range of interindividual differences in the hepatic clearance and/or the extent of transporter-mediated DDI. Human hepatocytes may represent an important experimental system in the near future for evaluating the genetic and environmental factors that may be responsible for the interindividual differences in transporter functions.

In the present study, CER was shown to be a substrate of human OATP2 (Fig. 3), like pravastatin (Hsiang et al., 1999; Nakai et al., 2001). OATP2-mediated uptake of [^{14}C]CER was also inhibited by CsA (Fig. 4), and the obtained K_i value (0.24 μM) was within the same range as the values obtained in the inhibition study using human hepatocytes (0.28–0.69 μM) (Fig. 2). These results suggest that the inhibition by CsA on the uptake of CER in human hepatocytes is partly due to OATP2-mediated transport. Since OATP2 accepts a wide variety of compounds as substrates (Abe et al., 1999; Hsiang et al., 1999), these substrates in addition to CER may possibly exhibit DDI. Indeed, a DDI between pravastatin, a substrate of OATP2, and CsA was reported, which could also be due to OATP2-mediated uptake in the liver (Regazzi et al., 1993). To avoid this kind of DDI, the characterization of transporters, which are responsible for the drug uptake, and their contributions to total hepatic uptake are very important (Kouzuki et al., 1999a,b; Kusuhara and Sugiyama, 2002; Mizuno and Sugiyama, 2002; Shitara et al., 2002). The increase in the plasma concentration of drugs associated with a transporter-mediated DDI may be quantitatively predicted from in vitro studies that determine the extent of inhibition of transport in hepatocytes and/or in transporter-expressing cells (Ueda et al., 2001).

We also examined the effect of CsA on the metabolism of [^{14}C]CER in human microsomes (Fig. 6a). CsA did not markedly reduce the metabolic rate of [^{14}C]CER up to a concentration of 3 μM, and 10 to 30 μM CsA reduced it only to 70% of the control value (Fig. 6a). On the other hand, 30 μM CsA markedly reduced testosterone 6β-hydroxylation, which was mediated by CYP3A4, to 30% of the control value (Fig. 6b). To explain this different effect of CsA on the metabolisms of CER and testosterone, we examined the effect of ketoconazole, a potent CYP3A4 inhibitor (Kawahara et al., 2000). As the K_i values of ketoconazole for the inhibition of CYP2C8 and CYP3A4 functions are 2.5 and 0.03 μM, respectively (Kawahara et al., 2000; Ong et al., 2000), 0.2 μM ketoconazole should be enough to inhibit most of the CYP3A4-mediated metabolism of [^{14}C]CER and have only a slight effect on that mediated by CYP2C8. Indeed, we have confirmed that 0.2 μM reduced the CYP3A4-mediated metabolism of testosterone to 7% of the control value. However, 0.2 μM ketoconazole reduced the metabolism of [^{14}C]CER only to 72% of the control (Fig. 6a). This study supports that CYP3A4 plays a limited role in the metabolism of CER as previously reported by Mück (2000), and CYP3A4 inhibitors, such as ketoconazole and CsA, reduce the metabolism of CER to only a limited extent. From the present study, the contribution of CYP3A4 to the total metabolism of CER is estimated to be approximately 38% (Fig. 6a). We also examined the effect of quercetin, a CYP2C8 inhibitor (Ohyama et al., 2000). As the K_i value of quercetin for the inhibition of CYP2C8-mediated metabolism is 1.3 μM (Rahman et al., 1994), 10 μM quercetin should be enough to inhibit most of the CYP2C8-mediated metabolism of [^{14}C]CER, although it also reduces the CYP3A4-mediated metabolism of testosterone to 50% of the control value (Fig. 6b). In the presence of 10 μM quercetin, the metabolism of [^{14}C]CER was reduced to 63% of the control value (Fig. 6a). This result supports a contribution of CYP2C8 to the metabolism of CER is less than 37%, considering that the CYP3A4-mediated metabolism is partly inhibited by 10 μM quercetin (Fig. 6b). The present study suggests that at low concentrations (<3 μM), CsA does not inhibit the metabolism of CER (Fig. 6a), although it does inhibit its transporter-mediated hepatic uptake with a much lower concentration (<1 μM) (Fig. 2). This confirms that it is less likely that the DDI between CER and CsA is due to the metabolism of CER.

Recently, a severe DDI between CER and gemfibrozil was reported, and in the USA, 31 deaths from severe rhabdomyolysis in patients taking CER were reported, of whom 12 were taking concomitant gemfibrozil (Charatan, 2001). This resulted in the withdrawal of CER from the market. It is still unknown whether this severe DDI is mainly due to the pharmacokinetic event (i.e., the change of the plasma concentration of CER caused by gemfibrozil) or due to the nonpharmacokinetic event (for example, an increased formation of toxic metabolites of CER or the effects on the energy of the cell, which may lead to rhabdomyolysis). In the present study, however, we tried to clarify the mechanism of the effect of CsA on the pharmacokinetics of CER and found that the inhibition of the OATP2-mediated hepatic uptake of CER by CsA was the major mechanism. There is also a report that the AUC of CER was increased 4.2-fold when coadministered with gemfibrozil (Mueck et al., 2001), which may be one mechanism of this serious DDI. At the time of this report, Prueksaritanont et al. (2002) reported that both oxidation and glucuronidation of CER in human liver microsomes were inhibited by gemfibrozil with IC_{50} values of 82 and 87 to 220 μM, respectively, which at least in part explains the mechanism of DDI between CER and gemfibrozil. However, considering the relatively high plasma protein binding of gemfibrozil (plasma unbound fraction of 1.4–3%; Todd and Ward, 1988), the unbound therapeutic concentration of gemfibrozil is estimated to be less than 7.5 μM (total C_{max}, up to 250 μM; Prueksaritanont et al., 2002), which is much lower than the recently reported IC_{50} values (80–220 μM) for CER metabolism. Therefore, the increase in the AUC of CER caused by the DDI may not necessarily be accounted for only by the inhibition of metabolism. Although there has been no report concerning the effect of gemfibrozil on the transporter-mediated hepatic uptake of CER, this should also be examined in a future study. In conclusion, we should pay more attention to DDI that may originate from the inhibition of transporter-mediated hepatic uptake, since it may occur with a large number of drug combinations when their elimination (metabolism and/or biliary excretion) takes place following transporter-mediated hepatic uptake.

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Involvement of Organic Cation Transporter 1 in Hepatic and Intestinal Distribution of Metformin

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ABSTRACT

Metformin, a biguanide, is widely used as an oral hypoglycemic agent for the treatment of type 2 diabetes mellitus. The purpose of the present study was to investigate the role of organic cation transporter 1 (Oct1) in the disposition of metformin. Transfection of rat Oct1 cDNA results in the time-dependent and saturable uptake of metformin by the Chinese hamster ovary cell line with K_m and V_{max} values of 377 μM and 1386 pmol/min/mg of protein, respectively. Buformin and phenformin, two other biguanides, were also transported by rOct1 with a higher affinity than metformin: their K_m values were 49 and 16 μM, respectively. To investigate the role of Oct1 in the disposition of metformin, the tissue distribution of metformin was determined in Oct1 gene-knockout mice after i.v. administration. Distribution of metformin to the liver in Oct1(−/−) mice was more than 30 times lower than that in Oct1(+/+) mice, and can be accounted for by the extracellular space. Distribution to the small intestine was also decreased in Oct1(−/−) mice, whereas that to the kidney as well as the urinary excretion profile showed only minimal differences. In conclusion, the present findings suggest that Oct1 is responsible for the hepatic uptake as well as playing a role in the intestinal uptake of metformin, whereas the renal distribution and excretion are mainly governed by other transport mechanism(s).

Metformin, a biguanide, has been used for the treatment of hyperglycemia in patients with type 2 diabetes mellitus. It was developed during the late 1950s, first marketed in Europe in 1959 and available in the United States in 1995. Metformin seems to ameliorate hyperglycemia by improving peripheral sensitivity to insulin, reducing gastrointestinal glucose absorption and hepatic glucose production (Caspary and Creutzfeldt, 1971; Hundal et al., 2000; Borst and Snellen, 2001), although the exact mechanism for its pharmacological action has not yet been fully determined. In recent years, metformin has also become available for the treatment of polycystic ovary syndrome (Velazquez et al., 1994; Nestler, 2001) and has been found to improve vascular function (Katakam et al., 2000), prevent pancreatic cancer (Schneider et al., 2001), and reverse fatty liver diseases (Lin et al., 2000) in experimental animals. Thus, a reevaluation of its pharmacological activity is now underway.

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Metformin is extensively eliminated from the kidney via glomerular filtration and tubular secretion, approximately 79 to 86% of an intravenous dose being recovered in urine in humans (Sirtori et al., 1978; Tucker et al., 1981). However, despite its long clinical usage, the mechanism underlying its systemic elimination is still unknown. Inhibition of the renal tubular secretion of metformin occurs during its coadministration with cimetidine, resulting in increased systemic exposure of metformin (Somogyi and Gugler, 1987). Cimetidine, a cationic compound, is also known to be cleared via tubular secretion (Somogyi and Gugler, 1983). Grundemann et al. (1999) reported that cimetidine is a substrate of rat organic cation transporters (rOct1 and rOct2), leading to the possibility of the involvement of organic cation-specific transporters in the urinary excretion of metformin.

Octs are a family of polyspecific organic cation transporters responsible, at least in part, for the uptake of organic cations, including xenobiotics and endogenous compounds from the systemic circulation, maintaining body fluid homeostasis, and acting as a defense system against toxic agents. Because rOct1 (Slc22a1) was first cloned from the kidney by Grundemann et al. (1994), at least two other members of the OCT

ABBREVIATIONS: Oct, organic cation transporter; rOct, rat organic cation transporter; CHO, Chinese hamster ovary; HPLC, high-performance liquid chromatography; TEA, tetraethylammonium; CL_{total}, systemic clearance; CL_{renal}, renal clearance; AUC, area under the concentration-time curve.

family, Oct2/OCT2 (Slc22a2/SLC22A2) and Oct3/OCT3 (Slc22a3/SLC22A3), have been identified in rats, mice, and humans (Koepsell et al., 1999; Inui et al., 2000). rOct1 is expressed in the liver and kidney, whereas rOct2 is expressed mainly in the kidney. Based on these findings, in the present study, we attempted to investigate the possible involvement of Oct1 in the disposition of metformin. Transfection of rOct1 cDNA results in the saturable uptake of metformin as well as two other biguanides, buformin and phenformin, in a Chinese hamster ovary (CHO) cell line, suggesting that these biguanides are substrates of rOct1. Because the Oct1 gene-knockout mouse has recently been developed by Jonker et al. (2001), further investigations with this mouse were also performed to determine the contribution of Oct1 to the tissue distribution of metformin.

Experimental Procedures

Chemicals and Materials. Metformin, phenformin hydrochloride, and cimetidine were purchased from Sigma-Aldrich (St. Louis, MO). Buformin hydrochloride, acetonitrile of high-performance liquid chromatography (HPLC) grade, and diethyl ether were purchased from Wako Pure Chemicals (Osaka, Japan). Saline used for infusion was purchased from Otsuka Pharmaceutical Co. Ltd. (Tokyo, Japan) and pentobarbital was from Dainippon Pharmaceutical (Osaka, Japan). Deionized and distilled water, successively purified by the Milli-Q system (Millipore, Bedford, MA), was used for reagent preparation. All other chemicals were analytical grade and commercially available.

Cell Culture and Transfection. The parent CHO-K1 cells (JCRB9018) purchased from Japanese Health Science Research Resources Bank (Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium/F-12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C with 5% CO_2 and 95% humidity. The cDNA fragment was amplified by reverse transcription-polymerase chain reaction with the total RNA of rat liver using an RNA-PCR kit (Takara Shuzo, Osaka, Japan). For polymerase chain reaction, the primers were prepared based on the sequence reported previously (Grundemann et al., 1994). The sequences of the forward and reverse primers were 5′-TTTCGCCTGCTGCAGGGCATGGTC-3′ and 5′-TGATGAAGGCCGCGGGGAATTCCA-3′, respectively. The amplified fragment was used for library screening. For cDNA screening, the cDNA libraries were constructed from Sprague-Dawley rat liver using the SuperScript Choice system (Invitrogen). Briefly, poly(A)$^+$ RNA was fractionated by sucrose density gradient and fractions containing RNA at approximately 2 kilobase pairs were used as a template for cDNA construction using reverse transcriptase and Oligo(dT) primer. After two rounds of screening, single positive colonies were isolated. After coinfection with the M13 helper phage (ExAssist; Stratagene, La Jolla, CA), the cDNA was excised in a pBluescript II SK(−) plasmid and rescued by SOLO strain. The insert was subcloned into the vector pcDNA3.1 (Invitrogen) cut with the restriction enzymes Xho-I and Sal-I. The nucleotide sequence of the subcloned cDNA insert was determined using a fluorescence DNA sequencer 377 (Applied Biosystems, Foster City, CA). The rOct1/pcDNA3.1 so prepared and pcDNA3.1 alone were transfected into CHO-K1 cells with LipofectAMINE (Invitrogen) according to the manufacturer's protocol, and stably transfected cells were selected by G418 (0.6 mg/ml) (Invitrogen) in the culture medium. Then, 7 to 14 days after transfection, several colonies were selected both by Northern blot analysis and by uptake of tetraethylammonium (TEA), a typical substrate of the OCT family. After selection, the cells were maintained by adding G418 (0.4 mg/ml) to the culture medium.

Uptake Study. Uptake of biguanides by the cells was measured using 12-well cell culture dishes (BD Biosciences, Franklin Lakes, NJ). Cells were seeded onto plates at a density of 1.0×10^5 cells/well. The incubation buffer for the uptake experiments contained 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, and 5 mM HEPES pH 7.4, and the uptake study was performed at 37°C. After removing the culture medium, cells were washed twice with incubation buffer and preincubated for 5 min. Then, substrate solution was added to the dish and, at designated times, uptake was terminated by adding 1 ml of ice-cold buffer. The medium was rapidly aspirated and cells were rinsed twice. Cells were lysed with 300 μl/well of 0.5% SDS at 37°C for 1 h, and 200 μl was used for HPLC determination after deproteination with 3 times volume of acetonitrile. The remaining 25 μl of cell lysate was used to determine the protein concentration by the method of Lowry (1951), with bovine serum albumin as a standard. Uptake was given as the ratio of the amount of ligand associated with the cells divided by the medium concentration. Because the initial velocity of uptake of metformin, buformin, and phenformin was linear up to 5 min, uptake was terminated at 5 min to estimate K_m and V_{max} by the following equation:

$$V/S = V_{max}/(K_m + S) \quad (1)$$

where V is the initial uptake velocity and S is the substrate concentration.

Tissue Distribution of Metformin. Female Oct1(−/−) and Oct1(+/+) FVB mice (12–16 weeks old) were used in the present study (Jonker et al., 2001). Mice were housed at a room temperature of 24 ± 1°C and allowed food and water ad libitum. After anesthesia with intraperitoneal sodium pentobarbital (50 mg/kg), the bladder was catheterized for urine collection. Metformin (5.0 mg/kg) was administered via the tail vein in a volume of 0.1 ml/10 g of body weight. Blood samples were collected by cardiac puncture from an angular vein at 1, 5, and 10 min after administration. Urine was collected by washing the bladder with 0.2 ml of saline. Mice were killed and liver, kidney, small intestine, and colon were removed immediately. Each segment of the small intestine and colon was rapidly washed twice with ice-cold saline to remove residues and then dried with filter paper. The duodenal segment was obtained from the first 5-cm portion of the intestine closest to the stomach. The ileum was obtained from the final 5 cm of the intestine, just proximal to the cecum. The middle 10 cm of the rest of the segment was designated as the jejunum. The colon was a segment 3 cm in length immediately after the cecum. All the tissues were weighed, and portions were then homogenized with 4 volumes of PBS. Both the homogenized tissues and blood samples were deproteinized with acetonitrile as described above and evaporated to dryness. Pellets were dissolved in 200 μl of water for HPLC analysis. In the other groups of mice, urine was collected up to 1 h at 20-min intervals. The systemic clearance (CL_{total}) and renal clearance (CL_{renal}) were estimated, respectively, from the following equations:

$$CL_{total} = Dose/AUC_{(0-\infty)} \quad (2)$$

$$CL_{renal} = X_{urine(0-60)}/AUC_{(0-60)} \quad (3)$$

where $AUC_{(0-\infty)}$ and $AUC_{(0-60)}$ are the area under plasma concentration-time curve up to infinity and 60 min, respectively, and these values were calculated by integration of the biexponential equation, which was obtained by fitting to the plasma concentration profile. $X_{urine(0-60)}$ is the cumulative urinary excretion up to 60 min.

Coinfusion of Metformin with Cimetidine. Male Sprague-Dawley rats (8 weeks old, 250–280 g of body weight; Charles River Japan, Kanagawa, Japan) were anesthetized with diethyl ether and both the femoral vein and bladder were catheterized with polyethylene tubing for infusion and urine collection, respectively. Infusion was performed by a basic syringe pump (Harvard Apparatus, Holliston, MA). Metformin dissolved in saline was administered at a loading dose of 0.5 mg/kg of body weight, followed by constant infusion at 0.9 mg/kg/h for 4 h. Cimetidine was administered at the

loading dose of 24 mg/kg and an infusion rate of 50 mg/kg/h. The administered volume of saline was 8.0 ml/kg/h. Blood samples were collected from the cervical vein at 60, 120, 180, and 240 min. Urine was collected by washing the bladder with 0.5 ml of saline at 1-h intervals. Blood samples, deproteinized with 4 volumes of acetonitrile, and urine were subjected to HPLC.

HPLC Analysis. The HPLC system involved a model L-7100 pump and a model L-7400 UV monitor (Hitachi, Tokyo, Japan) with 300- × 3.9-mm i.d. C_{18} μBondapak (10 μm) purchased from Waters (Milford, MA). The mobile phase consisted of 0.01 M phosphate buffer (pH 6.5)/acetonitrile (30:70) at the flow rate of 1 ml/min. The wavelength of the UV detection was at 236 nm. The volume used for HPLC was 50 μl.

Statistical Analysis. Statistical analysis was performed by Student's *t* test to identify significant differences between various treatment groups.

Results

Uptake of Biguanides in rOct1-Transfected CHO Cells. The time profile for the uptake of metformin (100 μM), buformin (10 μM), and phenformin (1.0 μM) by rOct1- and vector-transfected CHO cells is shown in Fig. 1, A, C, and E, respectively. Uptake was much higher in rOct1-transfected cells than in vector-transfected cells (Fig. 1, A, C, and E). Because the uptake of each compound was linear up to 5 min (Fig. 1, A, C, and E), the initial uptake was assessed as the uptake for 5 min at various substrate concentrations (Fig. 1, B, D, and F). Saturation of uptake was observed in rOct1-transfected cells, and an Eadie-Hofstee plot revealed a single saturable component for each compound (Fig. 1, B, D, and F). The kinetic parameters for the uptake of metformin, buformin, and phenformin are shown in Table 1. Phenformin has a much higher affinity, but a lower capacity, for rOct1 than metformin, and the intrinsic clearance (V_{max}/K_m) of phenformin was ~2 to 3 times higher than that of metformin (Table 1). TEA, used in the present study as a positive control, exhibited K_m and V_{max} values of 84 μM and 320 pmol/min/mg of protein, respectively. This K_m was not very different from that found in previous reports (47–100 μM; Urakami et al., 1998; Dresser et al., 2000).

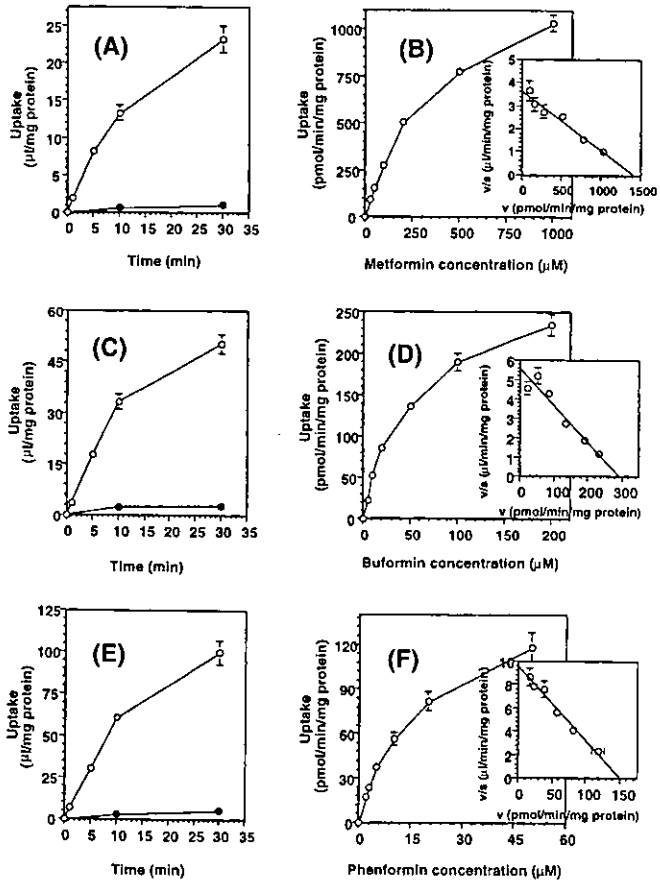

Fig. 1. Uptake of metformin in rOct1-transfected (open symbols) and pcDNA3.1-transfected (closed symbols) CHO cells. The time profile (A, C, and E) and concentration dependence (B, D, and F) of the uptake of metformin (A and B), buformin (C and D), and phenformin (E and F) are shown. The inset in B, D, and F represents Eadie-Hofstee plots. Results are mean ± S.E. (n = 3). Error bars for some points lie within the symbols.

Plasma Concentration and Tissue Distribution of Metformin in Oct1(−/−) and Oct1(+/+) Mice. The time profiles of the plasma concentration and tissue distribution of metformin at 10 min in Oct1(−/−) and Oct1(+/+) mice are shown in Fig. 2. The plasma concentration of metformin at 5 and 10 min was almost comparable for Oct1(−/−) and Oct1(+/+) mice, whereas a small (less than 2-fold) but significant difference was observed at 1 min (Fig. 2A). The difference at these earlier time points suggests that there is a smaller distribution volume in Oct1(−/−) mice than in Oct1(+/+) mice. The liver concentration of metformin was approximately 30 times higher in Oct1(+/+) mice than Oct(−/−) mice (Fig. 2B). The amount of metformin associated with the liver at 10 min was 24.0 ± 2.4 and 0.690 ± 0.065% of dose in Oct1(+/+) and Oct1(−/−) mice, respectively. A 3- to 7-fold higher distribution in Oct1(+/+) than Oct(−/−) mice was also observed in the duodenum, jejunum, and ileum (Fig. 2B). On the other hand, the distribution of metformin to the kidney was almost identical for the two types of mice and the difference in the distribution to the colon was minimal (Fig. 2B). The amount of metformin associated with the kidney was 34.2 ± 6.7 and 32.1 ± 7.0% of dose in Oct1(+/+) and Oct1(−/−) mice, respectively.

Urinary Excretion of Metformin in Oct1(−/−) and Oct1(+/+) Mice. The urinary excretion of metformin was chased in Oct1(−/−) and Oct1(+/+) mice (Table 2). In all, 55 to 70% of the dose was recovered in the urine up to 60 min after i.v. administration. There was only a minimal difference in both the CL_{renal} and CL_{total} between Oct1(−/−) and Oct1(+/+) mice (Table 2).

Coinfusion of Metformin with Cimetidine. To demonstrate the cation-specific renal excretion of metformin in rodents, the effect of coadministration of cimetidine on the urinary excretion of metformin was investigated in rats (Fig. 3). The plasma concentration of metformin was much higher in the presence of cimetidine than in its absence (Fig. 3A). On

TABLE 1

Kinetic parameters for the uptake of biguanide compounds in the rOct1-transfected CHO cell line

K_m and V_{max} values were determined by nonlinear regression analysis. Data are shown as mean ± S.E. (n = 3).

Compounds	K_m	V_{max}	V_{max}/K_m
	μM	pmol/min/mg protein	
Metformin	377 ± 38	1386 ± 101	3.68 ± 0.46
Buformin	48.8 ± 6.9	282 ± 25	5.78 ± 0.96
Phenformin	15.6 ± 1.1	150 ± 7	9.62 ± 0.81

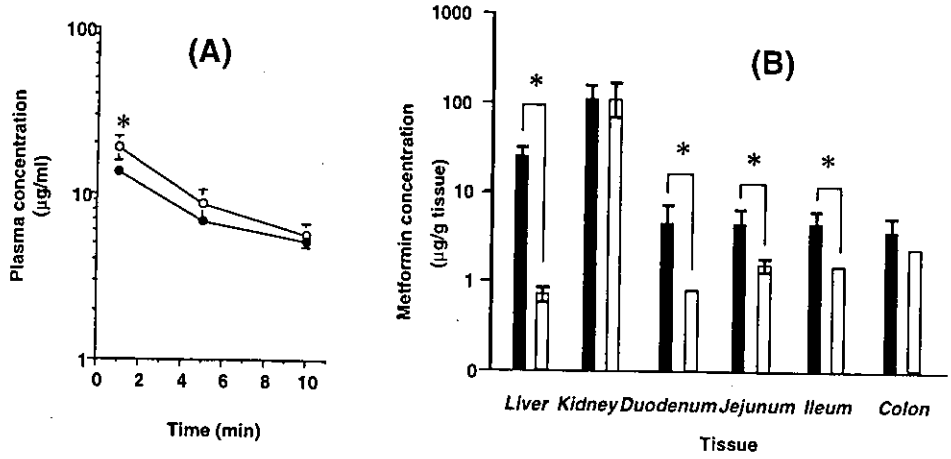

Fig. 2. Plasma concentration profile (A) and tissue distribution (B) of metformin in Oct1(−/−) (open symbols) and Oct1(+/+) mice (closed symbols). Metformin was injected intravenously at 5.0 mg/kg, and tissues were excised at 10 min. Results are mean ± S.E. (n = 3–4). *, significant difference ($p < 0.05$).

TABLE 2

Renal clearance analysis of metformin in Oct1(+/+) and (−/−) mice

Metformin was intravenously injected at a dose of 5.0 mg/kg. Data are shown as mean ± S.E. (n = 3).

	Recovery (% of dose)			$CL_{renal(0-60)}$	CL_{total}
	0–20 min	20–40 min	40–60 min		
				ml/min/kg	
Oct1(+/+)	38.3 ± 2.0	10.9 ± 3.4	5.73 ± 0.23	14.0 ± 3.1	18.3 ± 1.7
Oct1(−/−)	47.4 ± 3.5	13.8 ± 4.2	8.94 ± 0.19	14.7 ± 2.7	16.1 ± 1.7

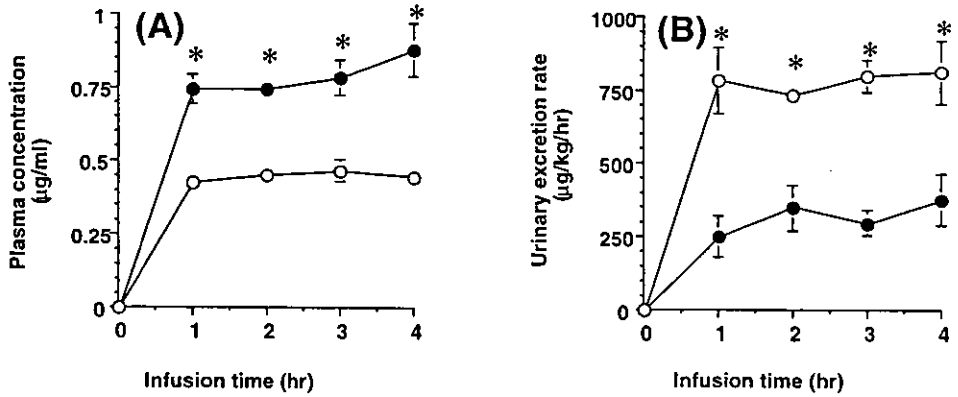

Fig. 3. Plasma concentration (A) and urinary excretion (B) of metformin in the presence and absence of cimetidine in rats. Metformin was infused intravenously at 0.9 mg/kg/h after a loading dose of 0.5 mg/kg with (closed symbols) or without (open symbols) coinfusion of cimetidine at 50 mg/kg/h after a loading dose of 24 mg/kg. Results are mean ± S.E. (n = 3–4). *, significant difference ($p <$ 0.05).

the other hand, the urinary excretion of metformin was significantly reduced in the presence of cimetidine (Fig. 3B).

Discussion

Despite the widespread use of the biguanide metformin in the treatment of hyperglycemia, the mechanism(s) underlying its disposition has not yet been clarified. Renal excretion is the major elimination pathway for metformin in humans and is much higher than the glomerular filtration rate (Pentikainen et al., 1979), suggesting involvement of tubular secretion systems, although the detailed mechanism has not yet been determined. Metformin exerts its antidiabetic effects, at least partly, via a direct and/or indirect inhibitory effect on complex 1 of the mitochondrial respiratory chain in hepatocytes (El-Mir et al., 2000). Considering that biguanide compounds are positively charged at physiological pH, there may be membrane transport system(s) for these compounds in the liver. Thus, it is important to clarify their disposition mechanism(s) to understand the factors that may affect the pharmacokinetics and pharmacodynamics of biguanides.

Gene knockout mice for Oct1, which accepts a variety of types of organic cations as substrates, have recently been established (Jonker et al., 2001). Considering that biguanides are also cationic compounds, the involvement of Oct1 in the disposition of biguanides was investigated in the present study using rOct1-transfected cells and knockout mice as the first step to clarify the pharmacokinetic mechanism(s). The increase in the saturable uptake of the three biguanides by rOct1 transfection suggests that these compounds are substrates of rOct1 (Fig. 1; Table 1). The distribution of metformin to the liver and three segments of the small intestine is much lower in Oct1(−/−) mice, compared with Oct1(+/+) mice, suggesting that Oct1 may be involved in the distribution to these tissues (Fig. 2). In the liver and duodenum of Oct1(−/−) mice, the K_p values of metformin were 0.13 and 0.14, respectively, these values being comparable with the extracellular volume of these tissues (Tsuji et al., 1983). Because the plasma concentration at this time point (5–10 μg/ml corresponding to 30–60 μM) is much lower than the K_m of metformin for Oct1, Oct1-mediated transport may not

be saturated. Thus, the distribution of metformin to these tissues seems to be mainly governed by Oct1 at least under linear conditions. These results are compatible with previous observations (Grundemann et al., 1994; Schweifer and Barlow, 1996; Jonker et al., 2001) and suggest that Oct1 localization in the basolateral membrane in the liver may explain its possible function as an uptake mechanism for organic cations (Meyer-Wentrup et al., 1998; Urakami et al., 1998; Karbach et al., 2000). Although the localization of Oct1 in the small intestine has not been clarified yet, the present findings suggest that it has a possible role in the basolateral uptake of biguanides, although further studies are needed to demonstrate this hypothesis.

On the other hand, neither the distribution of metformin to the kidney nor its renal excretion showed any clear difference between Oct1 (−/−) and Oct1 (+/+) mice (Fig. 2B; Table 2), suggesting that Oct1 is not the major transporter involved in the renal uptake of metformin. Because the intrinsic transport activity (V_{max}/K_m) of metformin by rOct1 is comparable with that of TEA, and the renal uptake of TEA is very similar to the blood flow rate, the renal uptake of metformin may also be limited by the blood flow, suggesting that the change in intrinsic renal uptake due to the lack of Oct1 may result only in a minimal difference in renal uptake in vivo. Therefore, the result obtained in vivo does not fully exclude the possible role of Oct1 in the renal uptake of metformin. Nevertheless, cimetidine inhibits the urinary excretion of metformin and increases its systemic exposure in rats (Fig. 3). A similar drug-drug interaction has also been reported in humans (Somogyi and Gugler, 1987), suggesting that a cation-specific mechanism(s) is involved in the urinary excretion of metformin, irrespective of the species. However, the difference between the dose of cimetidine and metformin was more than 50-fold in rats (Fig. 3), whereas the difference in humans was less than 2-fold (Somogyi and Gugler, 1987). Therefore, the mechanism underlying this drug interaction both in humans and rats should be discussed once additional studies have been performed. rOct2 and rOct3 are also expressed in kidney (Sugawara-Yokoo et al., 2000; Wu et al., 2000) and may be involved in the uptake and/or secretion of cationic compounds. rOct2 is also expressed in the basolateral side of the kidney (Karbach et al., 2000; Sugawara-Yokoo et al., 2000), although the substrate recognition of rOct2 has not yet been clearly distinguished from that of rOct1 (Urakami et al., 1998; Arndt el al., 2001). In a recent study using Oct3 (−/−) mice, no difference in the renal disposition of 1-methyl-4-phenylpyridinium was observed (Zwart et al., 2001). Based on this information, Oct2 is the most likely candidate for the control of the renal excretion of metformin, and additional studies are now being performed in our laboratory to test this hypothesis.

The present findings highlight the importance of Oct1 as the mechanism for the hepatic distribution of metformin. Its pharmacological actions include a reduction in glucose production and inhibition of the mitochondrial respiratory chain in hepatocytes (El-Mir et al., 2000; Hundal et al., 2000; Owen et al., 2000). The existence of the uptake system for metformin may be compatible with the previous finding that a lower metformin concentration is required to inhibit the respiratory chain in isolated hepatocytes than in isolated mitochondria (Owen et al., 2000). However, the metformin concentration (1–5 mM) that inhibits oxygen consumption in rat hepatocytes is higher than the K_m for rOct1 (Table 2), suggesting that rOct1 might be, at least partly, saturated at such an effective concentration. Therefore, further studies are required to clarify the functional relationship between the transport of biguanides by rOct1 and their pharmacological action in hepatocytes.

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Involvement of Organic Cation Transporter 1 in the Lactic Acidosis Caused by Metformin

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ABSTRACT

Biguanides are a class of drugs widely used as oral antihyperglycemic agents for the treatment of type 2 diabetes mellitus, but they are associated with lactic acidosis, a lethal side effect. We reported previously that biguanides are good substrates of rat organic cation transporter 1 (Oct1; *Slc22a1*) and, using Oct1(−/−) mice, that mouse Oct1 is responsible for the hepatic uptake of a biguanide, metformin. In the present study, we investigated whether the liver is the key organ for the lactic acidosis. When mice were given metformin, the blood lactate concentration significantly increased in the wild-type mice, whereas only a slight increase was observed in Oct1(−/−) mice. The plasma concentration of metformin exhibited similar time profiles between the wild-type and Oct1(−/−) mice, suggesting that the liver is the key organ responsible for the lactic acidosis. Furthermore, the extent of the increase in blood lactate caused by three different biguanides (metformin, buformin, and phenformin) was compared with the abilities to reduce oxygen consumption in isolated rat hepatocytes. When rats were given each of these biguanides, the lactate concentration increased significantly. This effect was dose-dependent, and the EC_{50} values of metformin, buformin, and phenformin were 734, 119, and 4.97 μM, respectively. All of these biguanides reduced the oxygen consumption by isolated rat hepatocytes in a concentration-dependent manner. When the concentration required to reduce the oxygen consumption to 75% of the control value (from 0.40 to 0.29 μmol/min/mg protein) was compared with the EC_{50} value obtained in vivo, a clear correlation was observed among the three biguanides, suggesting that oxygen consumption in isolated rat hepatocytes can be used as an index of the incidence of lactic acidosis.

Metformin, a biguanide, is used for the treatment of hyperglycemia in patients with type 2 diabetes mellitus. It was developed during the late 1950s, first marketed in Europe in 1959, and has been available in the United States since 1995 (Davidson and Peters, 1997). Metformin seems to ameliorate hyperglycemia by improving peripheral sensitivity to insulin, reducing gastrointestinal glucose absorption and hepatic glucose production (Caspary and Creutzfeldt, 1971; Hundal et al., 2000; Borst and Snellen, 2001). Recently, metformin has also become available for the treatment of polycystic ovary syndrome (Velazquez et al., 1994; Nestler, 2001) and has been found to improve vascular function (Katakam et al., 2000), prevent pancreatic cancer (Schneider et al., 2001), and reverse fatty liver diseases (Lin et al., 2000) in experimental animals. Thus, a re-evaluation of its pharmacological activity is now underway. Lactic acidosis is a severe adverse effect of biguanides, and phenformin was withdrawn from the market in the 1970s for this reason (Assan et al., 1975; Kwong and Brubacher, 1998). Lactic acidosis is a life-threatening condition characterized by low arterial pH (<7.35) and elevated arterial lactate levels (5.0 mEq/l in humans), and more than 50% of the patients died when lactic acidosis took place under phenformin administration (Brown et al., 1998; Kwong and Brubacher, 1998). Metformin also associated with lactic acidosis in the lower incidence of approximately 3 cases per 100,000 patients per year, compared with a 10- to 20-fold higher incidence for phenformin (Pearlman et al., 1996; Lalau and Race, 2000; Kruse, 2001). Lactic acidosis is observed in patients with renal dysfunction, and because renal secretion is the major elimination route of biguanides (Davidson and Peters, 1997), renal dysfunction will cause a significant increase in plasma biguanide concentration, resulting in lactic acidosis. Metformin has been shown to reduce the oxygen consumption and glucose production in isolated hepatocytes in a concentration-dependent manner (El-Mir et al., 2000; Owen et al., 2000). This has been explained by the inhibition of mitochondrial respiratory complex I, although whether

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ABBREVIATIONS: Oct1, organic cation transporter 1; rOct1, rat organic cation transporter 1; AUC, area under the concentration-time curve; HPLC, high-performance liquid chromatography.

this occurs via direct or indirect inhibition remains unknown (El-Mir et al., 2000; Owen et al., 2000). Excessive inhibition of mitochondrial respiration by biguanide may cause lactic acidosis.

Recently, we demonstrated that the biguanides are good substrates of rat organic cation transporter 1 (Oct1; *Slc22a1*) (Wang et al., 2002). Oct1 is a polyspecific transporter for small and hydrophilic organic cations such as tetraethylammonium and 1-methyl-4-phenylpyridinium (Grundemann et al., 1994; Dresser et al., 2000; Inui et al., 2000). Oct1 is abundantly expressed in the liver and, to a lesser extent, in the kidney, where it is localized in the basolateral membrane. Previously, we demonstrated that the order of the transport activity (V_{max}/K_m) of biguanides by rOct1 is phenformin > buformin > metformin. Although no significant reduction was observed in the urinary excretion of metformin, the hepatic uptake of metformin was reduced markedly in Oct1(−/−) mice, and the distribution volume of metformin in the liver of Oct1(−/−) was very close to the hepatic extracellular space (Wang et al., 2002). Therefore, the Oct1(−/−) mouse is a good animal model in which to examine whether the liver is a key organ in the increase of blood lactate concentration.

In the present study, the increase in blood lactate concentration was compared in wild-type and Oct1(−/−) mice when metformin was given by constant intravenous infusion. The increase in lactate concentration was investigated in rats after the infusion of biguanides (phenformin, buformin, and metformin) at different rates, and their potency was compared with that in the reduction in oxygen consumption in isolated rat hepatocytes.

Materials and Methods

Animals and Materials. Male Sprague-Dawley rats (8 weeks old, 250–280 g of body weight; Charles River Japan Inc., Kanagawa, Japan) and male Oct1(−/−) and wild-type FVB mice (12–16 weeks old) used in the present study (Jonker et al., 2001) were housed at a room temperature of 24 ± 1°C with food and water ad libitum. Metformin and phenformin were purchased from Sigma Chemical (St. Louis, MO). Buformin and perchloric acid (60%) were purchased from Wako Pure Chemicals (Osaka, Japan). Pentobarbital was from Dainippon Pharmaceutical (Osaka, Japan). The L(+)-lactate kit was purchased from Sigma Diagnostics (St. Louis, MO), and the model 5300 biological oxygen monitor was purchased from YSI Inc. (Yellow Springs, OH). All other chemicals were of analytical grade and were commercially available.

Lactic Acidosis Study of Metformin in Mice. Mice were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg), and the femoral vein was catheterized with polyethylene tubing for infusion. Infusion was performed using a basic syringe pump (Harvard Apparatus Inc., Holliston, MA). Metformin dissolved in saline was administered at a rate of 8.0 ml/h/kg and a dose of 150 mg/h/kg for 3.5 h. Blood samples used for the determination of metformin were collected from an angular vein at 90, 150, and 210 min. After centrifugation, the plasma samples were deproteinized with four times their volume of acetonitrile and then subjected to HPLC. Blood samples used for the determination of lactate concentration were collected from the tail vein. Whole blood was mixed with double the volume of perchloric acid (8%) and vortexed. After centrifugation, the upper solution was used for lactate determination according to the manufacturer's protocol. After sampling at 210 min, the mice were killed, and the liver and femoral muscles from the opposite femur that had not been catheterized were removed immediately. The removed liver and muscles were homogenized with 4 volumes of phosphate-buffered saline, deproteinized with acetonitrile, and evaporated to dryness. Pellets were dissolved in 200 μl of water for HPLC analysis.

Lactic Acidosis Study of Biguanides in Rats. Rats were anesthetized with diethyl ether, and the femoral vein was catheterized with polyethylene tubing for infusion. Infusion was performed using the basic syringe pump. Metformin, buformin, and phenformin dissolved with saline were administered for 4 h. The constant infusion doses were 250, 175, 100, 50, and 25 mg/h/kg for metformin, 50, 25, 12.5, 5, and 2.5 mg/h/kg for buformin, and 25, 12.5, 5, 2.5, and 1.0 mg/h/kg for phenformin, respectively. The administration rate of the saline was 8.0 ml/h/kg. Blood samples used for the determination of biguanides were drawn from the cervical vein at 150, 180, and 210 min. Sample treatment, determination of biguanides, and blood lactate concentrations were performed as described above. AUCs of the blood lactate concentration until 240 min were calculated by the linear trapezoidal rule, and EC_{50} values were estimated by the equation (E_{max} model) $AUC = AUC_{max}S/(EC_{50} + S)$, where S represents unbound biguanide plasma concentrations. The fitting was carried out by an iterative nonlinear least-squares method.

Respiratory Chain Inhibitory Effect of Biguanides in Isolated Rat Hepatocytes. Hepatocytes were isolated from rats by the procedure described previously (Yamazaki et al., 1992). The experiment was prepared from the report of El-Mir et al. (2000). Briefly, hepatocytes (final protein concentration of 1.0 mg/ml) were incubated in closed vials at 37°C in a shaking water bath in 5 ml of Krebs-bicarbonate buffer (120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 24 mM $NaHCO_3$, 1.3 mM $CaCl_2$, pH 7.4) saturated with a mixture of O_2/CO_2 (95%/5%) supplemented with 20 mM lactate, 2 mM pyruvate, 4 mM octanoate, and different concentrations of biguanides. Oxygen consumption for up to 30 min was monitored and used for data analysis. The concentration of biguanides caused a reduction of oxygen consumption to 0.29 μmol/min/mg protein of hepatocytes (75% of control value) was calculated by linear estimation.

HPLC Analysis. The HPLC system involved a model L-7100 pump and a model L-7400 UV monitor (Hitachi, Tokyo, Japan) with a 300 × 3.9 mm i.d. C_{18} μBondapak (10 μm) column purchased from Waters (Milford, MA). The components of mobile phase consisted of 0.01 M phosphate buffer, pH 6.5, and acetonitrile at the ratio of 30:70. The flow rate was 1 ml/min. The wavelength for UV detection was 236 nm. The sensitivity was 1.5 ng for metformin and 3 ng for buformin and phenformin. The retention time was approximate 14, 11, and 9 min for metformin, buformin, and phenformin, respectively. The reproducibility was almost 90%, and no internal standard was used in the study. Biguanides were detected directly.

Statistical Analysis. Statistical analysis was performed by one-way analysis of variance followed by Fisher's t test or Student's t test to identify significant differences between various treatment groups.

Results

Lactic Acidosis Induced by Metformin in Wild-Type and Oct1(−/−) Mice. The time profiles of the blood lactate concentrations in wild-type and Oct1(−/−) mice at a constant infusion of a dose of 150 mg/h/kg of metformin are shown in Fig. 1A, and the simultaneously determined plasma concentrations of metformin are shown in Fig. 1B. There was a marked difference in the response to metformin between wild-type and Oct1(−/−) mice after 180 and 210 min of intravenous infusion of metformin, although the plasma concentrations of metformin were comparable. At 210 min, the blood lactate concentration was 25.6 ± 2.2 mg/dl in metformin-treated wild-type mice, which was significantly increased compared with that in saline-treated mice (5.07 ± 1.05 mg/dl), whereas there was no statistical difference in the

blood lactate concentration between metformin and saline-treated Oct1(−/−) mice (10.2 ± 1.1 and 5.25 ± 0.17 mg/dl, respectively). The blood lactate concentration in metformin-treated wild-type mice was 2.5-fold greater than that in metformin-treated Oct(−/−) mice. After the mice were sacrificed, the concentration of metformin in the liver and skeletal muscle was determined (Table 1). In contrast to the significant reduction in metformin concentration in the liver of Oct1(−/−) mice, the concentration of metformin in muscle was similar in both groups.

Lactic Acidosis Induced by Biguanides in Rats. The time profiles of the lactate concentration induced by constant intravenous infusion of phenformin, buformin, and metformin in rats are shown in Fig. 2. Compared with administration of saline, the blood lactate concentration was increased after each dose of metformin, buformin, and phenformin (Fig. 2, A-C). The correlation between increase in lactate AUC and the steady-state unbound plasma concentration of biguanides is shown in Fig. 2D. Taking the highest blood lactate AUC as 100%, the EC_{50} values of the biguanides were determined to be 734 ± 168, 119 ± 18, and 4.97 ± 0.87 μM for metformin, buformin, and phenformin, respectively (Table 2). There were 7- and 140-fold differences in the EC_{50} values for lactic acidosis between phenformin and buformin and between phenformin and metformin, respectively (Fig. 2D) (Table 2).

Oxygen Consumption Inhibition Study in Isolated Rat Hepatocytes. The effect of metformin, buformin, and phenformin on the oxygen consumption was examined using isolated rat hepatocytes. The control value of oxygen consumption by isolated rat hepatocytes was 0.389 ± 0.098 μmol/min/mg protein of hepatocytes. The concentration dependence of the reduction in oxygen consumption is shown in Fig. 3A. Oxygen consumption was decreased in the presence of biguanides in a concentration-dependent manner. Concerning the reduction in oxygen consumption, phenformin was the most potent drug. The concentrations of biguanides causing a reduction in oxygen consumption to 75% of the control value (0.29 μmol/min/mg protein) of rat hepatocytes were 1020 ± 136, 173 ± 15, and 7.25 ± 1.58 μM, and they correlated with the EC_{50} values determined in vivo for the increase of blood lactate (Fig. 3B).

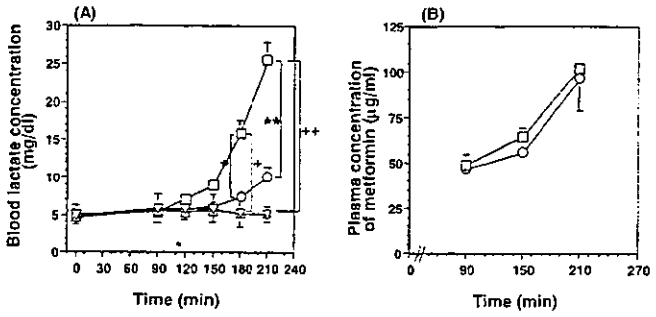

Fig. 1. Time profile of lactate concentration (A) and metformin plasma concentration (B) in wild-type and Oct1(−/−) mice during intravenous infusion of 150 mg/h/kg. Metformin was administered by constant intravenous infusion at a rate of 150 mg/h/kg. The whole blood lactate concentration in wild-type (□) and Oct1(−/−) mice (○) was compared with those in saline-treated wild-type and Oct1(−/−) mice (△, A). B, there was no marked difference in the metformin plasma concentration. Statistical analysis was performed by one-way analysis of variance followed by Fisher's *t* test. *, different tendency ($p = 0.1$; metformin-treated wild-type versus Oct1(−/−) mice at 180 min); significant difference at $p < 0.05$: + and ++, metformin-treated versus saline-treated wild-type mice at 180 and 210 min, respectively; **, metformin-treated wild-type versus Oct1(−/−) mice at 210 min. Results are shown as mean ± S.E. of three wild-type and four Oct1(−/−) mice.

Discussion

The present study investigated whether the liver is the key organ for lactic acidosis, and it examined whether oxygen consumption by isolated hepatocytes can be used as an index of the incidence of lactic acidosis. Lactate, produced in the gut, liver, and peripheral tissues such as erythrocyte and skin, is used to form glucose in the liver (Cori cycle) (Radziuk and Pye, 2001). Two possible mechanisms for lactic acidosis caused by biguanides have been proposed: 1) increased lactate production in the peripheral tissues, because metformin increases the glycolytic lactate production in peripheral tissue (Borst and Snellen, 2001); and 2) inhibition of lactate metabolism/transport in the liver and other tissues such as heart and muscle. Oct1(−/−) mice provide a good animal model to examine these possibilities because the hepatic concentration of metformin is drastically reduced in these mice at similar plasma concentrations. The importance of the intrahepatic concentration of metformin was shown by comparing the effect of metformin on the lactate concentration between wild-type and Oct1(−/−) mice (Fig. 1). At 210 min, the blood lactate concentration was significantly increased in metformin-treated wild-type mice compared with that in saline-treated mice, whereas there was no statistical difference in the blood lactate concentration between metformin and saline-treated Oct1(−/−) mice (Fig. 1A). The blood lactate concentration in metformin-treated wild-type mice was 2.5-fold greater than that in metformin-treated Oct(−/−) mice (Fig. 1A). The plasma concentration-time profiles of metformin were similar in wild-type and Oct1(−/−) mice (Fig. 1B). As summarized in Table 1, the hepatic concentration of metformin in Oct1(−/−) mice was much reduced, whereas its concentration in the skeletal muscle was comparable between Oct1(−/−) and wild-type mice. These results indicate the importance of the intrahepatic metformin, which may inhibit the lactate metabolism in the liver in causing lactic acidosis. Buformin and phenformin have been shown to be substrates of Oct1, with greater transport activity than metformin (Wang et al., 2002). Because Oct1 is responsible for the hepatic uptake of organic cations (Jonker et al., 2001; Wang et al., 2002), it is very likely that the hepatic uptake of buformin and phenformin is also accounted for by Oct1, and the lack of Oct1-mediated hepatic uptake will reduce their inducibility of blood lactate.

The study using Oct1(−/−) mice thus indicates that lactic acidosis should be ascribed to the effect of biguanides on the liver. El-Mir et al. (2000) demonstrated that metformin reduced the oxygen consumption in a concentration-dependent manner in isolated rat hepatocytes. The relationship between the inhibition of oxygen consumption by the biguanides and the inducibility of blood lactate was investigated to determine the correlation between them. An increase in blood lactate was investigated in rats that were given biguanides at different infusion rates. Increasing the infusion rate of metformin, buformin, and phenformin caused a significant increase in blood lactate concentration (Fig. 2). Phenformin was the most potent drug to cause an increase in blood

TABLE 1

Plasma concentration and tissue accumulation of metformin after i.v. infusion at the dose of 150 mg/h/kg for 210 min

Metformin was administered by constant intravenous infusion at a rate of 150 mg/h/kg. Plasma was obtained at the end of infusion at 210 min. After blood sampling, liver and muscle were isolated to determine metformin concentration. Data are shown as mean ± S.E.M. of three wild-type and four Oct1 (−/−). No significant differences were observed in metformin concentration and K_p in muscle.

	Liver			Muscle	
	C_{plasma}	C_{liver}*	K_p*	C_{muscle}	K_p
	$\mu g/ml$	$\mu g/g$		$\mu g/g$	
Wild-type	102 ± 11	417 ± 178	4.08 ± 1.80	153 ± 18	1.50 ± 0.24
Oct(−/−)	97.7 ± 15.1	49.3 ± 10.4	0.505 ± 0.132	107 ± 17	1.10 ± 0.24

* Significant difference, as determined by Student's t test, between wild-type and Oct1(−/−) mice ($p < 0.05$).

lactate concentration. The EC_{50} values of phenformin, buformin, and metformin were determined using their unbound plasma concentration at steady state and the AUC of blood lactate. As summarized in Table 1, there was a 7-fold and 140-fold difference in the EC_{50} values between phenformin and buformin and between phenformin and metformin, respectively. As shown in Fig. 3, biguanides reduced oxygen consumption in isolated rat hepatocytes in a concentration-dependent manner. The EC_{50} values determined in vivo using unbound plasma concentration of biguanides were compared with their concentration needed to cause 75% of control value of oxygen consumption in isolated rat hepatocytes (Fig. 3). There was a clear linear correlation among the three biguanides examined in this study (Fig. 3), indicating that the reduction of oxygen consumption can be used as an index of the incidence of lactic acidosis. It is possible that excessive

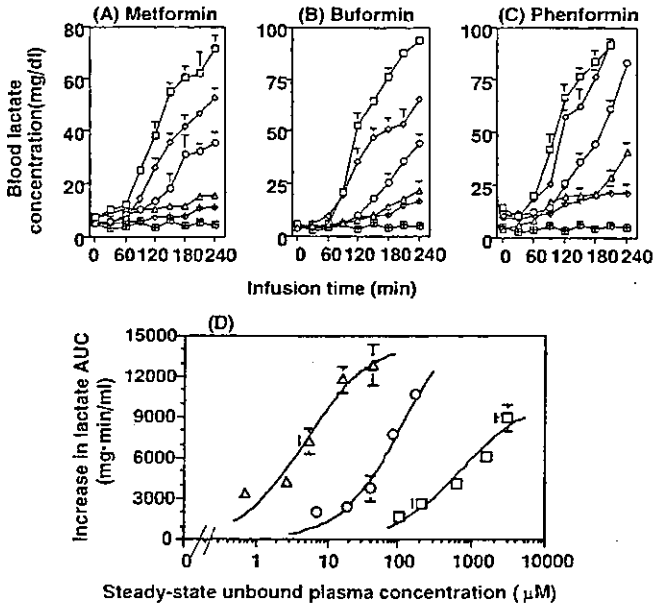

Fig. 2. Time profiles of the lactic acid increase induced in rats by metformin (A), buformin (B), and phenformin (C) and the relationship between the induced lactate AUC and the plasma concentration of biguanides (D). The whole blood lactate increase induced by metformin (A), buformin (B), and phenformin (C) is shown. The constant infusion doses were 25 (◁), 50 (△), 100 (○), 175 (◇), and 250 mg/h/kg (□) for metformin; 2.5 (◁), 5 (△), 12.5 (○), 25 (◇), and 50 mg/h/kg (□) for buformin; and 1 (◁), 2.5 (△), 5 (○), 12.5 (◇), and 25 mg/h/kg (□) for phenformin. The lactate was significantly increased in biguanide-treated animals compared with control (⊟), and this increase was dose-dependent. D, the increase in lactate AUC with regard to steady-state unbound plasma concentration of biguanides; △, phenformin; ○, buformin; □, metformin. Phenformin was the most potent inducer of blood lactate, and metformin was the least. Results are shown as the mean ± S.E. of three rats.

inhibition of mitochondrial respiration in the liver causes lethal reduction in the hepatic clearance of lactate. This should be examined in future studies, including an examination of the relationship between oxygen consumption and glucose production rate from lactate. Recently, human hepatocytes have become available for preclinical research, and these should allow the incidence of lactic acidosis to be predicted in humans.

The present study highlights two important issues, namely the transport activity of Oct1 and the saturation of hepatic uptake. Hepatic uptake is one of the main factors for determining the intrahepatic concentration of drugs. Basically, a biguanide that is transported more efficiently by Oct1 will achieve a higher intrahepatic concentration. Therefore, even though the IC_{50} value for the intrahepatic component, which plays a major role in lactic acidosis, is the same, a biguanide that is transported more efficiently by Oct1 will increase the blood lactate at lower plasma concentrations. Saturation of the hepatic uptake process may prevent increasing the intrahepatic concentration of biguanides to lethal levels. The IC_{50} values of metformin and phenformin for mitochondrial respiration that were determined with the use of isolated rat mitochondria were 15 mM and 50 μM, respectively (Owen et al., 2000). According to our previous report, the K_m values of metformin and phenformin for rOct1 were 377 and 16 μM, respectively (Wang et al., 2002). Taking the key role of Oct1 in the hepatic uptake of metformin into consideration, the K_m value determined in rOct1 expressing Chinese hamster ovary cells is very close to the K_m values for the uptake of biguanides by hepatocytes. The IC_{50} value of metformin for mitochondrial respiration is much greater than the K_m value for the hepatic uptake process, whereas the IC_{50} and K_m values of phenformin are quite similar. Increasing the plasma concentration of metformin saturates the hepatic uptake process initially, and, therefore, this limits the increase in intrahepatic concentration of metformin. However, in the case of phenformin, the K_m value and IC_{50} values are very similar, and even the concentration that causes saturation of the hepatic uptake process may lead to a significant reduction in oxygen consumption. The EC_{50} values of phenformin, buformin, and metformin obtained in this study are comparable

TABLE 2

EC_{50} of lactic acidosis induced by biguanide compounds in rats

Blood lactate concentration was determined after the administration of biguanides at five different doses by constant intravenous infusion. The EC_{50} was calculated from the increase in lactate AUC and steady-state unbound plasma concentration of biguanides.

Biguanide Compounds	Metformin	Buformin	Phenformin
EC_{50} (μM)	734 ± 168	119 ± 18	4.97 ± 0.87

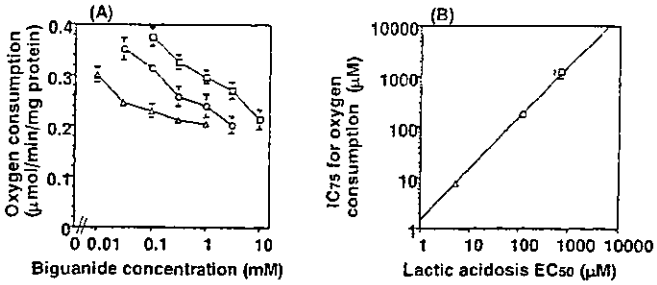

Fig. 3. Respiratory chain inhibitory effect in isolated rat hepatocytes (A) and the correlation between lactic acidosis (in vivo) and respiratory chain inhibition (in vitro) (B). The inhibitory effect of biguanides on the mitochondrial respiration was determined in isolated rat hepatocytes. The reduction in oxygen consumption was taken as the index of respiratory chain inhibition. The oxygen consumption was reduced by biguanides compared with the control value of 0.389 ± 0.098 μmol/min/mg protein of hepatocytes. Metformin (□) showed the lowest inhibitory effect and phenformin (△) the highest. Buformin (○) showed an inhibitory effect between those of metformin and phenformin. A correlation between the concentration of biguanides caused a reduction of oxygen consumption to 0.29 μmol/min/mg protein of hepatocytes (75% of control value), and their EC_{50} values for lactic acidosis is shown in B. Results are the mean ± S.E. of three determinations. Error bars for some points lie within the symbols.

with those of their K_m values for rOct1 (16, 50, and 377 μM, respectively). Taken from the results of the present study, two conditions need to be satisfied for the development of safer biguanides: 1) low transport activity by Oct1/OCT1, and 2) K_m value for hepatic uptake process that is smaller than the IC_{50} value for mitochondrial respiration.

In conclusion, the Oct1-mediated hepatic uptake of biguanides plays an important role in lactic acidosis. Oxygen consumption and OCT1 cDNA-transfected cells can perhaps be used to evaluate the incidence of lactic acidosis in vivo.

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26. トランスポーターを介した肝への取り込み過程で生じる薬物間相互作用

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Inhibition on the Transporter-mediated Hepatic Uptake as a Mechanism of Drug-drug Interaction

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KEY WORDS
Transporter, Drug-drug interaction

はじめに

薬物トランスポーターは，多くの薬物および内因性化合物の生体膜透過にかかわるタンパク質であり，多くの薬物の吸収，排泄，組織への分布などにおいて重要な役割を果たしている[1,2]。多くの薬物の排泄を司っている肝臓や腎臓においても，これらの薬物トランスポーターが，血中から臓器への薬物の取り込みおよび尿中または胆汁中への排泄過程において，重要な役割を果たしていることが知られている[1]。したがって，トランスポーターを介した膜透過過程は薬物の体外への消失速度を決定する重要な因子の一つであるといえる[3]。その一方で，薬物トランスポーターは，非常に幅広い基質認識性を示すことが報告されているため，同時に服用した薬物が互いのトランスポーターによる膜透過を阻害する可能性が考えられる[4]。このようなメカニズムでトランスポーターを介した膜透過過程に起因する薬物間相互作用が起こる可能性があると考えられる[4]。これまでにメカニズムが未解明であった薬物間相互作用は，こうした原因で説明できるかも知れない。

HMG-CoA 還元酵素阻害薬 cerivastatin (CER) は，Cytochrome P-450 (CYP) 2C8 と 3A4 による 2 種の異なる代謝経路をもつために（図1），一方の代謝経路が併用薬によって阻害された場合であっても，重篤な薬物間相互作用につながる可能性が低い薬物と考えられていた[5]。しかしながら，1999 年には免疫抑制薬 cyclosporin A (CsA) との相互作用により CER の血中濃度が上昇することが報告され，2001 年にはフィブラート系高脂血症治療薬 gemfibrozil

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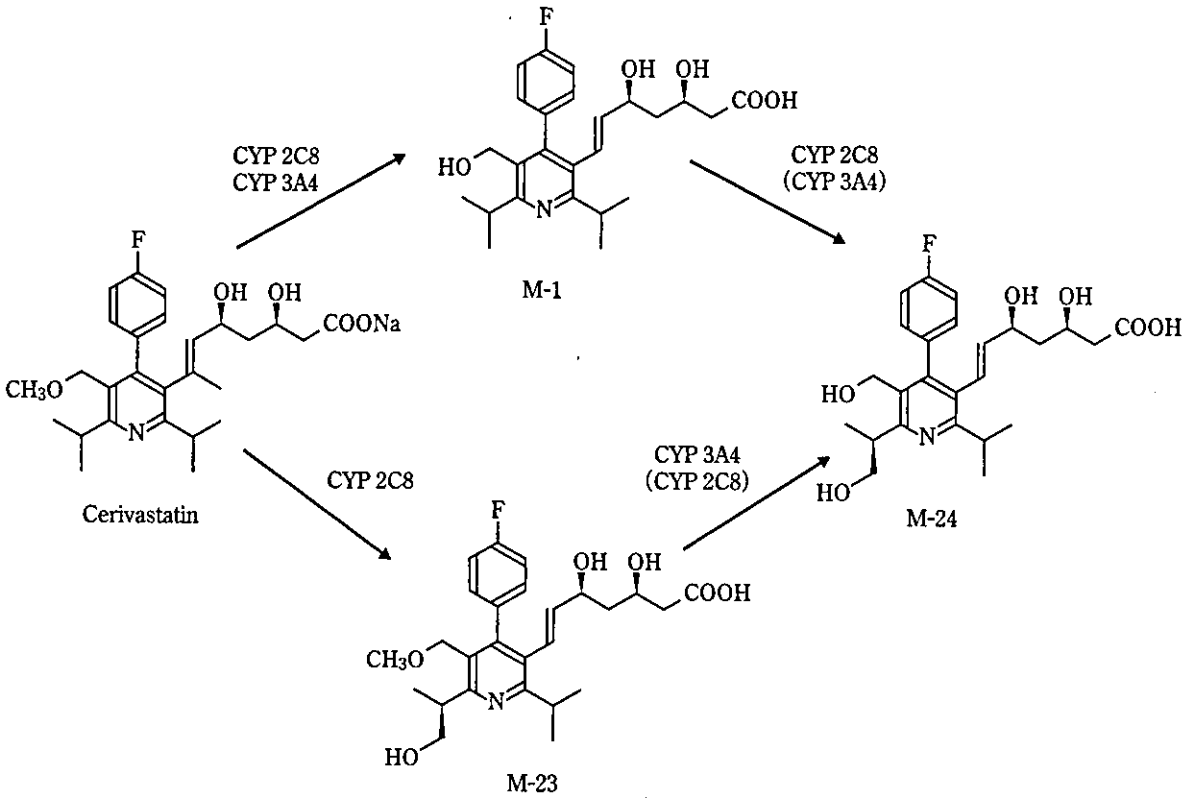

図 1 CER の代謝経路
CER の代謝経路の概略を示す。CER は CYP2C8 と CYP3A4 の二つによって代謝を受ける。

との相互作用で同様の例が報告されている[6,7]。特に後者の相互作用においては死亡症例を含む重篤な横紋筋融解症を起こした症例が報告された[8]。この相互作用が原因となって，CER は市場からの撤退を余儀なくされた[8]。これらの相互作用のメカニズムは不明である。私たちは，CER が非常に効率よく肝に取り込まれる[9]ことから，トランスポーターを介した肝への取り込み過程に起因するのではないかと考え，CER の肝取り込み過程に着目した相互作用のメカニズム解析を行った。なお，本記録は 2002 年 6 月 12 日に行われた「第 10 回肝病態生理研究会」における口演抄録で Journal of Pharmacology and Experimental Therapeutics に受理された内容を引用した。

I 実験方法

1 試薬・実験材料

[^{14}C]-CER および非標識体の CER は Bayer AG 社（Wuppertal, Germany）より供与された。凍結ヒト肝細胞は In Vitro Technologies 社（Baltimore, MD USA）より供与された[10]。ヒトミクロソームは第一化学薬品（東京）より入手した。Cyclolsporin A については Sigma-Aldrich（St. Louis, MO, USA）より入手した。

2 ヒト肝細胞を用いた取り込み実験

ヒト肝細胞は解凍した後，Krebs-Henseleit buffer で懸濁し，2×10^6 cells/mL に調製し，ガラス試験管に分注した。懸濁した細胞は実験開始直前まで 4°C で保存した。取り込み実験開始前に 37°C の恒温槽に移し，3 分間 preincubation を行った後，細胞懸濁液と等量の [^{14}C]-CER および阻害剤を含んだ薬液を加えることで，取り込み実験を開始した。取り込み実験開始後 0.5, 2 分後に，細胞懸濁液を採取して比重 1.015 に調製したオイルを入れたチューブに重層して，遠心分離することで薬液と細胞を分離し，細胞中に取り込まれた [^{14}C]-CER と薬液中に残った [^{14}C]-CER の放射活性を測定した。

3 ヒト OATP2 発現系を用いた取り込み実験

ヒト OATP2 発現系を用いた実験では，OATP2 を導入した MDCK II 細胞[11]を Cell Culture Insert™

（BD Biosciences, Bedford, MA）に播種し，2日後に培地を10 mM酪酸ナトリウムを加えた培地と置換し，その翌日に取り込み実験を行った。細胞をKrebs-Henseleit bufferで2回washした後，Krebs-Henseleit bufferを加えて10分間37℃でpreincubationを行い，[^{14}C]-CERおよび阻害剤を加えたbufferと置換することで取り込み実験を開始した。取り込み終了時には，bufferを吸い取り，細胞の表面を4℃ Krebs-Henseleit bufferで4回washした。細胞は0.1N NaOHを加えて1晩放置することにより溶解し，0.1N HClで中和した後，細胞に取り込まれた放射活性を測定した。また，細胞溶解液を用いて蛋白定量を行った。

4 ヒトミクロソームを用いた代謝実験

ヒトプールドミクロソームを3.3 mM $MgCl_2$，3.3 mM glucose-6-phosphate，0.4 U/mL glucose-6-phosphate dehydrogenase，1.3 mM NADPH，0.8 mM NADHを含む100 mMリン酸Kバッファーで，最終濃度0.5 mg protein/mLになるように希釈した。ミクロソーム懸濁液を10分間37℃でpreincubationしたあと，[^{14}C]-CER（final 1 μM）を加えることで代謝実験を開始した。45分後に等量のアセトニトリルを加えることで反応を止め，遠心分離後上清を採取して，TLC（Kieselgel 60F_{254}，Merck KGaA，Darmstadt，Germany）によって[^{14}C]-CERと代謝物の分離を行った。[^{14}C]-CERおよび代謝物は，TLCによって得られたバンドをBAS2000（富士フイルム，東京）で解析することにより定量した。

5 データ解析

[^{14}C]-CERの取り込みは，直線的な取り込みが見られる短時間で取り込まれた[^{14}C]-CERの放射活性（dpm/10^6 cellsまたはdpm/mg protein）を，incubation buffer中の放射活性（dpm/μL）で除することによって得られる取り込みクリアランス（μL/10^6 cellsまたはμL/mg protein）によって表した。速度論的解析では，[^{14}C]-CERの取り込み初速度を以下の式(1)に当てはめて，最小二乗法プログラムMULTI[12]を用いてfitting計算を行うことによって，各種パラメータを求めた。

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

ただし，vは取り込み初速度，Sは基質（CER）濃度，K_mはCERの取り込みに対するMichaelis定数，V_{max}は最大取り込み初速度，P_{dif}は非飽和性取り込みクリアランスを表す。

また，CsAによる阻害実験では，得られた取り込みクリアランスを次の式(2)に代入し，MULTIにより当てはめ計算を行うことで，阻害定数（K_i）を

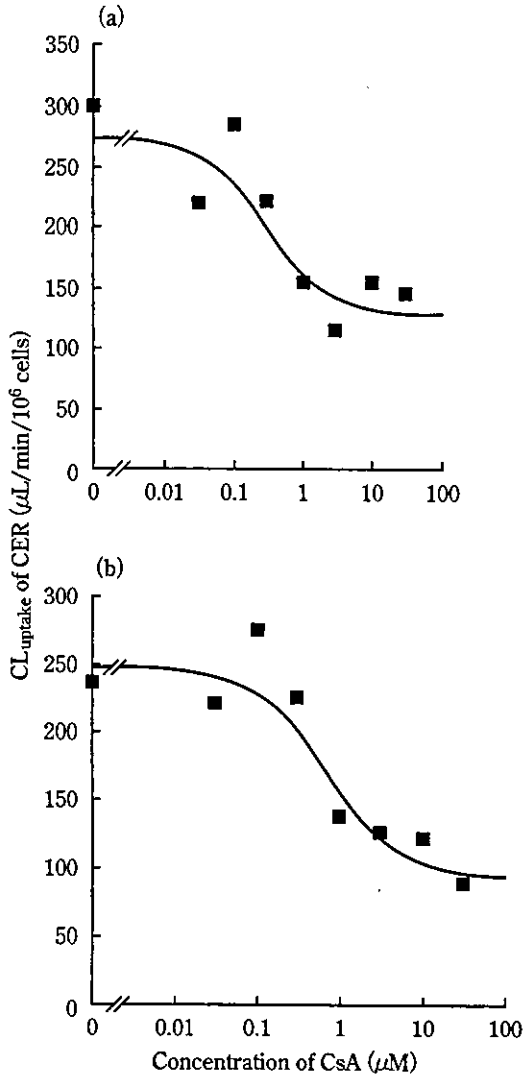

図2 ヒト肝細胞への[^{14}C]-CERの取り込みに対するCsAの阻害効果

ヒト肝細胞への[^{14}C]-CERの取り込みを観察し，それに対するCsAの阻害効果を示す。
(a)はロットHH-088を，(b)はロットHH-117を用いたときの結果を示す。
結果は，n=2の平均値を示す。実線はfitted lineを示す。
図は，Shitara Y, et al. J Pharmacol Exp Ther (accepted)を引用している。

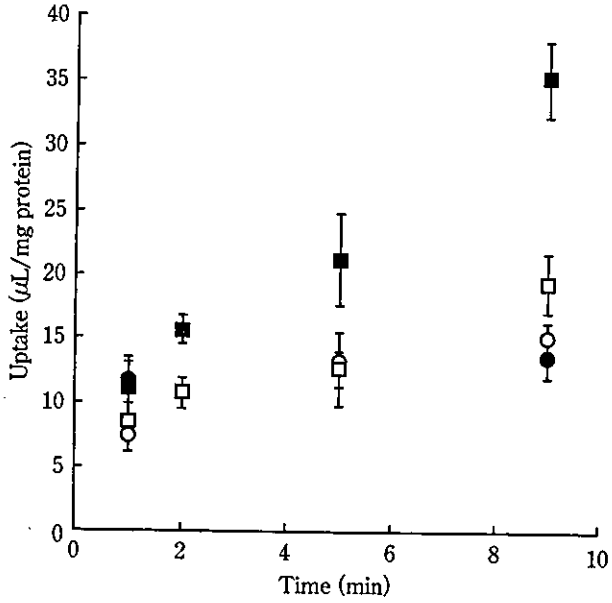

図 3 OATP2 発現細胞における[^{14}C]-CER の経時的取り込み

OATP2 発現 MDCK II 細胞における [^{14}C]-CER の取り込み（■, □）およびコントロールとして，OATP2 cDNA を含まない発現ベクターのみを導入した細胞への取り込み（●, ○）を示す。
図は 0.25 μM の [^{14}C]-CER を加えたときの取り込み（■, ●）および 30 μM の非標識 CER を加えたときの [^{14}C]-CER の取り込み（□, ○）を示す。
データは n=3 の平均値±SE である。
図は，Shitara Y, et al. J Pharmacol Exp Ther（accepted）を引用している。

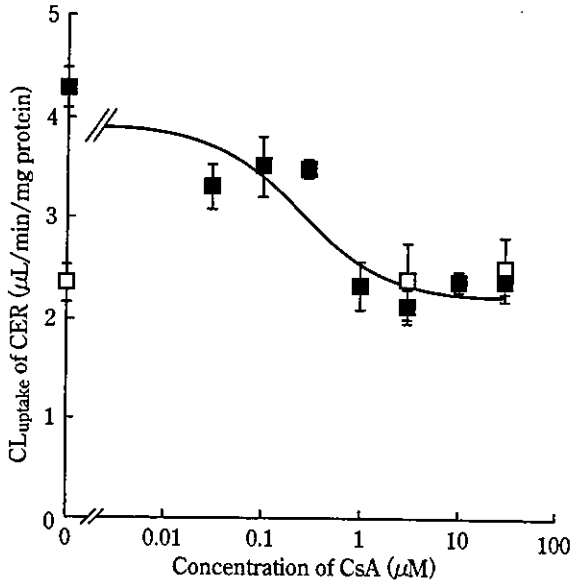

図 4 OATP2 を介した [^{14}C]-CER の取り込みに対する CsA の阻害効果

OATP2 発現細胞における [^{14}C]-CER の取り込みに対する CsA の阻害効果を示す。
■は OATP2 発現細胞，□はコントロールのベクターのみを導入した細胞への取り込みを示す。
データは n=3 の平均値±SE である。
図は，Shitara Y, et al. J Pharmacol Exp Ther（accepted）を引用している。

算出した。

$$CL_{uptake} = \frac{CL_{uptake}\ (control) - P_{dif}}{1 + I/K_i} + P_{dif} \cdots\cdots (2)$$

ただし，CL_{uptake}，CL_{uptake} (control) は，それぞれ阻害剤存在下および非存在下での取り込みクリアランス，I は阻害剤濃度を表す。

II 結　　果

1 ヒト肝細胞における [^{14}C]-CER の取り込みおよび CsA の影響

2 種の異なるドナー由来のヒト肝細胞（ロット HH-088, HH-117）を用いて取り込み実験を行った（図 2）。いずれのロットにおいても [^{14}C]-CER の経時的な取り込みが観察された。加える CER の基質濃度を変えることで，速度論的パラメータを算出したところ，K_m，V_{max}，P_{dif}の順に，HH-088 では，18.3±6.9 μM, 5200±1970 pmol/min/10^6 cells, 70.2±13.9 μL/min/10^6 cells, HH-117 では，3.72±1.29 μM, 362±120 pmol/min/10^6 cells, 41.7±3.4 μL/min/10^6 cells であった。これら二つのロットの肝細胞を用いて，CER の取り込みに対する CsA の影響を観察した。この結果，いずれのロットにおいても，濃度依存的な阻害効果がみられた。得られた K_i 値は，HH-088, -117 のそれぞれにおいて，0.280±0.215, 0.685±0.286 μM（mean±SE）であった。

2 ヒト OATP2 発現系における [^{14}C]-CER の取り込みおよび CsA の影響

OATP2 発現系における [^{14}C]-CER の取り込みを観察した（図 3）。この結果，OATP2 発現系においては，ベクターのみを導入したコントロール細胞に比べて有意に高い取り込みがみられた。そこで，OATP2 発現系における [^{14}C]-CER の取り込みに対する CsA による阻害効果を観察した。この結果，濃度依存的な阻害効果がみられた（図 4）。得られた

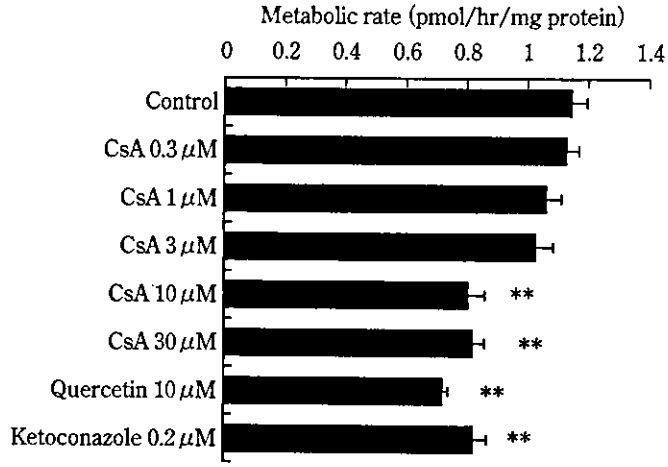

図 5 ヒト肝ミクロソームにおける [^{14}C]-CER の代謝に対する阻害剤の影響
各種阻害剤存在下で見積もったヒト肝ミクロソーム中での [^{14}C]-CER の代謝速度を示す。
は有意差を示す。(...$p<0.01$) データは $n=3$ の平均値±SE である。
図は，Shitara Y, et al. J Pharmacol Exp Ther (accepted) を引用している。

K_i値は 0.238±0.129 μM (mean±SE) であった。

3 ヒトミクロソームでの [^{14}C]-CER の代謝に対する CsA の影響

ヒトプールドミクロソームを用いて，[^{14}C]-CER の代謝を観察し，それに対する CsA の影響について観察した（**図 5**）。この結果，10 μM 以上の CsA を加えたときには阻害がみられるものの，IC_{50}値は 30 μM 以上で，非常に弱い阻害効果であった。

III 考　　察

今回の検討では，CER と CsA の相互作用のメカニズムを明らかにすることを目的として，特に CER のヒト肝細胞への取り込み過程に対する CsA の影響について検討を行った。ヒト肝細胞への [^{14}C]-CER の取り込みを観察したところ，今回用いたいずれのロットにおいても飽和性の取り込みが観察された。このことから，CER がトランスポーターを介してヒト肝細胞に取り込まれることが示唆された。V_{max}/K_mで表される飽和性輸送と P_{dif}で表される非飽和性の輸送との比較により，基質濃度が K_mより十分に小さいときには，70～80%は飽和性の輸送であることが明らかになった。CER の臨床血中濃度は nM オーダーであり[6]，臨床で用いられている CER の肝への取り込みに対してもトランスポーターが重要な役割を果たしていることが示唆された。

CsA の影響について観察したところ，肝細胞への取り込みに対して，非常に低い K_i (0.3～0.7 μM) で阻害がみられた。一方，CsA の臨床での循環血中濃度は C_{max}で約 1 μM である[6]。しかしながら，CsA は血中では約 90%が蛋白に結合しており[13]，蛋白非結合型の CsA のみがトランスポーターの機能を阻害すると考えた場合には，必ずしも臨床で報告されている薬物間相互作用を説明することはできない。しかしながら，薬物を経口投与したときには，肝入り口付近の血液中においては，循環血中の濃度よりも高くなっている可能性が考えられる[14,15]。このようなことを考慮すると，トランスポーターを介した取り込み過程に対する阻害が，臨床で報告されている薬物間相互作用の少なくとも一つの原因になりうる。

OATP2 発現系を用いて取り込み実験を行ったところ，ベクターのみを導入した細胞に比べて有意に高い取り込みが観察されたことから，CER が OATP2 の基質になることが示唆された。OATP2 を介した取り込みに対する CsA の影響について検討したところ，ヒト肝細胞に対する取り込みに対する阻害と同程度の K_i 値での阻害がみられた。これらのことから，ヒト肝細胞でみられた CER の取り込みが少なくとも部分的には OATP2 を介してなされていること，および CER と CsA の相互作用に OATP2

を介した取り込み過程が関与している可能性があることが示唆される。これまでに，多くの化合物がOATP2によって運ばれることが報告されている[16~19]。また，pravastatinのように医薬品がOATP2の基質となることも報告されている[17,20]。pravastatinもまたCsAと相互作用を受けて，血中濃度が上昇することが報告されている[21]が，これもまたOATP2を介した取り込み過程が原因となって起こっている可能性がある。同様にOATP2によって肝に輸送される薬物については，同じメカニズムによって相互作用が起こる危険性があるので注意が必要である。

以上の検討より，CERとCsAの薬物間相互作用においては，トランスポーターを介した取り込み過程での阻害が，少なくとも一つの要因になっていることがわかった。OATP2によって輸送される他の薬物についても同様の相互作用が起きる可能性は十分に考えられる。同種の薬物間相互作用を未然に防ぐためにも，薬物の輸送にかかわっているトランスポーターについて明らかにしておくことが必要である。

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