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G. 知的財産権の出願・登録状況

なし

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III. 研究成果の刊行物・別刷り

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Inhibition of Transporter-Mediated Hepatic Uptake as a Mechanism for Drug-Drug Interaction between Cerivastatin and Cyclosporin A

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ABSTRACT

The mechanism involved in the clinically relevant drug-drug interaction (DDI) between cerivastatin (CER) and cyclosporin A (CsA) has not yet been clarified. In the present study, we examined the possible roles of transporter-mediated hepatic uptake in this DDI. The uptake of [^{14}C]CER into human hepatocytes prepared from three different donors was examined. Kinetic analyses revealed K_m values for the uptake of [^{14}C]CER within the range of 3 to 18 μM, suggesting that more than 70% of the total uptake at therapeutic CER concentrations was accounted for by a saturable process, i.e., transporter-mediated uptake. This uptake was inhibited by CsA with K_i values of 0.3 to 0.7 μM. The uptake of [^{14}C]CER was also examined in human organic anion transporting polypeptide-2 (OATP2)-expressing Madin-Darby canine kidney cells (MDCKII). Saturable OATP2-mediated uptake of [^{14}C]CER was observed and was also inhibited by CsA, with a K_i value of 0.2 μM. These results suggest that the DDI between CER and CsA involves the inhibition of transporter-mediated uptake of CER and, at least in part, its OATP2-mediated uptake. The effect of CsA on the in vitro metabolism of [^{14}C]CER was also examined. The metabolism of [^{14}C]CER was inhibited by CsA with an IC_{50} value of more than 30 μM. From these results, we conclude that the DDI between CER and CsA is mainly due to the inhibition of transporter (at least partly OATP2)-mediated uptake in the liver.

The reduction of serum cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, a rate-determining enzyme in cholesterol synthesis, is an effective treatment for hypercholesterolemia (Moghadasian, 1999). Cerivastatin (CER) is a potent HMG-CoA reductase inhibitor (statin) with a high oral bioavailability, which makes it effective at low doses (Moghadasian, 1999). CER is extensively taken up into the liver and subsequently metabolized by two different enzymes, CYP2C8 and CYP3A4 (Mück, 2000). This dual metabolic pathway is a distinctive feature of CER among statins.

Patients who develop hypercholesterolemia after tissue transplantation are sometimes treated with combination therapy with statins and cyclosporin A (CsA). CsA is an inhibitor of CYP3A4, and therefore, this immunosuppressant is likely to cause a drug-drug interaction (DDI) with simvastatin, lovastatin, and atorvastatin, which are all substrates of CYP3A4 (Deseger and Horsmans, 1996). This DDI may also cause an increase in the plasma concentration of statins and result in myopathy and/or fatal rhabdomyolysis. Since CER can undergo metabolism via two pathways, the frequency of DDI was believed to be low. However, Mück et al. (1999) have reported that the plasma concentrations of CER are increased in kidney transplant patients following CsA treatment. That is, the area under plasma concentration-time curve (AUC) of CER was increased 4-fold by the coadministration of CsA compared with the control. The plasma concentrations of CER were not affected by coadministration of erythromycin, a potent mechanism-based inhibitor of CYP3A4 (Kanamitsu et al., 2000), suggesting that it is unlikely that the DDI between CER and CsA is due to CYP3A4-mediated metabolism (Mück et al., 1998). Moreover, the AUC of pravastatin, which is not a substrate of CYP3A4, is also increased approximately 20-fold by CsA (Regazzi et al., 1993). Until now, the mechanism of this DDI between CsA and these statins has remained unknown.

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ABBREVIATIONS: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; DDI, drug-drug interaction; CER, cerivastatin; CsA, cyclosporin A; OATP, organic anion transporting polypeptide; MDCK, Madin-Darby canine kidney; OAT, organic anion transporter; AUC, area under plasma concentration-time curve; CL, clearance.

Statins are taken up into the liver before undergoing metabolism. The hepatic uptake of some statins has already been studied. For example, in rats, the hepatic uptake of CER (Hirayama et al., 2000) and pravastatin (Komai et al., 1992) has been investigated, and their saturable transport systems have been studied. Pravastatin also exhibits saturable uptake in human hepatocytes (Nakai et al., 2001). However, the uptake of CER by human hepatocytes has not yet been investigated.

Recent studies of drug transport in the liver have provided detailed information on drug transporters, including substrate and inhibitor profiles. More recent studies clarifying the mechanism of drug uptake in the liver have used cloning to identify a number of transporters expressed at the sinusoidal membrane of hepatocytes. At present, organic anion transporting polypeptide-2 (OATP2/OATP-C; gene symbol, *SLC21A6*), OATP8 (*SLC21A8*), OATP-B (*SLC21A9*), and organic anion transporter-2 (OAT2; *SLC22A7*) are reported to be expressed in the human liver and involved in the hepatic uptake of a number of important substrates, including therapeutic drugs (Abe et al., 1999; Hsiang et al., 1999; Kok et al., 2000; König et al., 2000a,b; Tamai et al., 2000). Pravastatin has been shown to be a substrate of OATP2, and this transporter is at least partly responsible for its hepatic uptake (Hsiang et al., 1999; Nakai et al., 2001). As each of these transporters accepts a number of compounds as substrates, they may competitively inhibit the transport of other substrates. Moreover, CsA functions as an inhibitor of rat Oatp1 and Oatp2 (Shitara et al., 2002). It is therefore possible that CsA affects the plasma concentrations of substrates, leading to a clinically relevant DDI (Kusuhara and Sugiyama, 2001). In the present study, we examined the effect of CsA on the uptake of CER into human hepatocytes together with its metabolism to clarify the mechanism of their DDI.

Materials and Methods

Materials. [^{14}C]CER (2.03 GBq/mmol) and unlabeled CER were kindly provided by Bayer AG (Wuppertal, Germany). CsA was purchased from Sigma-Aldrich (St. Louis, MO), and all other reagents were of analytical grade.

Hepatocyte Preparation. The human hepatocytes used in the study were isolated from human livers donated for transplantation purposes but not used mainly due to the lack of appropriate recipients. All the donors were free of known liver diseases. All the livers were stored for less than 24 h in University of Wisconsin solution. The hepatocytes were isolated by perfusion using a two-step collagenase digestion procedure (Li et al., 1992). After enzymatic dissociation, the hepatocytes were further separated from nonparenchymal cells by centrifugation through 30% Percoll. The purified hepatocytes were cryopreserved (Li et al., 1999) in liquid nitrogen until analysis. Immediately before the uptake studies, the hepatocytes (1-ml suspension) were thawed at 37°C then immediately suspended in 10 ml of ice-cold Krebs-Henseleit buffer and centrifuged (50*g*) for 2 min at 4°C, followed by removal of the supernatant. This procedure was repeated to remove cryopreservation buffer, and then the cells were resuspended in the same buffer at a cell density of 2.0×10^6 viable cells/ml for the uptake studies.

Uptake of [^{14}C]CER into Hepatocytes. Prior to starting the uptake studies with [^{14}C]CER, the cell suspensions were prewarmed in an incubator at 37°C for 3 min. A pilot experiment confirmed that a 3-min preincubation was sufficient to raise the temperature of the cells to 37°C. The uptake studies were initiated by adding an equal volume of [^{14}C]CER solution containing various concentrations of unlabeled CER or CsA to the cell suspension. At 0.5 and 2 min, the reaction was terminated by separating the cells from the substrate solution. For this purpose, an aliquot of 100 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, 1.015; a mixture of silicone oil and mineral oil; Sigma-Aldrich), and subsequently, the sample tube was centrifuged for 10 s using a tabletop centrifuge (10,000*g*; Beckman Microfuge E; Beckman Coulter, Inc., Fullerton, CA). During this process, the hepatocytes pass through the oil layer into the alkaline solution. After an overnight incubation in alkali to dissolve the hepatocytes, the centrifuge tube was cut, and each compartment was transferred to a scintillation vial. The compartment containing the dissolved cells was neutralized with 50 μl of 2 N HCl, mixed with scintillation cocktail, and the radioactivity was determined in a liquid scintillation counter (LS6000SE; Beckman Coulter, Inc.).

Uptake Study of [^{14}C]CER in OATP2-Expressing Cells. The construction and culture of OATP2-expressing cells have been described previously (Sasaki et al., 2002). For the uptake study of [^{14}C]CER, MDCKII cells transfected with OATP2 or vector only as a control were seeded on cell culture inserts (BD Biosciences Discovery Labware, Bedford, MA). After 2 days, the culture medium was replaced with one containing 10 mM Na^+ butyrate for the induction of OATP2. After culturing for a further day, the culture medium was replaced with ice-cold Krebs-Henseleit buffer and washed twice with the same buffer, followed by preincubation at 37°C. The uptake study was initiated by replacing the buffer on the basal side of the cells with that containing [^{14}C]CER in the presence or absence of unlabeled CER or CsA. At the designated times, the reaction was terminated by aspirating the incubation buffer and washing four times with ice-cold buffer. Subsequently, the cells were dissolved in 0.75 ml of 0.1 N NaOH overnight, followed by neutralization with 0.75 ml of 0.1 N HCl. Then, 1.3-ml aliquots were transferred to scintillation vials, and the radioactivity associated with the cells and that in the medium was determined in a liquid scintillation counter (LS6000SE). The remaining 0.1-ml aliquots of the cell lysate were used for protein assay by the Lowry method with bovine serum albumin as a standard (Lowry et al., 1951).

Metabolism of [^{14}C]CER and Testosterone in Human Microsomes. To measure the effect of CsA on the metabolism of [^{14}C]CER and testosterone, its in vitro metabolism was examined. Prior to the metabolism study, human microsomes (final 0.5 mg of protein/ml; BD Gentest, Woburn, MA) were incubated at 37°C for 10 min in 100 mM potassium phosphate buffer, pH 7.4, containing 3.3 mM $MgCl_2$, 3.3 mM glucose 6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 1.3 mM NADPH, and 0.8 mM NADH. A 500-μl volume of incubation mixture was transferred into a polyethylene tube, and [^{14}C]CER (final 1 μM) or testosterone (final 30 μM; Wako, Osaka, Japan) were added to initiate the reaction with or without inhibitors. After incubation for a designated time, the reaction was terminated by the addition of 500 μl of ice-cold acetonitrile and 200 μl of ice-cold methanol for the metabolism of [^{14}C]CER and testosterone, respectively, followed by centrifugation. To measure the metabolic rate of [^{14}C]CER, the supernatant was collected and concentrated to approximately 20 μl in a centrifugal concentrator, followed by thin layer chromatography. The analytes were separated on silica gel $60F_{254}$ (Merck KGaA, Darmstadt, Germany) using a mobile phase (toluene/acetone/acetic acid, 70:30:5, v/v/v). The intensity of the bands for intact [^{14}C]CER separated by thin layer chromatography was determined by the BAS 2000 system (Fuji Film, Tokyo, Japan). To measure the metabolic rate of testosterone, 6β-hydroxytestosterone in the incubation mixture was determined by a high-performance liquid chromatography-UV detection method. To a 100-μl volume of supernatant, 100 μl of internal standard (10 μg/ml phenacetin) was added and subjected to a high-performance liquid chromatography system (VP-5; Shimadzu, Kyoto, Japan). The analyte was separated by a C_{18} column (Cosmosil $5C_{18}$-AR; 5-mm, 4.6-mm i.d. × 250 mm; Nakalai Tesque, Kyoto, Japan) at 45°C. The mobile

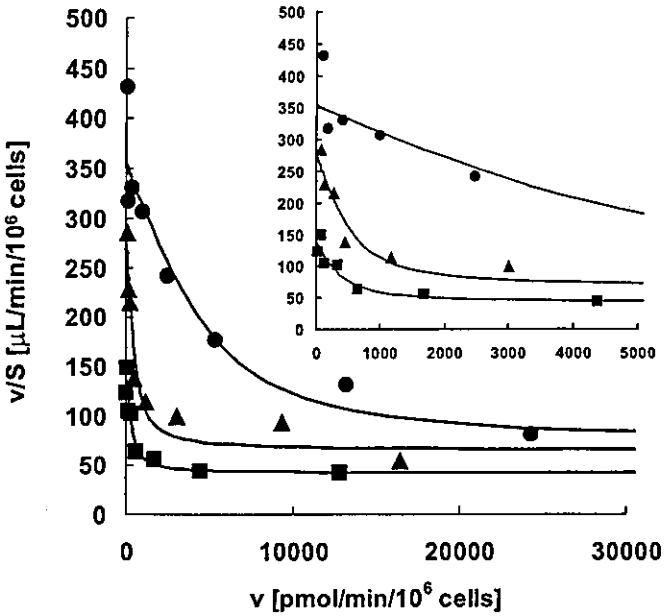

Fig. 1. Eadie-Hofstee plot of the uptake of [^{14}C]CER in cryopreserved human hepatocytes. The uptake of [^{14}C]CER was examined in three lots of cryopreserved human hepatocytes. Closed circles, triangles, and squares (●, ▲, and ■) represent the data for lot numbers HH-088, -106, and -117, respectively. Each symbol represents the mean value of two independent experiments. Solid lines represent the fitted lines.

TABLE 1
Kinetic parameters for the uptake of cerivastatin in cryopreserved human hepatocytes

Lot No.	K_m	V_{max}	V_{max}/K_m	P_{dif}
	μM	$pmol/min/10^6$ cells	$\mu l/min/10^6$ cells	$\mu l/min/10^6$ cells
HH-088	18.3 ± 6.9	5200 ± 1970	284 ± 108	70.2 ± 13.9
HH-106	2.61 ± 1.48	553 ± 161	212 ± 62	65.1 ± 8.3
HH-117	3.72 ± 1.29	362 ± 120	97.3 ± 32.3	41.7 ± 3.4

phase comprised solvent A (20% tetrahydrofuran and 80% water) and solvent B (methanol). A 20-min linear gradient from 20% B to 30% B was applied at a flow rate of 1.0 ml/min. The product was detected by its absorbance at 254 nm and quantitated by comparing with the absorbance of a standard curve for 6β-hydroxytestosterone.

Data Analysis. The time courses of the uptake of [^{14}C]CER into the hepatocytes were expressed as the uptake volume (microliters per 10^6 viable cells) of radioactivity taken up into the cells (dpm/10^6 cells) divided by the concentration of radioactivity in the incubation buffer (disintegrations per minute per microliter). The initial uptake velocity of [^{14}C]CER was calculated using the uptake volume obtained at 0.5 and 2 min and expressed as the uptake clearance (CL_{uptake}; microliters per minute per 10^6 cells). The kinetic parameters for the uptake of [^{14}C]CER were calculated using the following equation:

$$v_0 = \frac{V_{max} \cdot S}{K_m + S} + P_{dif} \cdot S \quad (1)$$

where v_0 is the initial uptake rate (picomoles per minute per 10^6 cells), S is the substrate concentration (micromolar), K_m is the Michaelis constant (μM), V_{max} is the maximum uptake rate (picomoles per minute per 10^6 cells), and P_{dif} is the nonsaturable uptake clearance (microliters per minute per 10^6 cells).

When the substrate concentration is much lower than the K_m value, the data obtained in the inhibition study of the uptake into isolated hepatocytes regardless of inhibitor type (i.e., competitive or

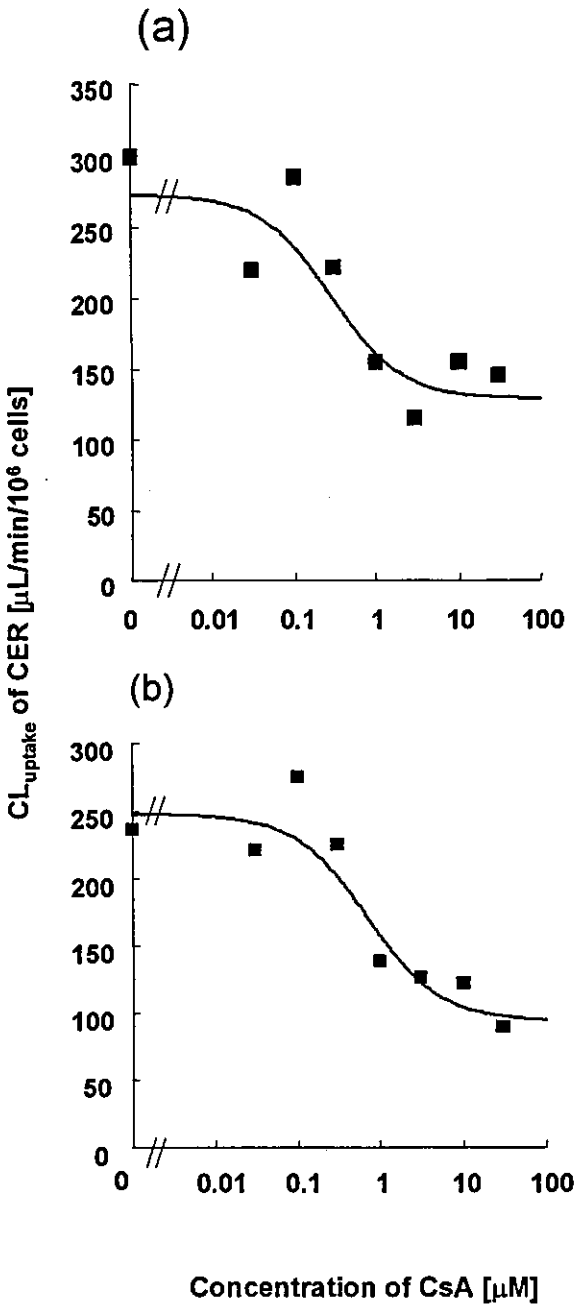

Fig. 2. Inhibitory effect of CsA on the uptake of [^{14}C]CER in cryopreserved human hepatocytes. The inhibitory effect of CsA on the uptake of [^{14}C]CER in lot numbers HH-088 (a) and HH-117 (b) of cryopreserved human hepatocytes was examined. Each symbol represents the mean value of two independent experiments. Solid lines represent the fitted lines.

noncompetitive inhibitor) can be fitted to the following equation to calculate the inhibition constant (K_i).

$$CL_{uptake}(+\text{inhibitor}) = \frac{CL_{uptake}(\text{control}) - CL_{uptake}(\text{resistant})}{1 + I/K_i} + CL_{uptake}(\text{resistant}) \quad (2)$$

where CL_{uptake}(+ inhibitor) is the CL_{uptake} estimated in the presence of inhibitor, CL_{uptake}(control) is the CL_{uptake} estimated in the absence of CsA, CL_{uptake}(resistant) is the CL_{uptake} that is not affected by CsA, and I is the CsA concentration. Using this equation, the K_i value of CsA for the uptake of [^{14}C]CER was calculated.

The data were fitted to these equations by a nonlinear least-squares method using a computer program, MULTI, to obtain the kinetic parameters or inhibition constant with computer-calculated S.E. values (Yamaoka et al., 1981). The input data were weighted as the reciprocal of the observed values, and the Damping Gauss-Newton method was used as the fitting algorithm. The uptake of [^{14}C]CER into OATP2-expressing MDCKII cells was also expressed as the uptake volume (microliters per milligram of protein) for the radioactivity in the cell lysate (disintegrations per minute per milligram of protein) divided by that in the incubation buffer (disintegrations per minute per milliliter).

Results

Uptake into Human Hepatocytes. Eadie-Hofstee plots for the uptake of [^{14}C]CER into human hepatocytes prepared from three donors are shown in Fig. 1. Both the saturable and nonsaturable components were observed in all of three lots (Fig. 1). The obtained kinetic parameters were 3 to 18 μM, 360 to 5200 pmol/min/10^6 viable cells, and 42 to 70 ml/min/10^6 viable cells for K_m, V_{max}, and P_{dif} (Table 1). The saturable component estimated by V_{max}/K_m ranged from 70 to 80% of the total uptake ($V_{max}/K_m + P_{dif}$) (Table 1). In lots HH-088 and HH-117, the inhibitory effect of CsA was examined (Fig. 2). In both lots, a concentration-dependent inhibitory effect was observed (Fig. 2), and the K_i values for HH-088 and -117 were 0.280 ± 0.215 and 0.685 ± 0.286 μM (mean ± computer-calculated S.E.), respectively.

Uptake Study in OATP2-Expressing MDCKII Cells. The time courses of uptake of [^{14}C]CER into human OATP2-expressing MDCKII cells and vector-transfected cells are shown in Fig. 3. The uptake of [^{14}C]CER into OATP2-expressing cells was 2.6 times higher at 9 min than that into vector-transfected cells (Fig. 3). In OATP2-expressing cells, the uptake of [^{14}C]CER observed in the presence of excess unlabeled CER (30 μM) was reduced to the same level as that in vector-transfected cells (Fig. 3). OATP2-mediated uptake of [^{14}C]CER was also inhibited by CsA in a concentration-dependent manner (Fig. 4). The K_i value for the OATP2-mediated uptake of [^{14}C]CER was 0.238 ± 0.129 μM (mean ± computer-calculated S.E.) (Fig. 4).

Metabolic Stability of [^{14}C]CER. The metabolic stability of [^{14}C]CER in human microsomes was examined. In Fig. 5, a time profile of the metabolic stability of [^{14}C]CER in pooled human microsomes is shown. As a linear metabolic rate in human microsomes was observed for up to 45 min (Fig. 5), the inhibitory effects of CsA, 10 μM quercetin (a CYP2C8 inhibitor; Ohyama et al., 2000), and 0.2 μM ketoconazole (a CYP3A4 inhibitor; Kawahara et al., 2000) on the metabolism of [^{14}C]CER were followed for 45 min. In Fig. 6a, the metabolic rates of [^{14}C]CER when incubated in human microsomes in the absence or presence of inhibitors are shown. CsA did not alter the metabolic rate of [^{14}C]CER up to a concentration of 3 μM and reduced it to, at most, 71% of the control value at 10 to 30 μM, whereas 10 μM quercetin and 0.2 μM ketoconazole reduced it to 63 and 72% of the control value, respectively (Fig. 6a). The effect of CsA on testosterone 6β-hydroxylation, which is mediated by CYP3A4, was also followed for 2 min (Fig. 6b). The metabolic rate of testosterone 6β-hydroxylation measured in the absence of inhibitors was 1560 pmol/min/mg of protein, and it was reduced to 30 and 5.9% of the control value in the presence of 3 and 30 μM CsA, respectively (Fig. 6b). It was also reduced to 6.5% of the control value by 0.2 μM ketoconazole and 52% by 10 μM quercetin (Fig. 6b).

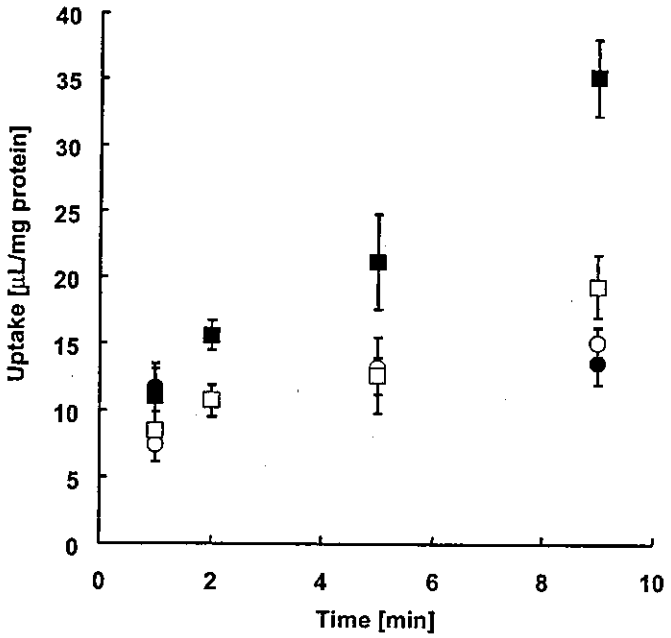

Fig. 3. Uptake of [^{14}C]CER in OATP2-expressing MDCKII cells. The uptake of [^{14}C]CER in MDCKII cells transfected with human OATP2 (■, □) or vector as a control (●, ○) was examined. The initial concentration of CER on the basal side of cells was 0.25 (■, ●) and 30 μM (□, ○). Each symbol represents the mean ± S.E. of three independent experiments.

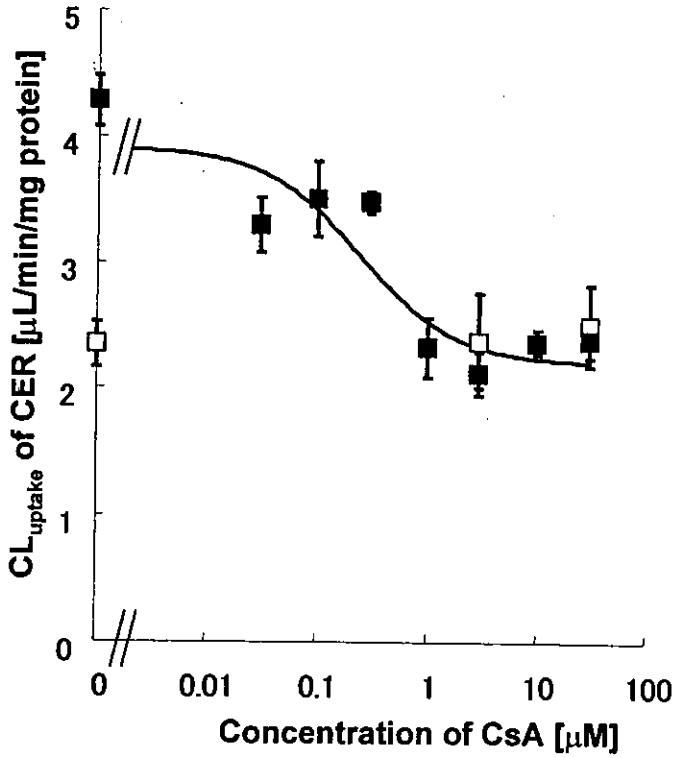

Fig. 4. Inhibitory effect of CsA on OATP2-mediated uptake of [^{14}C]CER. The inhibitory effect of CsA on the uptake of [^{14}C]CER in MDCKII cells transfected with human OATP2 (■) or vector (□) was examined. Each symbol represents the mean ± S.E. of three independent experiments. A solid line represents the fitted line for OATP2-mediated uptake of CER.

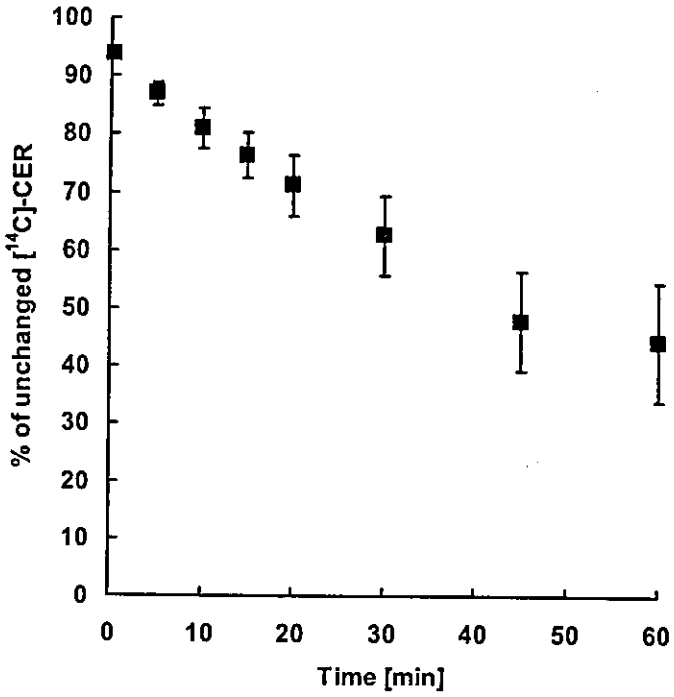

Fig. 5. Metabolic stability of [^{14}C]CER in pooled human microsomes. The metabolism of [^{14}C]CER was examined in pooled human microsomes at 37°C for 60 min. Data are shown as the percentages of unchanged [^{14}C]CER with respect to the total radioactivity. Each point represents the mean ± S.E. of three independent experiments.

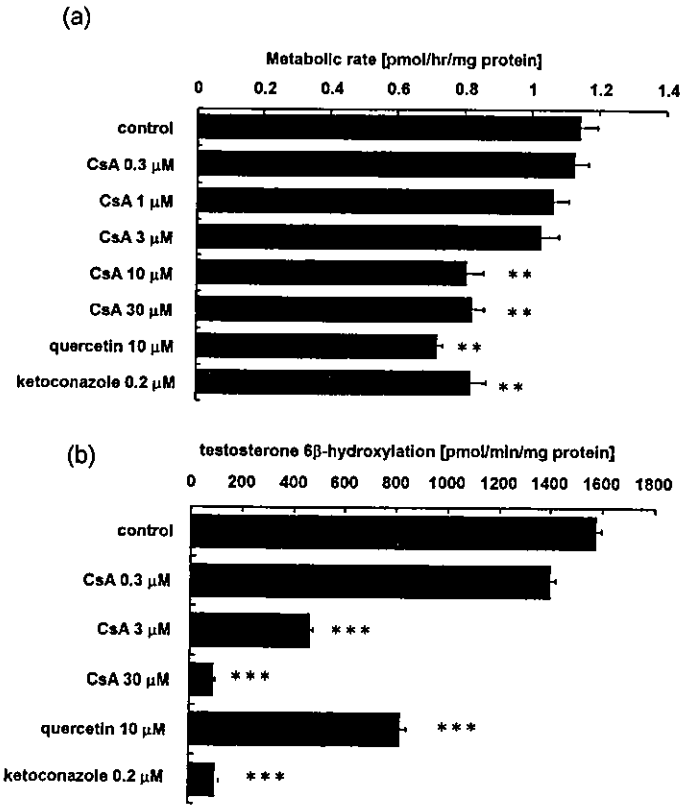

Fig. 6. The effect of CsA and other inhibitors on the metabolic rate of [^{14}C]CER (a) and testosterone 6β-hydroxylation (b). The metabolic rates of [^{14}C]CER (a) and testosterone 6β-hydroxylation in the absence or presence of CsA (0.3–30 μM), quercetin (10 μM), and ketoconazole (0.2 μM) were examined. Each bar represents the mean ± S.E. of three independent experiments. **, $p < 0.01$; ***, $p < 0.001$ significant difference by Student's t test.

Discussion

In kidney transplantation patients undergoing CsA treatment, the plasma concentrations of CER are increased (Mück et al., 1999) due to a DDI between the two drugs. In the present study, we examined the effect of CsA on the hepatic uptake and metabolism of CER, especially on its hepatic uptake, to clarify the mechanism underlying this DDI.

In vitro uptake studies in isolated hepatocytes revealed saturable transport of [^{14}C]CER in human hepatocytes (Fig. 1), suggesting the involvement of transporters in the uptake process. In this study, we found that transporter-mediated uptake accounted for 70 to 80% of the total hepatic uptake. In clinical situations, the maximum plasma concentration (C_{max}) of CER is approximately 4 nM (after a single oral dose of 0.2 mg; Mück et al., 1999), which is much lower than the K_m values (2.6–18 μM) obtained in the present study (Fig. 1 and Table 1), suggesting that the hepatic uptake of CER is largely mediated by transporters over the therapeutic range.

The present study revealed a concentration-dependent inhibition of transporter-mediated [^{14}C]CER uptake by CsA in human hepatocytes, with K_i values of 0.28 to 0.69 μM (Fig. 2). The obtained data may at least partly explain the clinically observed DDI (Mück et al., 1999). Mück et al. (1999) reported that the C_{max} and the AUC of CER in kidney transplant patients given CsA was increased 4- and 3-fold, respectively, when the C_{max} of CsA was approximately 1 μM. In the present study, the saturable component of the uptake of [^{14}C]CER was mostly inhibited in the presence of 1 μM CsA (Fig. 2). However, considering that approximately 90% of the CsA in blood is bound to plasma proteins that consist of mainly lipoproteins (Lemaire and Tillement, 1982), the clinically relevant unbound concentration of CsA is estimated to be 0.1 μM, which may not be enough to inhibit hepatic uptake of CER. This discrepancy may be explained by a number of factors. First, in the case of oral administration, the plasma concentration of CsA in the circulating blood and portal vein are different, and therefore, the concentration exposed to the liver may be much higher than that observed in the circulating blood (Ito et al., 1998; Sugiyama et al., 2002). Second, the increase in the plasma concentration of CER reported by Mück et al. (1999) could be partly due to the change in the intrinsic hepatic clearance associated with renal failure and/or kidney transplantation. In the present study, although the increase in the plasma concentration observed clinically cannot be fully predicted from the in vitro uptake study, the results suggest that the increase in the plasma concentration of CER is at least partly due to the interaction between CER and CsA involving transporter-mediated hepatic uptake.

The range of CL_{uptake} values for [^{14}C]CER observed among human hepatocytes from the three donors (Fig. 1; Table 1) may be due to the interindividual differences in the expression level and/or function of transporters, although it may be caused by other factors, such as the cell integrity being affected during the cryopreservation process. Indeed, the fact that the interindividual differences were greater for the V_{max}/K_m values, which reflect transporter-mediated uptake and can be affected by the expression level and/or intrinsic function of transporters, than for the P_{dif} values, which mainly represent passive diffusion, supports our hypothesis (Table 1). If this hypothesis is correct, there must be a wide