediated tel-glu uptake were 3.40 ±

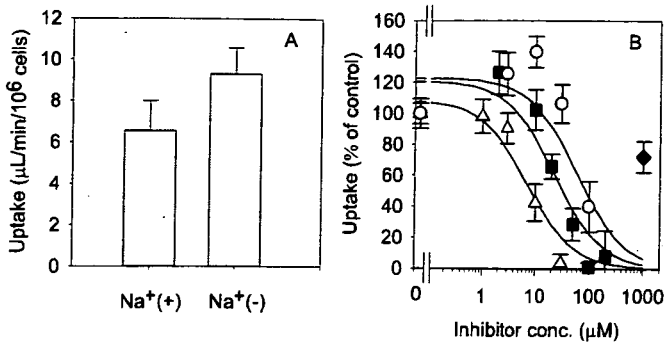

FIG. 3. Effect of Na^+ ion (A) and various compounds (B) on the uptake of tel-glu in isolated rat hepatocytes in the presence of 1% HSA. The concentration of tel-glu used was 10 μM. Saturable uptake of tel-glu by isolated rat hepatocytes was determined using eqs. 3 and 4. B, data are shown as the percentage of the saturable uptake of tel-glu in the absence of inhibitors. ■, △, ○, and ◆ represent the uptake of tel-glu in the presence of digoxin, taurocholate, pravastatin, and TEA, respectively. Solid lines represent the fitted curves obtained by nonlinear regression analysis. Each bar and vertical bar represents the mean ± S.E. of three separate determinations.

0.16 μM and 124 ± 4 pmol/min/mg of protein and 1.09 ± 0.10 μM and 22.3 ± 1.0 pmol/min/mg of protein, respectively.

Inhibitory Effect of E-sul on OATP1B3- and OATP2B1-Mediated Uptake of Tel-Glu in Transporter Expression Systems. We previously reported that E-sul is a selective inhibitor against OATP1B1 and that 30 μM E-sul completely inhibited OATP1B1-mediated $E_2$17βG uptake but did not inhibit OATP1B3-mediated CCK-8 uptake in transporter-expressing HEK293 cells (Ishiguro et al., 2006). In this study, we investigated the effects of E-sul on OATP1B3- and OATP2B1-mediated tel-glu uptake using OATP1B3- and OATP2B1-expressing HEK293 cells. E-sul hardly inhibited the OATP1B3- and OATP2B1-mediated tel-glu uptake with high IC_{50} values of 216 ± 28 and 223 ± 37 μM, respectively (Fig. 5).

Estimation of Relative Contributions of OATP1B1 and OATP1B3 to the Hepatic Uptake of Tel-Glu in Cryopreserved Human Hepatocytes. To confirm the minor contribution of OATP1B1 to overall hepatic tel-glu transport, three batches of cryopreserved human hepatocytes (Lots HH-OCF, HH-094, and HH-TDH) were used for the inhibition study. The uptake of 1 μM $E_2$17βG and 2 μM tel-glu by three different batches of cryopreserved human hepatocytes in the presence of 0.3% HSA was increased from 0.5 to 2 min [uptake of $E_2$17βG and tel-glu by cryopreserved human hepatocytes (HH-TDH): 3.40 ± 0.49 and 15.4 ± 2.5 μl/min/10^6 cells, respectively]. The uptake was reduced in the presence of an excess of unlabeled $E_2$17βG (200 μM) and tel-glu (50 μM) to 1.33 ± 0.30 and 4.05 ± 0.47 μl/min/10^6 cells, respectively (HH-TDH). The uptake of $E_2$17βG into human hepatocytes was inhibited by more than 50% by an E-sul concentration of 30 μM, whereas the uptake of tel-glu was hardly inhibited by 30 μM E-sul (Table. 1).

Expression of Human OATP1B3, MRP2, MDR1, and BCRP in MDCKII Cells. The expression of OATP1B3, MRP2, MDR1, and BCRP in the double-transfected MDCKII cell lines was confirmed by Western blot analysis (Fig. 6). In the parental MDCKII cells, no expression of OATP1B3, MRP2, MDR1, and BCRP was observed. The major band, which appeared at approximately 120 kDa, was detected in all kinds of OATP1B3-transfected cells (Fig. 6A), as

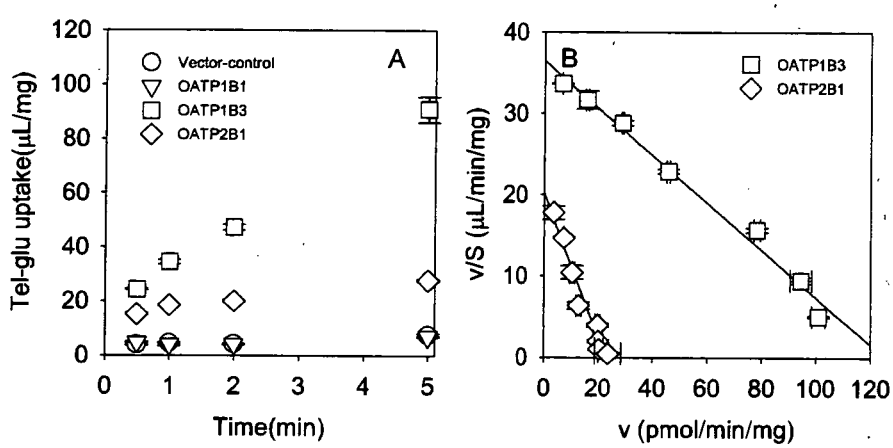

FIG. 4. Time profiles (A) and Eadie-Hofstee plots (B) of the uptake of tel-glu in transporter-expressing cells in the presence of 0.3% HSA. A, the concentration of tel-glu used was 2 μM. ▽, □, ◇, and ○ indicate the uptake of tel-glu by OATP1B1-, OATP1B3-, and OATP2B1-expressing cells and vector-transfected cells, respectively. B, uptake of tel-glu by OATP1B3-expressing cells (□) and OATP2B1-expressing cells (◇) was measured at a concentration between 0.2 and 20 μM for OATP1B3 and between 0.2 and 50 μM for OATP2B1. The transporter-mediated uptake was expressed by the difference in the uptake clearance between transporter-expressing cells and vector-transfected cells. Solid lines represent the fitted curves. Each point with bar represents the mean ± S.E. of three separate determinations.

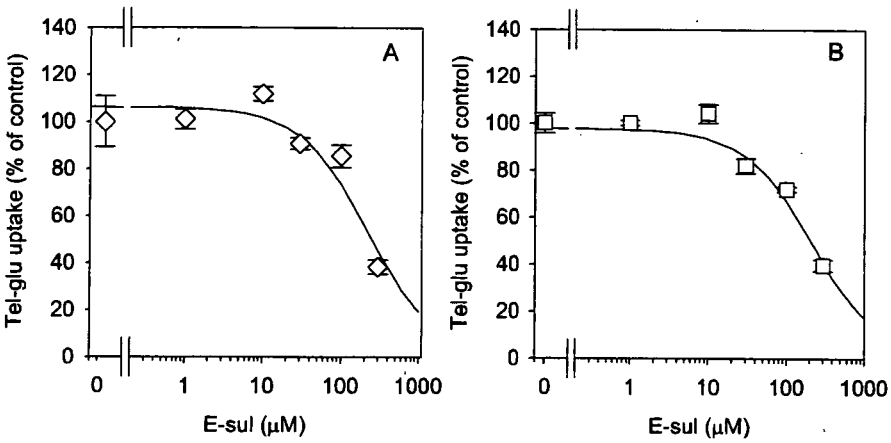

FIG. 5. The inhibitory effect of E-sul on OATP2B1 (A)- and OATP1B3 (B)-mediated tel-glu uptake in the presence of 0.3% HSA. The concentration of tel-glu used was 2 μM. The OATP2B1- and OATP1B3-mediated transport was obtained by subtracting the uptake in vector-transfected cells from that in OATP2B1- or OATP1B3-expressing cells for 2 min. The data are shown as the percentage of the OATP2B1- and OATP1B3-mediated tel-glu uptake in the absence of E-sul. The solid lines represent the fitted curves obtained by nonlinear regression analysis. Each point with bar represents the mean ± S.E. of three separate determinations.

TABLE 1

Effect of E-sul on the uptake of tel-glu and $E_2$17βG by cryopreserved human hepatocytes in the presence of 0.3% HSA

The substrate concentration used was 2 and 50 μM for tel-glu and 1 and 200 μM for $E_2$17βG. The saturable uptake of tel-glu and $E_2$17βG into cryopreserved human hepatocytes was determined after the subtraction of nonsaturable uptake (evaluated as the uptake clearance in the presence of 50 μM tel-glu or 200 μM $E_2$17βG). The term "% control" represents the ratio of the uptake clearance in the presence of 30 μM E-sul to that in its absence.

	HH-OCF		HH-094		HH-TDH	
	$E_2$17βG	Tel-Glu	$E_2$17βG	Tel-Glu	$E_2$17βG	Tel-Glu
			μl/min/10⁶ cells			
E-sul						
0 μM	5.66 ± 1.05[a]	12.8 ± 0.7	1.85 ± 0.72[a]	15.7 ± 0.7	2.08 ± 0.5	11.4 ± 2.5
30 μM	1.50 ± 0.75[a]	9.84	1.03 ± 0.76[a]	14.1 ± 1.0	0.0472 ± 0.1155	10.6 ± 0.4
% control	26.6%	76.9%	55.8%	89.9%	2.3%	93.0%

[a] Values from Ishiguro et al. (2006).

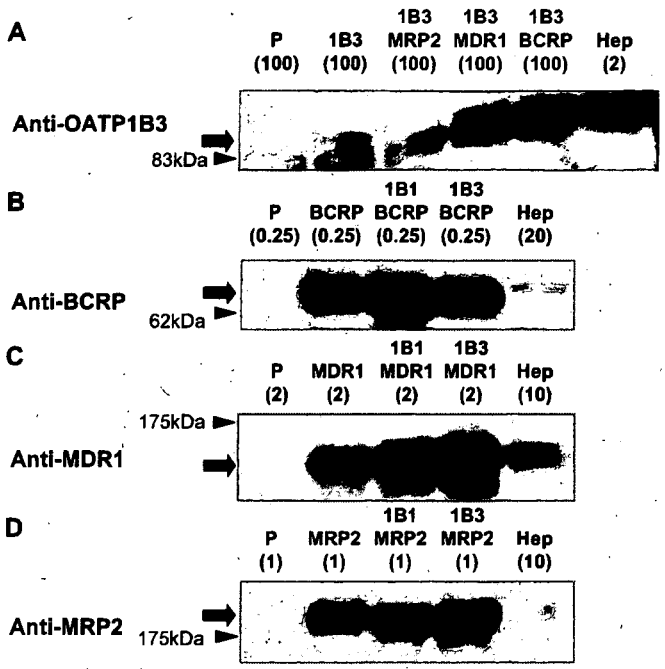

FIG. 6. Western blot analysis of OATP1B3 (A), BCRP (B), MDR1 (C), and MRP2 (D) in crude membrane vesicles obtained from MDCKII transfectants. Crude membrane prepared from MDCKII transfectants was separated by SDS-polyacrylamide gel electrophoresis, and each transporter was detected using antiserum or monoclonal antibody against respective transporter. The figures in parentheses represent the amount of protein applied to each lane (unit: micrograms). Arrows represent the specific bands for each transporter. P, parental MDCKII cells; Hep, hepatocytes (lot HH-OCF).

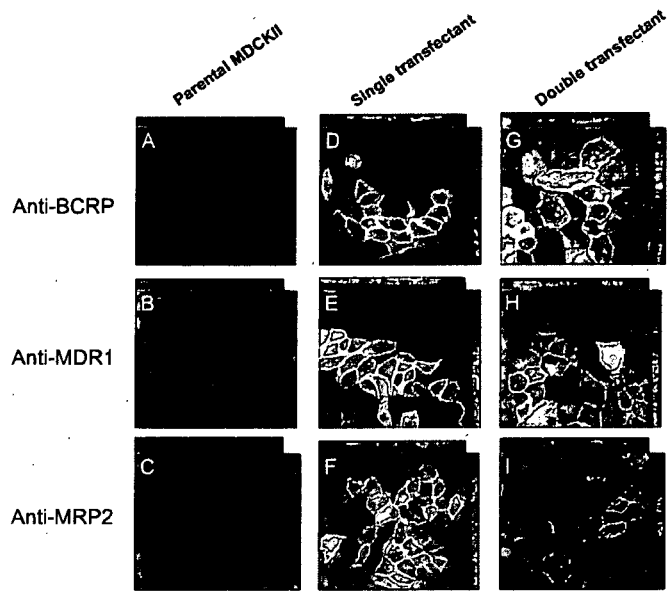

FIG. 7. Immunolocalization of recombinant OATP1B3, BCRP, MDR1, and MRP2 in MDCKII cells. MDCKII cells transfected with empty vector (A, B, and C), OATP1B3 (D, E, and F), both OATP1B3 and BCRP (G), both OATP1B3 and MDR1 (H), and both OATP1B3 and MRP2 (I) were stained with polyclonal antiserum against human OATP1B3 (green fluorescence, A–I), monoclonal antibody against human MRP2 (red fluorescence, C, F, and I), human MDR1 (red fluorescence, B, E, and H), and human BCRP (red fluorescence, A, D, and G). Nuclei were stained with TO-PRO-3 (blue fluorescence). Pictures are single optical sections (*x,y*) (center) with *xz* (top) and *yz* (right) projections, respectively.

shown previously (Hirano et al., 2004). The expression level of OATP1B3 in OATP1B3-transfected MDCKII cells per unit of protein was lower than that in cryopreserved human hepatocytes (Lot HH-OCF). We easily detected human MRP2, MDR1, and BCRP with apparent molecular masses of approximately 190, 170, and 70 kDa, respectively (Fig. 6, B–D). The MRP2, MDR1, and BCRP expression levels in double transfectants per unit of protein were almost comparable with those in single transporter-expressing cells and were much higher than those in cryopreserved human hepatocytes (Lot HH-OCF).

Localization of Recombinant Human OATP1B3, MRP2, MDR1, and BCRP. The cellular localization of the recombinant transporters in each transfectant was confirmed by confocal laser scanning microscopy (Fig. 7). OATP1B3 was correctly localized in the basolateral membrane of each transfectant expressing OATP1B3 (Fig. 7, D–I), whereas MDR1 and MRP2 were localized in the apical membrane (Fig. 7, H and I). BCRP was detected mainly in the apical membrane, but some fractions were also detected in the basolateral membrane (Fig. 7G).

Transcellular Transport of $E_2$17βG, CCK-8, and Tel-Glu across the MDCKII Monolayer Expressing Uptake and Efflux Transporters. To characterize the double-transfected MDCKII cells coexpressing OATP1B3 and MRP2, MDR1, and BCRP, we evaluated the transcellular transport of $E_2$17βG (OATP1B1/OATP1B3 bisubstrates) and CCK-8 and tel-glu (specific substrates of OATP1B3) across the MDCKII monolayer expressing uptake and efflux transporters (Figs. 8, 9, and 10). Significantly higher basal-to-apical transport of $E_2$17βG was observed in OATP1B1/MRP2, OATP1B1/MDR1, OATP1B1/BCRP, and OATP1B3/MRP2 double transfectants. However, such transport was not observed in parental MDCKII cells, single transfectants (OATP1B1, OATP1B3, MRP2, MDR1, and BCRP) and OATP1B3/MDR1 and OATP1B3/BCRP double transfectants (Fig. 8). Transcellular transport of the OATP1B3-selective substrate of CCK-8

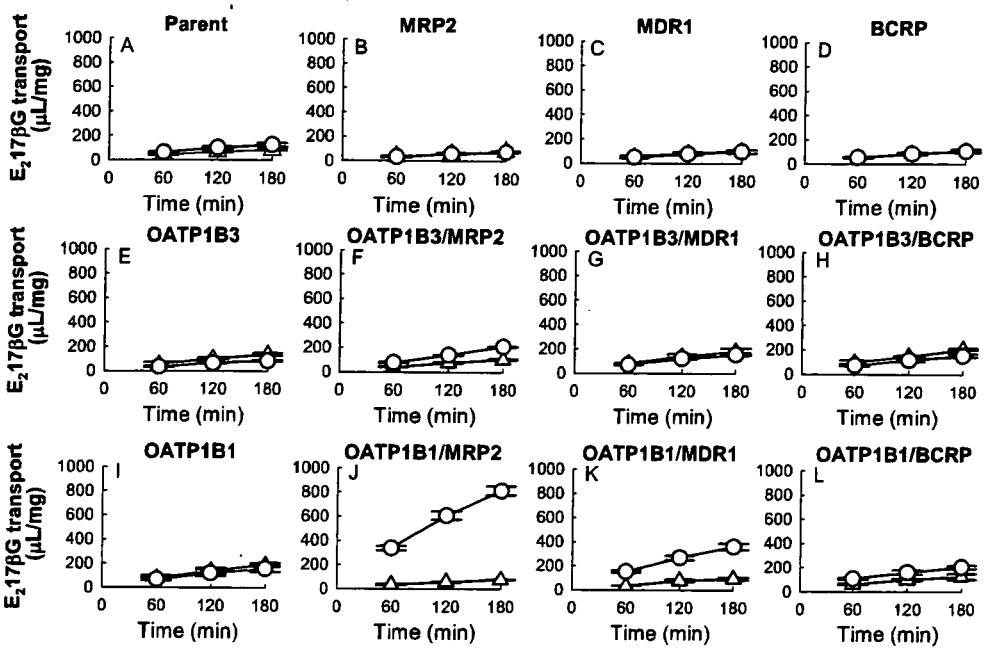

FIG. 8. Time profiles for the transcellular transport of $E_2$17βG across MDCKII monolayers. Transcellular transport of $E_2$17βG (0.1 μM) across MDCKII monolayers expressing MRP2 (B), MDR1 (C), BCRP (D), OATP1B3 (E), both OATP1B3 and MRP2 (F), both OATP1B3 and MDR1 (G), both OATP1B3 and BCRP (H), OATP1B1 (I), both OATP1B1 and MRP2 (J), both OATP1B1 and MDR1 (K), and both OATP1B1 and BCRP (L) was compared with that across the parental MDCKII monolayer (A). ○, transcellular transport in the basal-to-apical direction; △, transcellular transport in the apical-to-basal direction. Each point with vertical bar represents the mean ± S.E. of three determinations. Where vertical bars are not shown, the S.E. was contained within the limits of the symbol.

and tel-glu was also determined in the transporter-expressing MDCKII cells. The basal-to-apical vectorial transport of CCK-8 was significantly higher in the OATP1B3/MRP2 double transfectant than in the OATP1B3 single transfectant. However, no significant difference in vectorial transport was seen between OATP1B3-expressing cells and double transfectants expressing OATP1B3/MDR1 and OATP1B3/BCRP. In cell lines expressing OATP1B1 as an uptake transporter, no transcellular transport of CCK-8 was observed (Fig. 9). Before the transcellular transport study using tel-glu, the stability of tel-glu during the incubation was examined, and the decomposition from tel-glu into tel was negligible up to 3 h in the presence of 0.3% HSA. A higher basal-to-apical transport of tel-glu was found in all three cell lines expressing OATP1B3 and in the OATP1B1/MRP2 double transfectant. The vectorial transport in the double transfectants expressing OATP1B3 was higher than that in OATP1B3-expressing cells (Fig. 10).

Discussion

Although OATP1B1 and MRP2 are thought to play a major role in the hepatic transport of several organic anions (Evers et al., 1998; Cui et al., 1999; König et al., 1999; Vavricka et al., 2002; Hirano et al., 2004), the importance of OATP1B3, BCRP, and MDR1 has also recently been indicated. Previously we showed that double transfectants coexpressing OATP1B1 and an efflux transporter are a useful system to identify efflux transporters of organic anions in human liver (Matsushima et al., 2005). However, we found that some compounds such as fexofenadine and telmisartan are taken up into human hepatocytes mainly via OATP1B3 (Shimizu et al., 2005; Ishiguro et al., 2006). Telmisartan is excreted into bile predominantly as tel-glu (Wienen et al., 2000). To understand the pharmacokinetics of telmisartan, it is important to clarify the transport mechanisms of tel-glu as well as that of telmisartan. In the present study, we constructed a novel set of double-transfected cells expressing OATP1B3 and MDR1, MRP2, or BCRP and examined the transport mechanisms of tel-glu using rat and human hepatocytes, OATP transporter-expressing HEK293 cells, and double transfectants.

Previously, there were substantial difficulties to overcome in evaluating the transport of lipophilic telmisartan because of its extensive adsorption to cells (Ishiguro et al., 2006). Therefore, we examined whether tel-glu also exhibits this unfavorable property as does telmisartan. In the absence of HSA, the apparent uptake of tel-glu by isolated rat hepatocytes was very high and almost the same as that of telmisartan. To avoid the extensive adsorption of tel-glu to cells by HSA, we decided to use the same experimental conditions as for telmisartan, 1 and 0.3% HSA in the incubation media, for the further evaluation of tel-glu uptake by rat and human hepatocytes, respectively.

Because the involvement of Oatp isoforms on the hepatic uptake of tel-glu into isolated rat hepatocytes was anticipated by uptake experiments using isolated rat hepatocytes, we assessed which OATP transporters were involved in the human hepatic uptake of tel-glu by using cryopreserved human hepatocytes and transporter expression systems. Tel-glu was taken up by OATP1B3 and OATP2B1 but not by OATP1B1 (Fig. 4A). To confirm the minor contribution of OATP1B1 to the hepatic uptake of tel-glu, we performed an inhibition study using three batches of cryopreserved human hepatocytes and an OATP1B1-selective inhibitor, E-sul. Previously we reported that 30 μM E-sul can selectively inhibit the OATP1B1-mediated uptake in the presence of 0.3% HSA compared with OATP1B3 (Ishiguro et al., 2006). As a result, tel-glu was taken up into cryopreserved human hepatocytes in a saturable manner, and 30 μM E-sul did not significantly inhibit the uptake of tel-glu into all batches of cryopreserved human hepatocytes that we tested and OATP1B3- and OATP2B1-expressing HEK293 cells (Table 1; Fig. 5, A and B). These results confirmed the minor contribution of OATP1B1 to tel-glu uptake in cryopreserved human hepatocytes. The transport clearance (V_{max}/K_m) of tel-glu by OATP1B3-expressing cells was approximately 2-fold higher than that by OATP2B1-expressing cells. Hirano et al. (2004, 2006) reported that the ratio of the protein expression level of OATP2B1 in human hepatocytes to that in our expression system was less than 0.2, whereas for OATP1B3, that ratio was almost 1 by Western blot analysis. Our results suggested that the contribution of OATP2B1 to the hepatic uptake of tel-glu into human hepatocytes was at most one-tenth that of OATP1B3. Therefore, we concluded that tel-glu

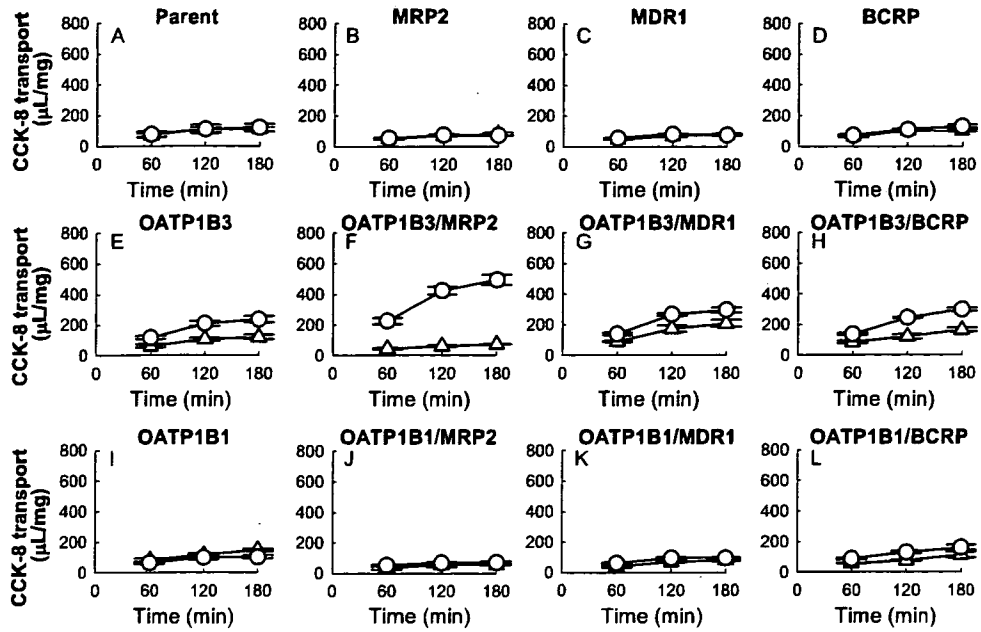

FIG. 9. Time profiles for the transcellular transport of CCK-8 across MDCKII monolayers. Transcellular transport of CCK-8 (0.1 μM) across MDCKII monolayers expressing MRP2 (B), MDR1 (C), BCRP (D), OATP1B3 (E), both OATP1B3 and MRP2 (F), both OATP1B3 and MDR1 (G), both OATP1B3 and BCRP (H), OATP1B1 (I), both OATP1B1 and MRP2 (J), both OATP1B1 and MDR1 (K), and both OATP1B1 and BCRP (L) was compared with that across the parental MDCKII monolayer (A). ○, transcellular transport in the basal-to-apical direction; △, transcellular transport in the apical-to-basal direction. Each point with vertical bar represents the mean ± S.E. of three determinations. Where vertical bars are not shown, the S.E. was contained within the limits of the symbol.

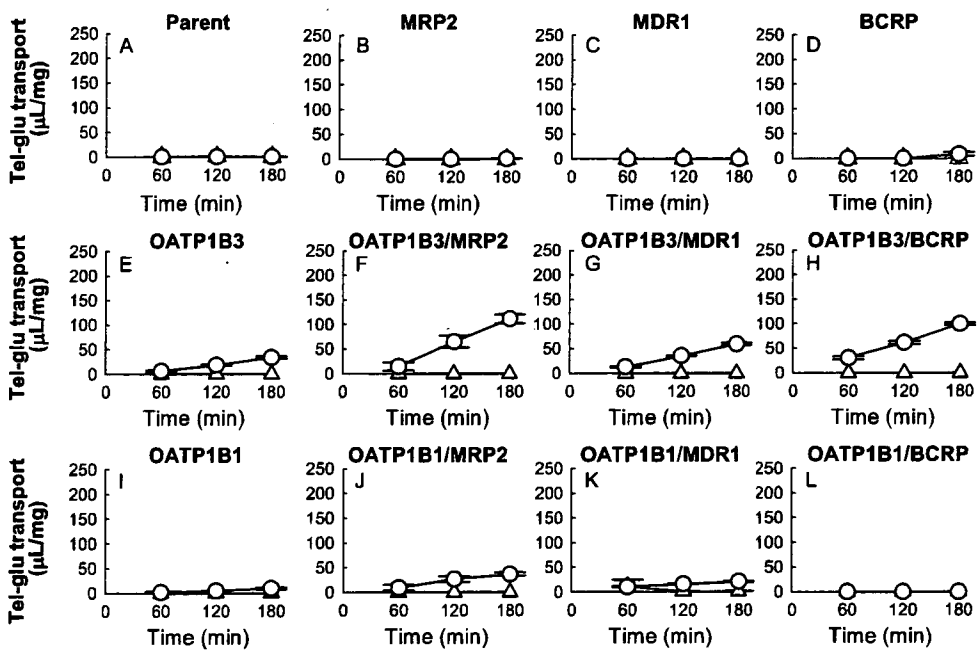

FIG. 10. Time profiles for the transcellular transport of tel-glu across MDCKII monolayers in the presence of 0.3% HSA. Transcellular transport of tel-glu (5 μM) across MDCKII monolayers expressing MRP2 (B), MDR1 (C), BCRP (D), OATP1B3 (E), both OATP1B3 and MRP2 (F), both OATP1B3 and MDR1 (G), both OATP1B3 and BCRP (H), OATP1B1 (I), both OATP1B1 and MRP2 (J), both OATP1B1 and MDR1 (K), and both OATP1B1 and BCRP (L) was compared with that across the parental MDCKII monolayer (A). ○, transcellular transport in the basal-to-apical direction; △, transcellular transport in the apical-to-basal direction. Each point with vertical bar represents the mean ± S.E. of three determinations. Where vertical bars are not shown, the S.E. was contained within the limits of the symbol.

is taken up into human hepatocytes predominantly by OATP1B3 as telmisartan.

Regarding the biliary excretion of tel-glu, we previously showed that tel-glu is transported by both MRP2 and another transporter(s) that are also expressed in Eisai hyperbilirubinemic rats (Nishino et al., 2000). Because of its extensive adsorption of tel-glu, it was not possible to assess its transport into canalicular membrane vesicles. CCK-8 is a recognized substrate of OATP1B3 and MRP2 (Ismair et al., 2001; Letschert et al., 2004, 2005), but the involvement of BCRP and MDR1 in its efflux remains to be investigated. To identify the efflux transporters of OATP1B3-selective substrates, CCK-8 and tel-glu, we established a set of novel double-transfected MDCKII cells coexpressing OATP1B3 and MDR1, MRP2, or BCRP and characterized their transcellular transports using several kinds of double transfectants.

Western blot and immunocytochemical analyses revealed that OATP1B3, MRP2, and MDR1 were expressed in MDCKII cells and localized correctly on the basolateral (OATP1B3) and apical membrane (MRP2 and MDR1), respectively. However, BCRP was localized mainly on the apical membrane and partially on the basolateral membrane (Figs. 6 and 7). This phenomenon was also observed in OATP1B1/BCRP double transfectants, but basal-to-apical transcellular transport of several compounds could be observed in these cell lines. This finding suggested that a minor distribution of BCRP on the basolateral side may not become a major concern to characterize efflux transport processes (Matsushima et al., 2005).

$E_2$17βG is a bisubstrate of OATP1B1 and OATP1B3, and its efflux is mediated by MRP2, MDR1, and BCRP (Hirano et al., 2004; Matsushima et al., 2005). No vectorial transport of $E_2$17βG was observed in MDCKII cells expressing only efflux transporter, whereas higher basal-to-apical transport was seen in double transfectants expressing OATP1B1 (Fig. 8) as had been reported previously (Matsushima et al., 2005). This result was most likely due to the limited access of $E_2$17βG inside the cells without the action of a suitable uptake transporter such as OATP1B1. Our findings showed that the transcellular transport of $E_2$17βG was significantly enhanced only in OATP1B3/MRP2 double transfectants among the three kinds of double transfectants expressing OATP1B3. This result can be explained by the previous findings that the transport clearance of $E_2$17βG by OATP1B3 is 7-fold lower than that by OATP1B1 (Hirano et al., 2004) and that $E_2$17βG is a good substrate of MRP2 compared with BCRP and MDR1 (Matsushima et al., 2005). In the case of CCK-8, a higher vectorial transport compared with the OATP1B3 single transfectant was observed only in OATP1B3/MRP2 double transfectants (Fig. 9). This finding indicated that CCK-8 was preferably transported by MRP2 rather than by MDR1 and BCRP, which was also in line with a previous report (Letschert et al., 2005).

In a following set of experiments, the vectorial basal-to-apical transcellular transport of tel-glu was observed in all kinds of double transfectants expressing OATP1B3, indicating that tel-glu was a substrate of MRP2, MDR1, and BCRP (Fig. 10). This result implies that other transporter(s), as was predicted by the previous study using Eisai hyperbilirubinemic rats (Nishino et al., 2000), might be MDR1 and/or BCRP. The vectorial transport of tel-glu was also seen in OATP1B1/MRP2 double transfectants (Fig. 10J) but not in MRP2-expressing cells (Fig. 10B), which was apparently inconsistent with the result that tel-glu was a specific substrate of OATP1B3. In our experiment, only a very small uptake of tel-glu by OATP1B1-expressing cells was observed, compared with that by vector-transfected cells in the absence of HSA (vector: 21.5 $\pm$ 0.9 μl/0.5 min/mg; OATP1B3: 193 $\pm$ 8 μl/0.5 min/mg; and OATP1B1: 36.2 $\pm$ 0.9 μl/0.5 min/mg). Because of the high sensitivity of the detection of transport in double-transfected cell lines, this observation may explain the apparent discrepancy.

Taken together, the results of this study using transporter expression systems and human hepatocytes suggested that tel-glu is taken up into human liver mainly via OATP1B3. For the more general purpose of the identification of efflux transporters of OATP1B3-selective substrates, we constructed a novel set of double transfectants coexpressing OATP1B3 and MDR1, MRP2, or BCRP. Telmisartan 1-*O*-acylglucuronide was excreted via MDR1, MRP2, and BCRP, although the relative quantitative contribution of each transporter has not been determined yet.

In consideration of the fact that OATP1B1 and OATP1B3 are responsible for the hepatic uptake of organic anions in human liver, a set of double transfectants expressing OATP1B3 combined with double transfectants expressing OATP1B1 can be used as a powerful tool for the rapid identification of efflux transporters of many organic anions that are substrates of OATP1B1 or OATP1B3.

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Involvement of multiple efflux transporters in hepatic disposition of fexofenadine

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Abbreviations: BCRP; breast cancer resistance protein; BSEP, bile salt export pump; CL, clearance; EG, estradiol-17β-D-glucuronide; EHBR, Eisai hyperbilirubinemic rat; FEX, fexofenadine; GFR, glomerular filtration rate; LUI, liver uptake index; Mate, multidrug and toxin compound extrusion; MDR, multidrug resistance; MPA, metaphosphoric acid; MRP, multidrug resistance-associated protein; OATP, organic anion transporting polypeptide; P-gp, P-glycoportein; TC, taurocholate

Abstract

Fexofenadine (FEX) is mainly eliminated from the liver into bile in unchanged form. We previously demonstrated that organic anion transporting polypeptide (OATP) 1B1 and OATP1B3 are involved in the hepatic uptake of FEX. However, little is known about the mechanisms controlling the hepatic efflux of FEX from the liver to bile and blood. In the present study, the involvement of hepatic efflux transporters in the pharmacokinetics of FEX was investigated in both *in vitro* and *in vivo* studies. Vectorial transport of FEX was observed in OATP1B3/human bile salt export pump (hBSEP)-double transfectants, but not in OATP1B3/human breast cancer resistance protein (hBCRP)-double transfectants, which indicates the possible contribution of hBSEP to the biliary excretion of FEX in humans. In multidrug resistance associated protein 2 (Mrp2) (-/-) mice, the biliary excretion clearance based on the plasma concentration and the liver-to-plasma concentration ratio significantly decreased, whereas the biliary excretion clearance based on the liver concentration decreased only with 20%, suggesting the minimum contribution of Mrp2 to its biliary excretion. ATP-dependent transport of FEX was observed in hMRP3-enriched membrane vesicles, but not hMRP4. In Mrp3 (-/-) mice, the biliary excretion clearance based on both the plasma and liver concentration, and the liver-to-plasma concentration ratio increased, suggesting the significant contribution of Mrp3 to its sinusoidal efflux and the up-regulation of its biliary excretion in Mrp3 (-/-) mice. On the other hand, pharmacokinetics of FEX remained unchanged in Mrp4 (-/-) mice. This information provides a novel insight into the transporters important for FEX disposition.

Introduction

Fexofenadine (FEX) is an orally active nonsedating histamine H_1-receptor antagonist that is prescribed for oral treatment of allergic rhinitis and chronic idiopathic urticaria. After oral administration of [^{14}C]FEX to healthy volunteers, 80% of the dose was recovered in feces and 12% in urine, in unchanged form (Lippert et al., 1995). Since the absolute oral bioavailability of FEX is reported to be 33% (product information, Hoechst Marion, Roussel, Laval, Quebec, Canada), it follows that two-thirds of the bioavailable FEX is excreted into bile. Accordingly, hepatic transport of FEX is one of the determinants for its systemic clearance.

Previously, we demonstrated that human organic anion transporting polypeptide 1B1 (hOATP1B1/SLCO1B1) and hOATP1B3 (SLCO1B3) contribute to the hepatic uptake of FEX in humans (Shimizu et al., 2005; Matsushima et al., *submitted*). On the other hand, the transporters involved in its biliary excretion have not been clarified yet. In the canalicular membrane, several ATP-binding cassette transporters such as multidrug resistance-associated protein 2 (MRP2/ABCC2), P-glycoprotein/multidrug resistance 1 (P-gp/MDR1/ABCB1), breast cancer resistance protein (BCRP/ABCG2), and bile salt export pump

(BSEP/ABCB11), are involved in the excretion of several compounds. MRP2 is responsible for the biliary excretion of a wide variety of organic anions including glutathione- and glucuronide-conjugates and drugs such as pravastatin (Suzuki and Sugiyama, 1998). MDR1 preferentially accepts hydrophobic cationic and neutral compounds (Hoffmann and Kroemer, 2004), while BCRP accepts various kinds of organic anions (Hirano et al., 2005b; Merino et al., 2005; Suzuki et al., 2003; van Herwaarden et al., 2003). Though BSEP was thought to accept only bile salts (Byrne et al., 2002), recent studies indicate that BSEP transports some drugs such as vinblastine and pravastatin (Hirano et al., 2005a; Lecureur et al., 2000).

It has been shown that FEX is a substrate of P-gp and hMRP2 (Cvetkovic et al., 1999; Matsushima et al., *submitted*), whereas nobody has checked whether FEX is a substrate of hBCRP and hBSEP. Tahara et al. (2005) investigated biliary excretion of FEX using Eisai hyperbilirubinemic rats (EHBRs/Mrp2-deficient rats), and Mdr1a/1b (-/-) and Bcrp1 (-/-) mice. Surprisingly, these transporters didn't have an effect on FEX biliary excretion clearance based on the liver concentration (Tahara et al., 2005). These results suggest that the biliary excretion of FEX is mediated by unknown transporters

distinct from rat Mrp2 (rMrp2), mouse Mdr1a/1b (mMdr1a/1b) and mBcrp1. However, there may be a species difference in the mechanisms of FEX biliary excretion between rats and mice. Recently, Mrp2 (-/-) mice have been established and the impact of mMrp2 on the pharmacokinetics of some drugs and toxins has been characterized (Chu et al., 2006; Vlaming et al., 2006). Therefore, to clarify the biliary excretion mechanisms of FEX in greater detail, we investigated whether FEX is accepted by hBCRP and hBSEP/rBsep in *in vitro* studies and demonstrated the importance of mMrp2 in its biliary excretion using Mrp2 (-/-) mice.

On the other hand, it has become clear that MRP3 (ABCC3) and MRP4 (ABCC4) are important transporters in sinusoidal efflux (Borst et al., 2007). MRP3 can transport a wide variety of organic anions, such as glucuronides, glutathione-conjugates, bile acids and methotrexate (Hirohashi et al., 1999; Hirohashi et al., 2000; Kool et al., 1999; Zelcer et al., 2001; Zeng et al., 2001). Because rMrp3 is expressed at low levels in normal rat liver and its expression markedly increases in EHBRs (Hirohashi et al., 1998), the physiological role of rMrp3 has been thought to be the protection of hepatocytes from intrahepatic toxins such as bile acids only under pathological conditions (*e.g.* cholestatis).

Recent *in vivo* studies using Mrp3 (-/-) mice suggest that mMrp3 contributes to sinusoidal efflux of various glucuronide conjugates (Borst et al., 2007). The substrate specificity of MRP4 overlaps with that of MRP3, but it is somewhat distinguished from MRP3 by its ability to transport nucleotide analogues (Reid et al., 2003; van Aubel et al., 2002). The physiological function of MRP4 in hepatocytes is considered to protect hepatocytes from bile acids under cholestatic conditions as if it is upregulated during cholestasis (Keitel et al., 2005; Mennone et al., 2006). Since both hMRP3 and hMRP4 are expressed in human liver under physiological conditions (Konig et al., 1999; Rius et al., 2003), these transporters may also be involved in the hepatic distribution of drugs. Accordingly, we investigated the role of MRP3 and MRP4 in the sinusoidal efflux of FEX using hMRP3- and hMRP4-enriched membrane vesicles and Mrp3 (-/-) and Mrp4 (-/-) mice.