Carnitine/organic cation transporter OCTN2 (Slc22a5) is responsible for renal secretion of cephaloridine in mice

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**OCTN2-mediated renal secretion of cephaloridine**

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**Abbreviations:**

OCTN2, carnitine/organic cation transporter 2; CER, cephaloridine; CMD, cefamandole; CPM, cefpiramide; CL<sub>tot</sub>, total clearance; CL<sub>renal,p</sub>, renal clearance normalized by plasma concentration; CL<sub>renal,k</sub>, renal clearance normalized by kidney concentration; I, infusion rate; C<sub>ss,plasma</sub>, steady-state plasma concentration; V<sub>ss,urine</sub>, steady-state urinary excretion rate; C<sub>ss,kidney</sub>, steady-state kidney concentration; CL<sub>sec</sub>, secretion clearance; K<sub>p,kidney</sub>, kidney-to-plasma concentration ratio
ABSTRACT

Carnitine/organic cation transporter OCTN2 (SLC22A5) plays a pivotal role in renal tubular reabsorption of carnitine, a vitamin-like compound, on apical membranes of proximal tubules, but its role in relation to therapeutic drugs remains to be clarified. The purpose of the present study was to elucidate the involvement of OCTN2 in renal disposition of a β-lactam antibiotic, cephaloridine (CER), based on experiments with juvenile visceral steatosis (jvs) mice, which have a functional deficiency of octn2 gene. Renal clearance of CER during constant intravenous infusion in wild-type mice was much higher than could be accounted for by glomerular filtration, but was decreased by increasing the infusion rate with minimal change in kidney-to-plasma concentration ratio, suggesting the existence of saturable transport mechanism(s) across the apical membranes. The plasma concentration profile and kidney-to-plasma concentration ratio after intravenous injection in jvs mice were higher than those in wild-type mice, whereas renal clearance in jvs mice was much lower than that in wild-type mice and could be accounted for by glomerular filtration. Uptake of CER by mouse OCTN2 was demonstrated in *Xenopus laevis* oocytes expressing mouse OCTN2. The CER transport by OCTN2 exhibited saturation with Km of ~3 mM, which is similar to the renal CER concentration exhibiting saturation in renal clearance *in vivo*. The OCTN2-mediated CER transport was inhibited by carnitine, and independent of Na⁺ replacement in the medium. These results demonstrate that OCTN2 on apical membranes of proximal tubules plays a major role in renal secretion of cephaloridine in mice.
INTRODUCTION

OCTN2 (SLC22A5), which is a member of the carnitine/organic cation transporter (OCTN) family, is clinically important because deficiency of the OCTN2 gene in humans causes systemic carnitine deficiency (SCD) (Nezu et al., 1999). Carnitine is a vitamin-like compound essential for β-oxidation of fatty acids, and is transported by OCTN2 in a Na⁺-dependent manner. Because OCTN2 has also been reported to transport other substrates, including therapeutic agents such as quinidine, verapamil and some β-lactam antibiotics (Ohashi et al., 1999; Ganapathy et al., 2000), it may have a significant influence on the disposition, efficacy and side effects of such drugs.

Many studies have been conducted to clarify the roles of OCTN2 in vivo. OCTN2 is expressed ubiquitously (Tamai et al., 1998, 2000; Wu et al., 1999), and is involved in carnitine transport in kidney (Tamai et al., 1999; Yokogawa et al., 1999), small intestine (Yokogawa et al., 1999; Kato et al., 2006), blood brain barrier (Kido et al., 2001; Inano et al., 2003), placenta (Shekhawat et al., 2004; Grude et al., 2005) and heart (Iwata et al., 2008). The juvenile visceral steatosis (jvs) mouse model is a particularly useful tool for elucidating the physiological and pharmacological roles of OCTN2, because jvs mice have genetic mutation in the octn2 gene and exhibit fatty liver, hyperammonemia and hypoglycemia, which are common phenotypes in humans with SCD (Treem et al., 1988; Breningstall 1990; Nezu et al., 1999).

Compared with the well-established fundamental role of OCTN2 in the disposition of its endogenous substrate carnitine, quite limited information is available on possible involvement of OCTN2 in the disposition of other substrates. In mouse kidney, OCTN2 is localized on apical membranes of proximal tubules (Tamai et al.,
2001) and it was suggested to be involved in renal secretion of its cationic substrate, tetraethylammonium (TEA), based on a study with jvs mice (Ohashi et al., 2001). Taking into account that genetic deficiency in OCTN2 reduces reabsorption of carnitine in renal tubules, OCTN2 may play essential roles not only in homeostasis of its endogenous substrate carnitine, but also in elimination of xenobiotics in the kidney. However, the role of OCTN2 in renal secretion has been clarified only for TEA, and nothing is known about the effects in renal secretion of other substrates in vivo. Further studies are necessary to elucidate the roles of OCTN2 in kidney.

Cephaloridine (CER), one of the prototype cephalosporin antibiotics, has been used to elucidate the mechanisms of nephrotoxicity induced by cephalosporin antibiotics. Regardless of route of administration, CER is mainly accumulated in the renal cortex as the nonmetabolized form (Atkinson et al., 1966; Barza, 1978), and renal tubular secretion seems to be an important pathway for its elimination. Ganapathy et al. (2000) have already clarified that CER is a substrate of human OCTN2, based mainly on an inhibition study using carnitine as a substrate. However, possible involvement of OCTN2 in renal tubular secretion remains to be established. On the other hand, several in vivo and in vitro studies have focused on the renal transport mechanisms of CER, and it has been suggested that organic anion transporters, OAT1/SLC22A6 and OAT3/SLC22A8, are involved in the renal uptake of CER on basolateral membranes of proximal tubules (Takeda et al., 1999; Motohashi et al., 2002; Jung et al., 2002; Ueo et al., 2005). CER is a substrate of human OAT1 and OAT3, the uptake of CER by OAT3 being much greater than that by OAT1 (Ueo et al., 2005). Uptake experiments in vitro are also consistent with the idea that rat OAT1 and OAT3 are responsible for the basolateral uptake of CER (Takeda et al., 1999; Jung et al., 2002).
The purpose of the present study was to examine the pharmacological role of OCTN2 in the renal disposition of CER in mice. We first performed a constant infusion study to see whether a saturable transport mechanism is involved in renal secretion of CER at apical membranes. Then, the pharmacokinetics of CER was compared *in vivo* between wild-type and *jvs* mice to confirm the involvement of OCTN2. An *in vitro* transport study using *Xenopus laevis* oocytes expressing mouse OCTN2 directly demonstrated that CER is a substrate of OCTN2 in mice.
METHODS

Materials

CER and cefamandole (CMD) were gifts from Shionogi & Co., Ltd. (Osaka, Japan). Cefpiramide (CPM) was purchased from Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). TEA bromide was obtained from Wako Pure Chemical Industries (Osaka, Japan). L-Carnitine inner salt was obtained from Sigma-Aldrich Japan K.K. (Tokyo, Japan). The pcDNA3 vector was obtained from Invitrogen (Carlsbad, CA).

Animals

Jvs mice, originally found among mice of the C3H.OH strain (Koizumi et al., 1988), and control (wild-type) mice C3H/HeJ (Japan SLC, Hamamatsu, Japan) were used. By mating heterozygous male mice with heterozygous female mice, we obtained wild-type (+/+) and homozygous mutant (-/-) mice. This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals in Takara-machi Campus of Kanazawa University.

Pharmacokinetics studies

Male C3H/HeJ mice (8 weeks old, 20-23 g body weight) were anesthetized with pentobarbital, and the bladder was catheterized with polyethylene tubing for urine collection. CER was infused via the jugular vein with a basic syringe pump (Harvard Apparatus, Holliston, MA). CER dissolved in PBS was administered at loading doses of 0.75, 7.5 or 75 mg/kg body wt, followed by constant infusion at 1.74, 17.4 or 174 mg/kg/h for 2 h, respectively. Blood samples were collected from the jugular vein at 60, 90 and 120 min. Urine was collected by washing the bladder with 0.2 ml of saline at the
same intervals as the blood sampling. Mice were decapitated at 2 h after the start of infusion. The kidney was then quickly excised, rinsed with ice-cold saline, blotted dry and weighed. Samples were stored at -30°C until analysis.

For the bolus injection study, eight-week-old wild-type and jvs mice were anesthetized with pentobarbital and administered with 10 or 50 mg/kg of CER via the jugular vein or orally by gavage, respectively. Blood samples were then collected from the intraorbital vein using heparinized capillary tubes at designated time intervals. All blood samples were immediately centrifuged to obtain plasma. Urine samples were collected by washing the bladder with saline (0.2 mL) through a catheter.

**Plasma protein binding of CER**

The free fraction of CER in plasma (fₚ) was determined in vitro by ultrafiltration. CER dissolved in phosphate buffer (50 mM, pH 7.4) was diluted 10 times with plasma to give a final concentration of 10 µg/mL (24 µM). The mixture was incubated at 37 °C for 30 min, and a 40 µL aliquot was then taken for the determination of total plasma concentration. Next, the plasma was placed in an ultrafiltration apparatus (Centrifree, Amicon Inc., Beverly, MA) with a molecular mass cut-off of 13 kD and centrifuged at 3,000 rpm (Tomy RL-100, Tokyo, Japan) for 10 min. After centrifugation, the concentration in the filtrate was also measured for determination of unbound concentration. The fₚ was calculated by dividing the unbound concentration by the total plasma concentration. All binding values were normalized with respect to the filter blank.

**Determination of CER**
The quantification of CER in plasma, urine and kidney was performed by high-performance liquid chromatography (HPLC). The HPLC system consisted of a constant-flow pump (JASCO PU-2080 Plus, JASCO International Co., Ltd., Tokyo, Japan), a UV detector (JASCO UV-2075 Plus), an automatic sample injector (JASCO AS-2057 Plus) and an integrator (Chromatopac C-R7A, Shimadzu Corporation, Kyoto, Japan). The analytical column was COSMOSIL 5C18-AR-II (4.6 x 150 mm; Nacalai Tesque, Kyoto, Japan) for plasma and urine samples, and COSMOSIL 5C4-MS (4.6 x 150 mm) for kidney samples. Plasma or 50-fold diluted urine were mixed with sterile distilled water (30 μL) containing CMD as an internal standard, and deproteinized with acetonitrile (30 μL), followed by centrifugation at 15,000 g for 15 min at 4 °C. The supernatants were diluted with an equal volume of mobile phase and again centrifuged at 15,000 g for 15 min at 4 °C. The supernatants were then subjected to HPLC analysis.

To extract CER from kidney, kidney samples were mixed with 4 volumes of phosphate buffer, spiked with IS, and homogenized. After centrifugation at 15,000 g for 15 min at 4 °C, 150 μL of the supernatant was deproteinized by adding 20 μL of 10% perchloric acid. The supernatant was collected after centrifugation and neutralized with 50 μL of phosphate buffer (pH 9). The supernatant was filtered through HLC-DISK (0.45 μm; Kanto Chemical Co. Ltd., Tokyo, Japan), and a 30 μL aliquot of the filtrate was subjected to HPLC analysis. Detection was performed at 254 nm.

**Calculation of pharmacokinetic parameters**

In the infusion study, total clearance (CL\text{tot}), renal clearance with respect to plasma concentration (CL\text{renal,p}) and renal clearance with respect to kidney concentration (CL\text{renal,k}) were calculated as follows:
\[ \text{CL}_{\text{tot}} = \frac{I}{C_{\text{ss,plasma}}} \quad (1) \]

\[ \text{CL}_{\text{renal,p}} = \frac{V_{\text{ss,urine}}}{C_{\text{ss,plasma}}} \quad (2) \]

\[ \text{CL}_{\text{renal,k}} = \frac{V_{\text{ss,urine}}}{C_{\text{ss,kidney}}} \quad (3) \]

where \( I, C_{\text{ss,plasma}}, V_{\text{ss,urine}} \) and \( C_{\text{ss,kidney}} \) represent the infusion rate (\( \mu \text{g/min/kg} \)), steady-state plasma concentration (\( \mu \text{g/ml} \)), steady-state urinary excretion rate (\( \mu \text{g/min/kg} \)), and steady-state kidney concentration (\( \mu \text{g/ml} \)), respectively. \( C_{\text{ss,plasma}} \) was estimated as plasma concentration at 120 min. \( V_{\text{ss,urine}} \) was estimated as the mean value of excretion rate from 60 to 90 min and that from 90 to 120 min. \( C_{\text{ss,kidney}} \) was estimated as kidney concentration at 120 min on the assumption that the specific gravity of the kidney is unity. Renal secretion clearance (\( \text{CL}_{\text{sec}} \)) and kidney-to-plasma concentration ratio (\( K_{\text{p,kidney}} \)) were estimated as follows:

\[ \text{CL}_{\text{sec}} = \text{CL}_{\text{renal,p}} - f_p \times \text{GFR} \quad (4) \]

\[ K_{\text{p,kidney}} = \frac{C_{\text{ss,kidney}}}{C_{\text{ss,plasma}}} \quad (5) \]

where GFR is glomerular filtration rate (ml/min/kg), which was estimated as creatinine clearance. Creatinine in plasma and urine was measured using the Jaffé methods (Creatinine Test WAKO; Wako Pure Chemical Industries) with a U-2001 spectrophotometer (Hitachi, Tokyo, Japan) at 520 nm wavelength.

In the bolus injection study, the area under the plasma concentration-time curve (AUC), the steady-state distribution volume (\( V_{\text{dss}} \)), mean residence time (MRT) and \( \text{CL}_{\text{tot}} \) were estimated according to model-independent moment analysis (Yamaoka et al., 1978). The \( \text{CL}_{\text{renal,p}} \) was estimated as:

\[ \text{CL}_{\text{renal,p}} = \frac{X_{\text{urine}}}{\text{AUC}(0-120)} \quad (6) \]

where \( X_{\text{urine}} \) and \( \text{AUC}(0-120) \) are the amount of urinary excretion, which was estimated as that up to 120 min after injection, and AUC from 0 to 120 min, respectively. The
**Kp,kidney** was estimated as the ratio of kidney concentration to plasma concentration of CER at 120 min.

**Uptake study in *Xenopus laevis* oocytes injected with cRNA encoding OCTN2**

The full-length cDNA of mouse OCTN2 was subcloned into the BamHI site of pcDNA3 (Tamai *et al.*, 2000). The capped cRNA of OCTN2 was synthesized by means of a mCAP™ RNA capping kit (Stratagene, La Jolla, CA). *Xenopus laevis* oocytes were prepared and injected with 50 nL of cRNA (25 ng) or water as described previously (Ito *et al.*, 2007). Three days after the cRNA injection, the oocytes were transferred to ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2·6H2O, 5 mM HEPES, pH 7.4) and pre-incubated at 25 °C for 15 min. Uptake was initiated by replacing the solution with fresh ND96 solution (25 °C) containing CER. At the designated times, the oocytes were rinsed five times with ice-cold ND96 solution, then 50% methanol containing 0.5 μg/mL CPM as an internal standard (35 μL) and acetonitrile (35 μL) were added to each oocyte. The samples were disrupted with a tip sonicator, and the lysate was cleared by centrifugation at 15,000 g for 15 min at 4 °C. The supernatants were diluted with an equal volume of sterile distilled water and centrifuged at 19,000 g for 15 min at 4 °C. The supernatants were then subjected to LC-MS/MS analysis. The uptake of [3H]carnitine (83 Ci/mmol) was determined as radioactivity associated with the oocytes in a liquid scintillation counter (LSC-5100; Aloka, Tokyo, Japan) with Clearsol I (Nacalai Tesque) as a scintillation fluid. Cell-to-medium ratio was obtained by dividing the cellular uptake amount by the concentration of test compound in the medium. Kinetic parameters for transport activity
were estimated by nonlinear least-squares fitting of the data to the following equation using the MULTI program:

\[ V = \frac{V_{\text{max}} \cdot S}{K_m + S} \]  \hspace{1cm} (7)

where \( V \), \( S \), \( K_m \) and \( V_{\text{max}} \) represent the initial uptake velocity, substrate concentration, Michaelis constant and maximum uptake velocity, respectively.

Concentration-dependent inhibition of OCTN2-mediated uptake of CER by carnitine and TEA was fitted to the following equation:

\[ R = \frac{I}{I + IC_{50}} \]  \hspace{1cm} (8)

where \( R \), \( I \) and \( IC_{50} \) are the uptake normalized by the control (without inhibitor) value, the inhibitor concentration and the inhibition constant, respectively.

**Measurement of CER using LC-MS/MS**

CER associated with oocytes was measured with a LC-MS/MS system equipped with a constant flow pump (Agilent 1200 series G1312A, Agilent Technologies, Tokyo, Japan), an automatic sample injector (G1367B; Agilent Technologies), a column oven (G1316A; Agilent Technologies) and a mass spectrometer (API 3200, Applied Biosystems, Tokyo, Japan). The analytical column was COSMOSIL 3C18-MS-II (2.0 x 50 mm; Nacalai Tesque). The mobile phase consisted of 18:82 (v/v) methanol and 0.1% formate. Chromatography was isocratically performed at a flow rate of 0.2 mL/min at 40 °C. The multiple reaction monitor was set at 416.2 to 152.0 m/z for CER and 613.0 to 257.0 m/z for CPM.

**Statistical analysis**
Data were expressed as mean ± S.E.M. Statistical analysis was performed with Student's \( t \) test to identify significant differences between various treatment groups, with \( p < 0.05 \) as the criterion of significance.
RESULTS

Saturable transport mechanism for CER on renal apical membranes

To investigate saturable transport mechanisms operating in renal disposition of CER, CER was administered at three doses by constant intravenous infusion, and the plasma concentration and renal excretion were measured. At all three doses, CL_{tot} was close to CL_{renal,p} (Fig. 1A), indicating that urinary excretion is a major elimination pathway for CER. The CL_{tot} and CL_{renal,p} of CER were reduced in mice at the highest infusion rate (Fig. 1A). The CL_{renal,p} at the highest infusion rate approached the glomerular filtration rate for CER (f_p \cdot GFR) (Fig. 1A), which was estimated in the bolus injection study, as described below (Table 1). At the end of the infusion, the kidney concentration of CER was also measured, and the renal clearance with respect to the kidney concentration (CL_{renal,k}) and the kidney-to-plasma concentration ratio (K_{p,kidney}) were determined. The CL_{renal,k} was reduced at the highest infusion rate (Fig. 1B), whereas the K_{p,kidney} was not changed (Fig. 1B), suggesting saturation of permeation across the apical membrane of renal tubules.

Comparison of the urinary excretion of CER between wild and jvs mice

To examine possible roles of OCTN2 in the renal disposition of CER, the pharmacokinetics of CER after intravenous bolus administration (10 mg/kg body wt) was compared between wild-type and jvs mice of both genders. The plasma concentration profile in jvs mice after intravenous administration was higher than that in wild-type mice (Fig. 2A, 2E). On the other hand, the cumulative urinary excretion of CER in jvs mice was reduced compared with that of wild-type mice (Fig. 2B, 2F). At 120 min after administration, the kidney concentration of CER was also measured, and
$K_{p,kidney}$ was estimated. The kidney concentration and the $K_{p,kidney}$ of CER in $jvs$ mice were much higher than those in wild-type mice (Fig. 2C, 2D, 2G, 2H). The plasma concentration profile, cumulative urinary excretion and $K_{p,kidneys}$ of CER were almost the same in male and female mice in both strains (Fig. 2), indicating minimal gender difference in renal handling of CER.

Pharmacokinetic parameters in male mice are summarized in Table 1. Both $CL_{tot}$ and $CL_{renal,p}$ in $jvs$ mice were lower than those in wild-type mice, whereas GFR, $V_{dss}$, and $f_p$ were similar in the two strains (Table 1). Taking the $f_p$ of CER and GFR into consideration, the $CL_{renal,p}$ in wild-type mice was higher than glomerular filtration clearance for CER ($f_p \cdot GFR \sim 5.4 \text{ mL/min/kg}$), yielding secretion clearance ($CL_{sec}$) for CER (Table 1). On the other hand, the $CL_{renal,p}$ in $jvs$ mice was slightly lower than $f_p \cdot GFR$ ($\sim 4.7$ mL/min/kg, Table 1).

The plasma concentration profile after oral administration of CER (50 mg/kg body wt) was also measured and compared between wild-type and $jvs$ mice. Plasma concentration profile and AUC in $jvs$ mice were higher than those in wild-type mice (Fig. 3, Table 1). The elimination half-life after oral administration was much longer than that after intravenous administration (Figs. 2, 3), suggesting that gastrointestinal absorption still continues during the elimination phase. Therefore, the absolute bioavailability cannot be precisely estimated in the present study.

**Uptake of CER by oocytes expressing mouse OCTN2**

CER was previously reported to be a substrate for human OCTN2 (Ganapathy et al., 2000), but it is not known whether it is transported by the mouse ortholog or not. In addition, detailed information on the transport properties (saturation, inhibition,
sodium dependence, etc) of OCTN2-mediated CER uptake is not available. Therefore, we performed an uptake study for CER in *Xenopus laevis* oocytes injected with cRNA for mouse OCTN2. The uptake of CER by oocytes injected with mouse OCTN2 cRNA increased in a time-dependent manner and was higher than that in oocytes injected with water (Fig. 4A). The uptake of CER was decreased in the presence of OCTN2 substrates, carnitine and TEA (Fig. 4B). This inhibition was concentration-dependent, with IC50 values of 14.8 ± 6.0 µM and 0.677 ± 0.162 mM for carnitine and TEA, respectively.

Concentration-dependent uptake of CER was then measured in the oocytes. Saturation was observed in the uptake of CER by oocytes injected with mouse OCTN2 cRNA (Fig. 5A). An Eadie-Hofstee plot for mouse OCTN2-mediated CER uptake, which was obtained by subtracting the uptake in water-injected oocytes from that in mouse OCTN2-injected oocytes, exhibited monophasic saturation (Fig. 5B). The Km and Vmax values were estimated to be 3.04 ± 0.49 mM and 34.5 ± 3.6 pmol/oocyte/90 min (Fig. 5B). The uptake of CER by mouse OCTN2-injected oocytes was similar in the presence and absence of Na+, whereas the uptake of carnitine was reduced in the absence of Na+ (Fig. 6).
DISCUSSION

Mutation in the OCTN2 gene is directly associated with SCD in humans, as well as mice (Nezu et al., 1999). Reduced reabsorption of carnitine in kidney is considered to cause SCD. Thus, OCTN2 in kidney has been characterized as a homeostatic regulator of carnitine. On the other hand, the influence of OCTN2 on the pharmacokinetics of therapeutic agents in kidney remains to be understood. In the present study, we first demonstrated that saturable transport mechanism(s) play an essential role in renal secretion of CER by means of a constant intravenous infusion study in wild-type mice (Fig. 1). The fact that the CL_{renal,p} values were higher than glomerular filtration clearance for CER (fp • GFR) at low infusion rates or after intravenous bolus administration suggests that renal secretion of CER does occur in wild-type mice (Fig. 1, Table 1). Since saturation was observed in CL_{renal,p} and CL_{renal,k}, but not in K_{p,kidney} (Fig. 1), the saturable transport may occur at the apical membranes in kidney. Finally, the higher plasma and kidney concentration and lower urinary excretion of CER in jvs mice, compared with wild-type mice (Fig. 2) suggest a predominant role of OCTN2 in renal secretion of CER. The uptake study in Xenopus laevis oocytes indicates that CER is transported by mouse OCTN2 with Km of ~3 mM (Fig. 5), which is close to the steady-state kidney concentration of CER in vivo exhibiting saturation in CL_{renal,k} (Fig. 1B), supporting the idea that OCTN2 is responsible for the saturable secretory transport of CER. This is the first demonstration that OCTN2 is involved in renal secretion of a therapeutic agent in vivo.

Ganapathy et al. (2000) have previously demonstrated that CER is transported by human OCTN2, proposing that OCTN2 mediates renal reabsorption of CER across the apical membranes. During chemotherapy, CER may compete with renal
reabsorption of carnitine via OCTN2, leading to carnitine deficiency in humans. On the other hand, the present finding indicates that OCTN2 is involved in renal secretion of CER, at least in mice. This secretion may result in protection of the kidney from the accumulation of CER. Thus, OCTN2 may play a beneficial role in terms of nephrotoxicity caused by CER. However, the present finding does not necessarily exclude the possible involvement of OCTN2 in nephrotoxicity, because genetic variants or single nucleotide polymorphisms (SNPs) of OCTN2 that cause severe impairment of OCTN2 function have been reported (Koizumi et al., 1999; Amat di San Filippo and Longo, 2004; Urban et al., 2006). Therefore, these SNPs may increase the accumulation of CER in kidney. Further clinical studies are required to determine the toxicological relevance of OCTN2 to CER-induced nephrotoxicity.

Grigat et al. (2008) recently reported that OCTN2 does not transport CER, whereas the present findings suggest the involvement of OCTN2 in renal secretion of CER. One of the possible explanations for such discrepancy would be the species difference in the function of OCTN2, since they examined OCTN2 orthologues from human, rat and chicken (Grigat et al., 2008), whereas we checked mouse OCTN2 (Fig. 5, 6). The other possible explanation would be that quite small transport activity observed in vitro does play a significant role in renal secretion in vivo. Even in the present study, the uptake of CER by OCTN2 was quite small and just 1/1,000 that of carnitine (Fig. 6). Nevertheless, if the permeation of CER across the renal apical membranes by the mechanisms other than OCTN2 is also quite small, it would be possible that transport activity of OCTN2 may affect renal secretion of CER in vivo. Thus, the present findings may propose that the minimal transport activity observed in
vitro does not necessarily mean minor contribution of the transporter to membrane permeation in vivo.

Both OAT1 and OAT3 are considered to be responsible for basolateral uptake of CER, and have been reported to be associated with nephrotoxicity (Takeda et al., 1999; Motohashi et al., 2002; Jung et al., 2002; Ueo et al., 2005). Therefore, these transporters may also affect renal secretion of CER. Although we did not examine the renal expression of OAT1 or OAT3 in jvs mice, the $K_{p,kidney}$ in jvs mice was much higher than that in wild-type mice (Fig. 2D, 2H), whereas renal excretion in jvs mice was lower compared with wild-type mice (Fig. 2B, 2F). This cannot be explained by the difference in the permeation of CER across basolateral membranes. The present findings thus suggest the decrease in apical membrane transport of CER in jvs mice, even if OATs are involved in renal transport of CER.

Possible saturation of basolateral transporters may also need to be considered at the highest infusion rate examined in the present study. Although the $IC_{50}$ value of CER for mouse OATs has not yet been reported, Jung et al. (2002) have reported $IC_{50}$ values for rat OAT1 and OAT3 (1.3 and 1.1 mM, respectively), and close to the plasma concentration of CER at the highest infusion rate (~1 mM, Fig. 1A, 1C). Therefore, these uptake transporters may be partially saturated at this plasma concentration. At the highest infusion rate, the $CL_{renal,k}$ remarkably decreased (Fig. 1B), whereas the $K_{p,kidney}$ exhibited minimal change (Fig. 1C). Such minimal change in the $K_{p,kidney}$ can be explained by the saturation of both apical membrane transporters and basolateral ones. However, there may also be species difference in the affinity of CER to OATs, and further studies are required to clarify details in the kinetics of CER in the kidney.
In addition to OAT1 and OAT3, various transporters, including urate transporter 1, sodium/phosphate cotransporter 1, multidrug resistance-associated protein 4 and multidrug and toxic compound extrusion (MATE), have been proposed to be involved in the renal tubular secretion of various substrates across the brush-border membranes (Uchino et al., 2000; Enomoto et al., 2002; Otsuka et al., 2005), suggesting that the apical secretory transport system consists of multiple transporters. Whether CER is transported by these transporters or not remains to be elucidated. On the other hand, the \( CL_{\text{renal,p}} \) in jvs mice was slightly lower than \( f_p \cdot GFR \) (Table 1), suggesting that CER is reabsorbed, at least when OCTN2 is functionally defective. Oatp1a1 and Oatp1a3 are expressed on apical membranes of proximal tubules in rats (Bergwerk et al., 1996), and lower expression of Oatp1a1 in female rats was proposed to be relevant to the higher urinary excretion of its substrates (Gotoh et al., 2002; Kato et al., 2002). In mouse kidney, gene expression of Oatp1a1 is also higher in males than females (Cheng et al., 2006). In the present study, however, no gender difference was observed in plasma and kidney concentrations, or urinary excretion of CER (Fig. 2). The molecular mechanism(s) of CER reabsorption require further study.

Among xenobiotic transporters, the transport properties of OCTN2 are unique, since OCTN2 can be involved in the physiological coupling of secretion of xenobiotics and uptake of nutrients: Ohashi et al. (2001) demonstrated in HEK293 cells transfected with mouse OCTN2 that uptake of carnitine by OCTN2 is stimulated by preloading of cells with TEA, whereas the efflux of TEA is also stimulated in the presence of carnitine in the medium. In the present study, we failed to observe a similar trans-stimulation effect between CER and carnitine, using Xenopus laevis oocytes (data not shown). However, almost complete loss of renal secretion of CER in jvs mice (Fig.
2, Table 1) indicates that CER is pumped out from tubular cells into the lumen in kidney. Considering that OCTN2 is essential for reabsorption of carnitine, CER secretion and carnitine uptake may be coupled in kidney. In addition, it is noteworthy that the $V_{dss}$ of CER is 0.2-0.3 L/kg, which is close to the extracellular volume (Table 1). On the other hand, OCTN2 is functionally expressed and is involved in uptake of carnitine into various organs, including heart, lung, liver, brain, gut, spleen and muscle, since OCTN2 is expressed ubiquitously, and distribution of carnitine in these organs after intravenous administration was reduced in $jvs$ mice (Yokogawa et al., 1999). If OCTN2 expressed in these organs is involved in uptake (influx) of CER, such a small $V_{dss}$ value is unlikely. Thus, OCTN2 in these organs may not play a role as an influx transporter for CER.

OCTN2 is also expressed on apical membranes of small intestine and is involved in the absorption of carnitine (Kato et al., 2006). In the present study, we examined the pharmacokinetics of CER after oral administration (Fig. 3) in order to clarify whether small intestinal OCTN2 is involved in the influx and/or efflux of CER or not. The $AUC_{po(0-240)}$ value in $jvs$ mice was approximately three times higher than that in wild-type mice (Table 1), but this can be almost fully explained by the three times lower value of systemic clearance ($CL_{tot}$) in $jvs$ mice (Table 1). Plasma concentration of CER after oral administration was actually quite low, compared with that after intravenous administration (Figs. 2, 3). Thus, a prominent role of OCTN2 in gastrointestinal absorption or secretion of CER could not be suggested in the present study.
ACKNOWLEDGEMENT

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REFERENCES


FOOTNOTES

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LEGENDS TO FIGURES

Fig. 1  Saturation of plasma and renal clearances during intravenous infusion of CER in wild mice

CER was intravenously infused at doses of 1.74, 17.4 and 174 mg/hr/kg body wt in wild-type male mice. In panel A, total clearance (CL\text{tot}, ○) and renal clearance with respect to plasma concentration (CL\text{renal,p}, ●) are shown. The broken line indicates the clearance that can be accounted for by glomerular filtration (f_p \cdot GFR). In panels B and C, renal clearance with respect to kidney concentration (CL\text{renal,k}) and kidney-to-plasma concentration ratio (K_{p,kidney}) are shown, respectively. Each point represents the mean ± S.E.M. (n = 3 - 4).

Table 1.  Pharmacokinetic parameters of CER in wild-type and jvs mice a)

Fig 2.  Comparison of plasma disappearance, urinary excretion and renal distribution of CER between wild and jvs mice.

CER (10 mg/kg body wt) was intravenously administered to male (A, B, C, D) and female (E, F,G, H) mice of wild-type (●) and jvs strains (○), and the plasma concentration profile (A, E) and cumulative urinary excretion (B, F) were determined. At 120 min after the administration, kidney concentration of CER was also measured (C, G) and is shown as kidney-to-plasma concentration ratio (K_{p,kidney}) in panels D and H. Each point represents the mean ± S.E.M. (n = 4~6 for males and 3 for females).

Fig. 3  Gastrointestinal absorption of CER in wild-type and jvs mice.

CER (50 mg/kg body wt) was orally administered to wild-type (●) and jvs male mice (○), and the plasma concentration profile was measured. Each point represents the mean ± S.E.M. (n = 4).
Fig. 4  Time profile (A) and inhibition by carnitine and TEA (B) of the uptake of CER by *Xenopus laevis* oocytes expressing mouse OCTN2

In panel A, oocytes injected with cRNA for mouse OCTN2 (●) or water alone (○) were incubated with 1 mM CER, and uptake for designated periods was determined. The uptake (pmol/oocyte) was normalized by the medium concentration (mM) and shown as cell-to-medium concentration ratio (nL/oocyte). In panel B, oocytes were incubated with both 1 mM CER and designated concentration of carnitine (○) or TEA (●) for 90 min. Uptake of CER was then measured, and OCTN2-mediated uptake was obtained by subtracting the uptake by water-injected oocytes from that by oocytes expressing OCTN2. The straight lines are those fitted based on Eq. (8). Each point represents the mean ± S.E.M. (n = 4 - 6).

Fig. 5  Concentration dependence of CER uptake by *Xenopus laevis* oocytes expressing mouse OCTN2.

Panel A shows uptake of CER (50 – 10,000 μM) for 90 min in oocytes injected with cRNA for mouse OCTN2 (●) or water (○). Panel B shows an Eadie-Hofstee plot of OCTN2-mediated uptake calculated by subtracting the uptake by water-injected oocytes from that by oocytes expressing OCTN2. The straight line represents the best fit. Each point represents the mean ± S.E.M. (n = 5 - 7).

Fig. 6  Different Na⁺ dependences in mouse OCTN2-mediated transport of CER and carnitine.
Uptake of CER (1 mM, A) or [³H]carnitine (0.1 µM, B) for 90 min was determined in oocytes injected with cRNA for mouse OCTN2 (closed bars) or water (open bars) in the presence or absence of Na⁺. The uptake (pmol/oocyte) was normalized by the medium concentration (mM or µM) and shown as cell-to-medium concentration ratio (nL or µL, respectively/oocyte). Each value represents the mean ± S.E.M. (n = 5 - 6).
Table 1. Pharmacokinetic parameters of CER in wild-type and jvs mice

<table>
<thead>
<tr>
<th></th>
<th>wild</th>
<th>jvs</th>
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<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>AUC_{po(0-240)} (mg·min/mL)(^b)</td>
<td>0.507 ± 0.036</td>
<td>1.64±0.27(^*)</td>
</tr>
<tr>
<td>CL(_{tot}) (mL/min/kg)(^c)</td>
<td>9.46±1.5</td>
<td>3.32±0.25(^*)</td>
</tr>
<tr>
<td>CL(_{renal,p}) (mL/min/kg)(^d)</td>
<td>8.49±0.71</td>
<td>2.44±0.33(^*)</td>
</tr>
<tr>
<td>CL(_{sec}) (mL/min/kg)(^e)</td>
<td>3.10±0.71</td>
<td>N.D.(^f)</td>
</tr>
<tr>
<td>GFR (mL/min/kg)(^g)</td>
<td>7.22±0.49</td>
<td>6.18±0.90</td>
</tr>
<tr>
<td>MRT (min)(^h)</td>
<td>34.2±18.8</td>
<td>81.5±10.1(^*)</td>
</tr>
<tr>
<td>V(_{dss}) (L/kg)(^i)</td>
<td>0.286±0.021</td>
<td>0.265±0.025</td>
</tr>
<tr>
<td>f(_p)(^j)</td>
<td>0.747 ± 0.028</td>
<td>0.767±0.029</td>
</tr>
</tbody>
</table>

a) CER (10 or 50 mg/kg body wt) was administered intravenously or orally, respectively, to male mice.
b) Area under the plasma concentration curve after oral administration from 0 to 240 min
c) Total clearance
d) Renal clearance with respect to the plasma concentration
e) Renal secretion clearance
f) Not determined because CL\(_{renal,p}\) was lower than f\(_p\)•GFR
g) Creatinine clearance determined as glomerular filtration rate
h) Mean residence time
i) Steady-state distribution volume
j) Unbound fraction in plasma

\(^*\), Significantly different from wild mice
Fig. 1

A) Plasma concentration of CER (µM)

B) Kidney concentration of CER (µM)

C) Plasma concentration of CER (µM)

CL_\text{tot} \text{ or } CL_{\text{renal,}p} (\text{mL/min/kg})

CL_{\text{renal,k}} (\text{mL/min/kg})

K_{p,\text{kidney}}
**Fig. 2**

- **A** Plots of plasma concentration (µg/mL) over time (min) for two different conditions.
- **B** Plots of urinary excretion (% of Dose) over time (min) for two different conditions.
- **C** Bar graph showing kidney concentration (µg/g tissue) for two different conditions.
- **D** Comparison of kidney concentration (µg/g tissue) for two different conditions.

The graphs and bar charts compare plasma and urinary excretion data, as well as kidney concentration, across different conditions.
Fig. 3

Plasma concentration (µg/mL) vs. Time (min)

- 0 60 120 180 240
- 100
- 10
- 1
Fig. 4

A

Uptake of CER (nL/oocyte)

0 2 4 6 8
0 30 60 90 120

Time (min)

B

Uptake of CER (% of control)

0 20 40 60 80 100
0 0.1 10 100 1000 10000

Inhibitor (µM)
Fig. 5

Panel A: Uptake of CER (pmol/oocyte/90 min) vs. CER concentration (mM).

Panel B: V/S (nL/oocyte/90 min) vs. V (pmol/oocyte/90 min).
Fig. 6

A

Uptake of CER (nL/oocyte)

OCTN2 water OCTN2 water

+Na+ -Na+

B

Uptake of carnitine (µL/oocyte)

OCTN2 water OCTN2 water

+Na+ -Na+