kidney, but not by the hepatic organic cation transporter hOCT1. In addition, the transport of creatinine by hOCT2 was inhibited by cationic drugs at around their clinical concentrations.[17] We also clarified that the transepithelial transport of creatinine across LLC-PK_1 cell monolayers was directional from the basolateral to apical side, and the characteristics of creatinine's uptake across basolateral membranes was comparable to that demonstrated in hOCT2-expressing HEK293 cells.[18]

DX-619 is a novel des-fluoro(6) quinolone highly active against gram positive bacteria.[19-24] DX-619 has two dissociation constants, pKa1 = 6.4 and pKa2 = 8.3, showing that the drug is a zwitterion in the physiological environment. Fukuda *et al.*[24] reported that the area under the concentration-time curve (AUC)/MIC ratio in the lungs for DX-619 was significantly higher than that for sitafloxacin and ciprofloxacin when tested in mice. However, a phase I clinical trial of DX-619 has not been completed yet, so very little is known about the pharmacokinetic properties of DX-619. We have previously clarified that levofloxacin (LVFX) is excreted into urine *via* tubular secretion in addition to glomerular filtration *via* specific transport system.[25-27] In addition, the tubular secretion of LVFX should be partly mediated by P-glycoprotein.[28] Furthermore, we clarified in cultured epithelial cells derived from pig kidney that LVFX interacts with the organic cation transport system at apical membranes but not at basolateral membranes at its therapeutic concentrations.[29-31] However, no report has been made so far regarding the interactions of fluoroquinolone antibacterials with hOCT2-mediated transport of creatinine. In the present study, we investigated interactions of DX-619 and LVFX with the hOCT2-mediated transport of creatinine, and then referred to its clinical significance.

Materials and Methods

Materials: DX-619, (−)7-[(3R)-3-(1-aminocyclopropyl)pyrrolidin-1-yl]-1-[(1R,2S)-2-fluoro-1-cyclopropyl]1,4-dihydro-8-methoxy-4-oxoquinoline-3-carboxylic acid, and LVFX (**Fig. 1**) were provided by Daiichi pharmaceuticals Co. Ltd. (Tokyo, Japan). [2-^{14}C]Creatinine hydrochloride (55 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Creatinine was obtained from Nacalai Tesque (Kyoto, Japan). 1-Methyl-4-phenylpyridinium (MPP) iodide was purchased from Sigma-Aldrich (St. Louis, MO, USA). All other compounds used were of the highest purity available.

Cell culture: hOCT2-expressing HEK293 cells (hOCT2-HEK293) and mock-transfectants established by the transfection of the plasmid vector pCMV6-XL4 into HEK293 cells (VEC-HEK293) in our previous study[17] were cultured in complete medium consisting of

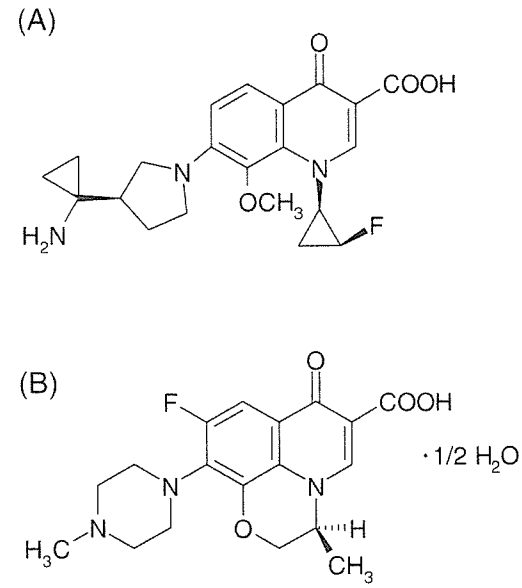

Fig. 1. Chemical structures of DX-619 (A) and LVFX (B).

Dulbecco's modified Eagle's medium with 10% fetal bovine serum in an atmosphere of 5% CO_2/95% air at 37°C. For uptake experiments, the cells were seeded onto poly-D-lysine-coated 24-well plates at a density of 2.0×10^5 cells per well. The cell monolayers were used at day 3 of culture for uptake experiments. In the present study, cells were used between the 73rd and 78th passages.

Uptake experiments using HEK293 transfectants: The uptake of [^{14}C]creatinine into cells was measured with monolayer cultures of hOCT2-HEK293 and VEC-HEK293 cells grown on poly-D-lysine-coated 24-well plates. The cells were preincubated with 0.2 mL of incubation medium for 10 min at 37°C. The medium was removed, and 0.2 mL of incubation medium containing 5 μM [^{14}C]creatinine was added. The composition of the incubation medium was as follows (in mM): 145 NaCl, 3 KCl, 1 $CaCl_2$, 0.5 $MgCl_2$, 5 D-glucose, and 5 HEPES (pH 7.4). The medium was aspirated off at the end of the incubation, and the monolayers were rapidly rinsed twice with ice-cold incubation medium. The cells were solubilized in 0.5 mL of 0.5 N NaOH, and then the radioactivity in aliquots was determined by liquid scintillation counting using a Packard TRI-CARB 1900CA (PerkinElmer, Wellesley, MA, USA). The protein content of the solubilized cells was determined by the Bradford method using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) with bovine γ-globulin as a standard.[32] The uptake of [^{14}C]creatinine was determined in the presence of various concentrations of DX-619 and LVFX. The apparent IC_{50} values were calculated from inhibition plots based on the equation, $V = V_0/[1 + (I/IC_{50})^n]$, by a

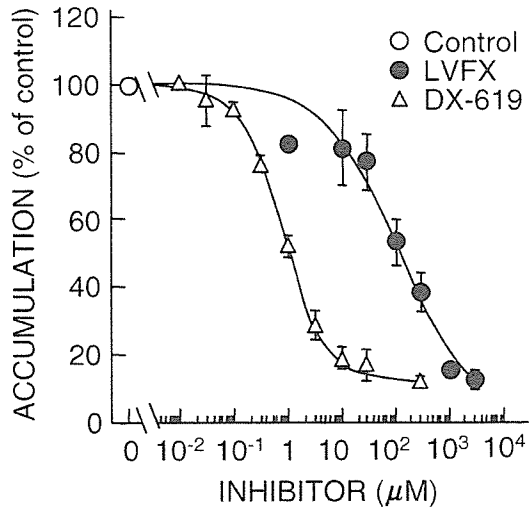

Fig. 2. Inhibition of hOCT2-mediated uptake of [^{14}C]creatinine by various concentrations of DX-619 and LVFX. HEK293 cells transfected with hOCT2 were incubated at 37°C for 2 min with 5 μM [^{14}C]creatinine (pH7.4) in the presence of DX-619 (in μM: 0.1, 0.3, 1, 3, 10, 30) or LVFX (in mM: 0.01, 0.03, 0.1, 0.3, 1, 3). Each point represents the mean ± SE of three separate experiments using three monolayers. When not shown, SE is included within the symbols.

nonlinear least squares regression analysis with Kaleidagraph Version 3.5 (Synergy Software, Reading, PA, USA).[17] V and V_0 are the uptake of [^{14}C]creatinine in the presence and absence of inhibitor, respectively. I is the concentration of inhibitor, and n is the Hill coefficient. For a Dixon plot, the uptake of [^{14}C]creatinine (5, 10 and 15 μM) was analyzed in the absence and presence of DX-619 (1 μM and 3 μM).

Statistical analyses: Data was analyzed statistically with Dunnett's test. P values of less than 0.05 were considered to be significant.

Results

Inhibition of hOCT2-mediated [^{14}C]creatinine uptake by various concentrations of DX-619 and LVFX: In order to assess the interaction of DX-619, a novel fluoroquinolone antibacterial, and LVFX with the hOCT2-mediated transport of creatinine, we measured the uptake of [^{14}C]creatinine by hOCT2-HEK293 and VEC-HEK293 cells in the absence and presence of various concentrations of DX-619 and LVFX. The hOCT2-HEK293 cells were confirmed to be able to transport [^{14}C]tetraethylammonium, a typical substrate for hOCT2, as well as [^{14}C]creatinine (data not shown). As demonstrated in our previous study,[17] the initial uptake rate of 5 μM [^{14}C]creatinine was obtained over a 2-min period with hOCT2-HEK293 cells. In the present study, both DX-619 and LVFX inhibited the uptake of 5 μM [^{14}C]creatinine for 2 min in a dose-dependent manner (**Fig. 2**). The IC_{50} values for the inhibition were 1.29 ± 0.23 μM and 127 ± 27 μM, respectively. **Table 1** gives a summary of the uptake in the absence (control) and presence of DX-619 (300 μM), LVFX (3 mM) and MPP (1 mM). Unlike in hOCT2-HEK293 cells, the uptake of 5 μM [^{14}C]creatinine in VEC-HEK293 cells was not inhibited by either of these fluoroquinolones.

Table 1. Effect of DX-619, LVFX and MPP on the uptake of [^{14}C]creatinine by VEC-HEK293 and hOCT2-HEK293 cells. The uptake of 5 μM [^{14}C]creatinine by VEC-HEK293 and hOCT2-HEK293 cells was evaluated for 2 min in the absence (control) and presence of DX-619 (300 μM), LVFX (3 mM) or MPP (1 mM). Data are expressed as the mean ± SE from three separate experiments. **, $p < 0.01$ compared to the control by Dunnet's test.

	VEC-HEK293 (nmol/mg protein/min) (% of control) n = 3	hOCT2-HEK293 (nmol/mg protein/min) (% of control) n = 3
Control	2.11 ± 0.37 (100.0)	16.58 ± 4.50 (100.0)
DX-619 (300 μM)	1.78 ± 0.18 (84.7)	1.93 ± 0.29** (11.7)
LVFX (3 mM)	1.61 ± 0.09 (76.4)	1.95 ± 0.16** (11.7)
MPP (1 mM)	1.60 ± 0.22 (76.0)	1.92 ± 0.24** (11.5)

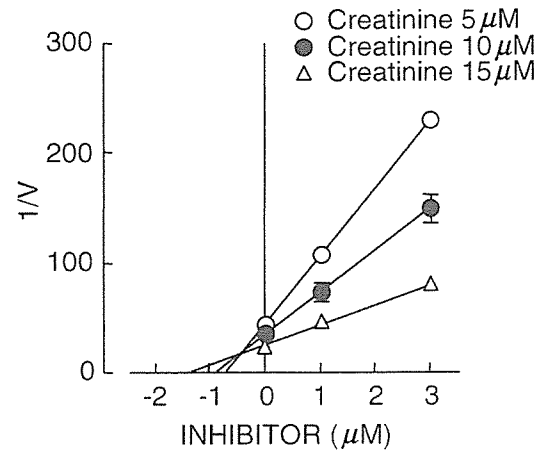

Fig. 3. Dixon plot of the inhibition of DX-619 of hOCT2-mediated transport of [^{14}C]creatinine. HEK293 cells transfected with hOCT2 were incubated at 37°C for 2 min with [^{14}C]creatinine (5 μM, open circle; 10 μM, closed circle; 15 μM, open triangle) in the absence and presence of DX-619 (1 μM or 3 μM). Each point represents the mean ± SE for three monolayers. When not shown, SE is included within the symbols.

Dixon plot of the interaction of DX-619 with hOCT2-mediated [^{14}C]creatinine transport: Next, we made a Dixon plot to clarify the type of interaction of the fluoroquinolones with the hOCT2-mediated transport of [^{14}C]creatinine. Cellular uptake of [^{14}C]creatinine (5 μM, 10 μM and 15 μM) was measured for 2 min in the absence and presence of DX-619 (1 μM and 3 μM). The Dixon plot clearly indicated that the inhibition by DX-619 of the hOCT2-mediated transport of [^{14}C]creatinine was competitive (**Fig. 3**).

Discussion

In the present study, both DX-619 and LVFX inhibited the transport of [^{14}C]creatinine in hOCT2-expressing HEK293 cells. Neither fluoroquinolone had an effect in mock-transfected HEK293 cells, suggesting that DX-619 interrupted the hOCT2-mediated transport of [^{14}C]creatinine specifically.

Serum creatinine is the most commonly used clinical marker of kidney function, because its clearance is mostly mediated by glomerular filtration. It is generally regarded that the serum creatinine level can be raised by reducing the glomerular filtration rate in patients with decreased renal functions. However, the usefulness of serum creatinine as a marker is limited by factors such as the age, sex, and amount of muscle of the patients as well as the method by which the level of creatinine is determined. Blood urea nitrogen (BUN) is also used as a clinical marker of renal function, although its value can be affected by the amount of protein ingested, protein catabolism, bleeding in the digestive tract, and the urea synthesis rate. Verho *et al.*[33] reported that the peak plasma concentration of LVFX was 6.4 μM after the oral administration of a 200-mg tablet in 6 healthy volunteers. In the present study, the peak plasma concentration was much lower than the IC_{50} of LVFX to inhibit the transport of [^{14}C]creatinine (127 ± 27 μM), suggesting that LVFX should not inhibit creatinine transport *via* hOCT2 at therapeutic concentrations. Given that similar peak plasma concentrations are obtained by administration of DX-619, the drug should markedly inhibit the transport of creatinine by hOCT2, because the IC_{50} value of DX-619 to inhibit the transport of [^{14}C]creatinine (1.29 ± 0.23 μM) was lower than the expected peak plasma concentration of DX-619. Actually, when DX-619 was administered to the healthy volunteers, an elevated serum creatinine level was observed with no apparent change in the level of BUN. In addition, the elevated serum creatinine level returned to normal with the systemic elimination of DX-619, suggesting that the tubular secretion of creatinine may be inhibited by DX-619 (unpublished observation in the clinical study).

In conclusion, DX-619 and LVFX inhibited the hOCT2-mediated transport of [^{14}C]creatinine in a competitive manner. These results suggest that the elevated serum creatinine levels after the administration of cationic drugs may be caused by the inhibition of tubular secretion of creatinine mediated in part by hOCT2.

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Induction of intestinal peptide transporter 1 expression during fasting is mediated via peroxisome proliferator-activated receptor α

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Shimakura, Jin, Tomohiro Terada, Hirofumi Saito, Toshiya Katsura, and Ken-ichi Inui. Induction of intestinal peptide transporter 1 expression during fasting is mediated via peroxisome proliferator-activated receptor α. *Am J Physiol Gastrointest Liver Physiol* 291: G851–G856, 2006. First published June 1, 2006; doi:10.1152/ajpgi.00171.2006.—We previously demonstrated that starvation markedly increased the amount of mRNA and protein levels of the intestinal H^+/peptide cotransporter (PEPT1) in rats, leading to altered pharmacokinetics of the PEPT1 substrates. In the present study, the mechanism underlying this augmentation was investigated. We focused on peroxisome proliferator-activated receptor α (PPARα), which plays a pivotal role in the adaptive response to fasting in the liver and other tissues. In 48-h fasted rats, the expression level of PPARα mRNA in the small intestine markedly increased, accompanied by the elevation of serum free fatty acids, which are endogenous PPARα ligands. Oral administration of the synthetic PPARα ligand WY-14643 to fed rats increased the mRNA level of intestinal PEPT1. Furthermore, treatment of the human intestinal model, Caco-2 cells, with WY-14643 resulted in enhanced PEPT1 mRNA expression and uptake activity of glycylsarcosine. In the small intestine of PPARα-null mice, augmentation of PEPT1 mRNA during fasting was completely abolished. In the kidney, fasting did not induce PEPT1 expression in either PPARα-null or wild-type mice. Together, these results indicate that PPARα plays critical roles in fasting-induced intestinal PEPT1 expression. In addition to the well-established roles of PPARα, we propose a novel function of PPARα in the small intestine, that is, the regulation of nitrogen absorption through PEPT1 during fasting.

Caco-2; SLC15A1; starvation; glycylsarcosine; small intestine

DIETARY PROTEINS ARE DEGRADED into a mixture of free amino acids and small peptides. A large number of studies have provided evidence that the absorption of protein digestion products in the small intestine occurs primarily in the form of small peptides rather than amino acids (22). Thus intestinal peptide transport is of major nutritional significance for the effective absorption of dietary amino nitrogen. Cellular uptake of di- and tripeptides is mediated via H^+-coupled peptide cotransporter 1 (PEPT1, SLC15A1), which is primarily expressed in the small intestine and slightly in the kidney (5). Because of its broad substrate specificity, PEPT1 can accept several peptidelike drugs such as oral β-lactam antibiotics (39) and plays important roles not only as a nutrient transporter but also as a drug transporter. A large number of functional studies using heterologous expression systems have demonstrated the molecular nature of its transport characteristics (6, 15, 20, 24). Furthermore, many studies have also been directed toward the regulation of PEPT1. For example, it has been reported that intestinal PEPT1 is regulated by various factors (1), including dietary conditions (28, 35, 42), hormones [insulin (10), thyroid hormone (2)], epidermal growth factor (27), some pharmacological agents (3, 9), and diurnal rhythm (29).

Among these factors, the dietary regulation of intestinal PEPT1 has been extensively investigated (14, 26, 28, 30, 31, 35, 42). It has been reported that short-term starvation markedly increased the amount of PEPT1 mRNA and protein expression (14, 30, 42) and uptake activity of dipeptides (42). The induction of PEPT1 expression might be an adaptive response against fasting for efficient absorption immediately after food is given again. This fasting-induced expression of PEPT1 altered the pharmacokinetic profiles of some drugs. Fasting increased the transport of the β-lactam antibiotic cefadroxil in an in situ loop experiment (26) and also increased pharmacokinetic parameters such as maximum plasma concentration and area under the plasma concentration-time curve of the β-lactam antibiotic ceftibuten in vivo (30).

Although these studies demonstrated the functional aspects of PEPT1 augmentation, the regulatory mechanisms remain to be clarified. In the present study, we assume that some metabolic signals direct this regulation. In the liver, an adaptive response to fasting has been well characterized (43). During fasting, lipolysis of stored triglycerides in adipose tissue is strongly activated, resulting in marked increase in plasma free fatty acid level. The released fatty acids are delivered to the liver, where they undergo β-oxidation for energy production. The peroxisome proliferator-activated receptors (PPARα, -β/δ, and -γ) are a family of nuclear receptors activated by fatty acid ligands (7, 12). PPARα acts as a nutritional state sensor and plays a pivotal role in the control of this adaptive response (16, 21) by inducing the transcription of genes such as the peroxisomal and mitochondrial β-oxidation pathways. Accordingly, PPARα is principally expressed in organs with a high capacity for fatty acid oxidation, such as heart, skeletal muscle, liver, and kidney. However, PPARα is also expressed in the small intestine (7) and is increased by fasting (8). This raises the possibility that PPARα might be responsible for the augmentation of PEPT1 during fasting. In the present study, we have investigated this possibility by examining PPARα activation through synthetic ligand and expression profiles of intestinal PEPT1 in fed and fasted PPARα-null mice.

MATERIALS AND METHODS

Materials. WY-14643 was purchased from Cayman Chemical (Ann Arbor, MI). Pioglitazone and GW-501516 were purchased from

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Alexis Biochemicals (Lausen, Switzerland) and Calbiochem (Darmstadt, Germany), respectively. [^{3}H]glycylsarcosine ([^{3}H]Gly-Sar; 18.5 GBq/mmol) was obtained from Moravek Biochemicals (Brea, CA). All other chemicals used were of the highest purity available.

Cell culture, treatment with PPAR ligands, and uptake study. Caco-2 cells were obtained from the American Type Culture Collection (ATCC CRL-1392) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% nonessential amino acids. Caco-2 cells were plated into 24-well plates (*day 1*) and, 2 or 9 days later, treated with the PPAR ligand WY-14643, pioglitazone, GW-501516, or DMSO (0.1%, as a control) for 24 h. The uptake experiment using [^{3}H]Gly-Sar with Caco-2 cells in a 24-well plate after WY-14643 treatment was performed according to our previous studies (38, 40).

Animal studies. Animal studies were performed in accordance with the Guidelines for Animal Experiments of Kyoto University. All protocols were previously approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University (MedKyo 05189). Male Wistar rats (8 wk old) were obtained from Japan SLC. Male PPARα-null mice (B6.129S4-Pparα^{tm1Gonz} N12) and wild-type mice (C57BL/6) (8 wk old) were purchased from Taconic (Germantown, NY). The animals were fed a normal chow ad libitum except for the fasting experiments. In all experiments, the animals had free access to water. For determination of the effects of fasting, food was removed from cages 48 h before the animals were killed. For determination of the effect of PPARα ligand, fed rats were treated with WY-14643 (50 mg·5 ml^{-1}·kg^{-1}·day^{-1}, suspended in 0.5% methyl cellulose solution) or vehicle (5 ml/kg) by oral gavage for 5 days and killed after an additional 24 h. The small intestine (duodenum and upper part of the jejunum) was removed from the rats or mice under anesthesia. The kidney cortex was also removed from mice. The scraped intestinal mucosa and kidney cortex were rapidly frozen in liquid nitrogen for later preparation of total RNA.

Measurement of level of blood glucose and serum free fatty acids. Blood glucose level was quantified with the FreeStyle blood glucose monitoring system (Nipro, Osaka, Japan). Serum free fatty acid level was quantified with an enzymatic colorimetric assay (free fatty acid, Half-micro test; Roche Diagnostics, Penzberg, Germany).

Real-time PCR. Total RNA was isolated from Caco-2 cells, intestinal mucosa of rats and mice, and mice kidney cortex with the RNeasy Mini Kit (Qiagen, Hilden, Germany). Isolated total RNA (250 or 500 ng) was reverse transcribed, and the reaction mixtures were used for real-time PCR. Real-time PCR was performed with an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) in a total volume of 20 μl containing a 2-μl aliquot of cDNA, 0.5 μM forward and reverse primers, 0.1 μM TaqMan probe, and 10 μl of TaqMan Universal PCR Master Mix (Applied Biosystems) under the following conditions: 50 cycles of 95°C for 15 s and 60°C for 60 s. The forward and reverse primers for mouse PEPT1 were 5′-CGTGCACGTAGCACTGTCCAT-3′ (positions 388–408) and 5′-GGCTTGATTCCTCCTGTACCA-3′(positions 433–453), respectively. The forward and reverse primers for rat PEPT1 were 5′-TGCACGTAGCACTGTCCATGA-3′ (positions 359–379) and 5′-CAGGGCTTGATTCCTCCTGTAC-3′ (positions 425–404), respectively. The sequence of the TaqMan probe was 5′-(6-FAM)TTGGCCTGGCCCTGATAGCCC(TAMRA)-3′, corresponding to positions 411–432 for mice, and 5′-(6-FAM)CGGCCTGGCCCTGATAGCCCT(TAMRA)-3′, corresponding to positions 381–404 for rats. The primer probe sets used for human PEPT1 (41) and rat Na^+-glucose cotransporter 1 (SGLT1) (13) were previously designed. The primer probe sets used for mouse and rat PPARα, mouse acyl-CoA oxidase (ACOX), rat Sp1, and rat Cdx2 were predeveloped TaqMan Assay Reagents (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was also measured as an internal control with GAPDH Control Reagent (Applied Biosystems).

Data analysis. Data are expressed as means ± SE. The statistical significance of differences between the groups was analyzed with one-way ANOVA followed by Scheffé *F* post hoc testing in the experiment using Caco-2 cells and multiple concentrations of WY-14643 as a ligand. In other experiments, the nonpaired *t*-test was used. Two or three experiments were conducted, and representative results are shown.

RESULTS

Fasting-induced expression of intestinal PEPT1 is accompanied by increased mRNA level of PPARα in rats. In general, energy depletion by fasting causes a shift in whole body fuel utilization from glucose and fat in the fed state to almost exclusively fat. Under our experimental conditions, 48-h fasting led to reductions in body weight and blood glucose level and dramatic increment in serum free fatty acid level, reflecting the metabolic switching mentioned above (Fig. 1). Under this condition, we determined mRNA levels of intestinal PEPT1 and PPARα (Fig. 2). The transcription factor Sp1 and caudal-related homeobox protein Cdx2 were also measured because we recently demonstrated that PEPT1 is transcriptionally regulated by Sp1 and Cdx2 (33, 34). As expected, fasting significantly induced mRNA levels of PEPT1, consistent with our previous result (30). The expression level of PPARα was increased to twofold by fasting, whereas the increments in Sp1 and Cdx2 were smaller than that in PPARα.

Expression of PEPT1 mRNA is induced by PPARα ligand WY-14643 in Caco-2 cells. To assess the potential involvement of PPARα in PEPT1 expression, we treated an intestinal model system, Caco-2 cells just before and after confluence, for 24 h with the PPARα ligand WY-14643 and measured the expression levels of PEPT1 mRNA and the activity of [^{3}H]Gly-Sar uptake (Fig. 3). PEPT1 mRNA expression levels and the activity of [^{3}H]Gly-Sar uptake were significantly increased in response to WY-14643 in the cells of both stages. Thus it was suggested that the induction of PEPT1 mRNA by WY-14643 led to a concomitant increase of the transport function. Next,

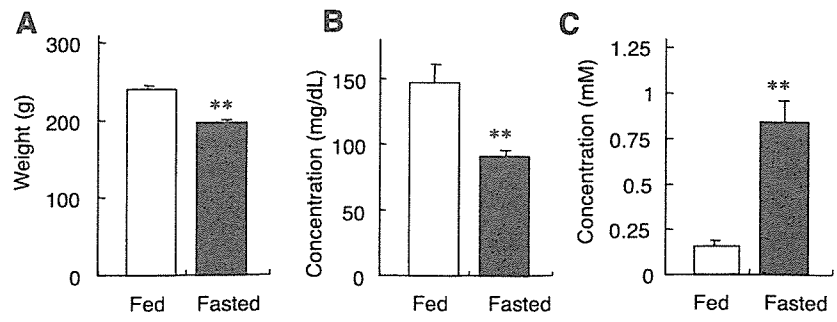

Fig. 1. Effects of fasting on body weight (*A*), blood glucose (*B*), and serum free fatty acids (*C*) in rats. Blood glucose and serum free fatty acid levels in 48-h fasted or fed rats were determined as described in MATERIALS AND METHODS. Data are means ± SE for 5 rats. **Significantly different from fed rats ($P < 0.01$).

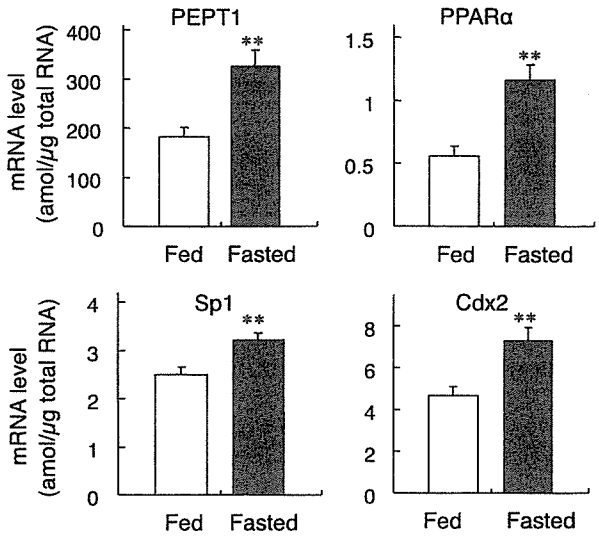

Fig. 2. Effects of fasting on mRNA levels of peptide cotransporter 1 (PEPT1), peroxisome proliferator-activated receptor (PPAR)α, Sp1, and Cdx2 in rat small intestine. Total RNA was isolated from the small intestine of 48-h fasted or fed rats, transcribed to cDNA, and subjected to real-time PCR analysis. The results corrected by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels are means ± SE for 5 rats. **Significantly different from fed rats ($P < 0.01$).

Caco-2 cells were treated with the PPARα ligand WY-14643 and increasing concentrations of the PPARγ ligand pioglitazone and the PPARβ/δ ligand GW-501516 (Fig. 4). The activity of [^{3}H]Gly-Sar uptake increased only when PPARα ligand WY-14643 was administered.

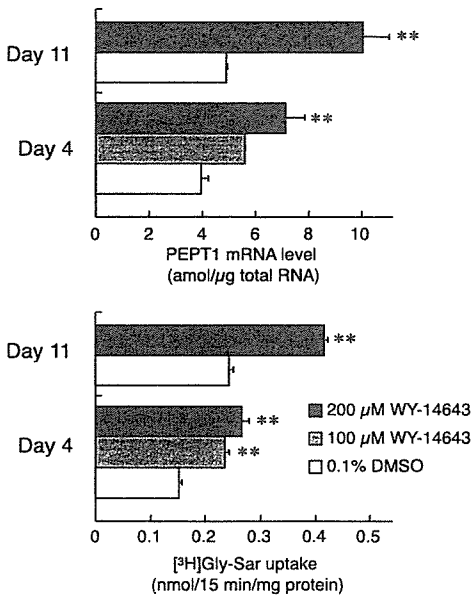

Fig. 3. Expression of PEPT1 mRNA and [^{3}H]glycylsarcosine ([^{3}H]Gly-Sar) uptake in Caco-2 cells treated with WY-14643. Twenty-four hours after WY-14643 treatment, Caco-2 cells were subjected to total RNA isolation or [^{3}H]Gly-Sar uptake experiment on *day 4* and *day 11*. The concentrations of WY-14643 were set to 100 and 200 μM on *day 4* and 200 μM on *day 11*. Total RNA was transcribed to cDNA and subjected to real-time PCR analysis. For the uptake experiment, Caco-2 cells were incubated with 25 μM [^{3}H]Gly-Sar for 15 min at 37°C. Data are means ± SE for 3 monolayers. Data of mRNA analysis are corrected by GAPDH levels. **Significantly different from control (0.1% DMSO) ($P < 0.01$).

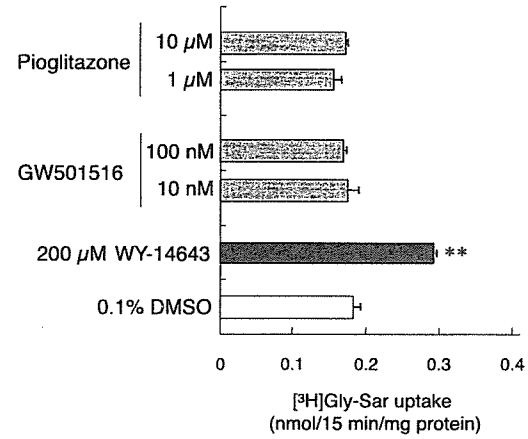

Fig. 4. [^{3}H]Gly-Sar uptake in Caco-2 cells treated with PPAR ligands for 24 h. WY-14643, pioglitazone, and GW-501516 were used as the ligands for PPARα, -γ, and -β/δ, respectively. The day after treatment (*day 4*), Caco-2 cells were incubated with 25 μM [^{3}H]Gly-Sar for 15 min at 37°C. Data are means ± SE for 3 monolayers. **Significantly different from control (0.1% DMSO) ($P < 0.01$).

Effect of WY-14643 on expression levels of intestinal PEPT1 mRNA in rats. We subsequently administered WY-14643 (50 mg/kg) orally to fed rats for 5 days to examine the effect of PPARα ligand on PEPT1 expression in vivo. For comparison, the mRNA levels of SGLT1 were also determined. SGLT1 is expressed at the brush-border membranes of intestinal epithelial cells (11) as PEPT1 but is reported to show no significant change in its expression level during fasting (14). As expected, mRNA levels of PEPT1 were significantly increased by WY-14643, whereas those of SGLT1 were not changed (Fig. 5).

Effect of fasting on expression levels of intestinal PEPT1 in PPARα-null mice. To confirm the contribution of PPARα to fasting-induced PEPT1 expression, we measured mRNA levels of PEPT1 in the small intestine of wild-type and PPARα-null mice (Fig. 6). Fasting induced the expression of PEPT1 and PPARα in wild-type mice as in rats, although the induction of PPARα was not statistically significant. In contrast, this fasting response of PEPT1 was completely abolished in PPARα-null mice, indicating the critical role of PPARα in this response.

Effect of fasting on expression levels of renal PEPT1 in PPARα-null mice. PEPT1 is also expressed in the kidney, although its expression level is much lower than that in the small intestine. Thus we investigated the effect of fasting on expression levels of PEPT1 and PPARα in the kidney (Fig. 7). For comparison, mRNA levels of ACOX, which is a peroxisomal β-oxidation enzyme and a representative PPARα target gene, were simultaneously determined. In contrast to the intestine, renal PEPT1 was not induced by fasting in either wild-type or PPARα-null mice, which is in agreement with our previous result in rats (30). ACOX expression was markedly enhanced, although the PPARα level did not show significant elevation. These results suggested that the lack of fasting-induced response of renal PEPT1 was not due to insufficient levels of endogenous ligands or PPARα.

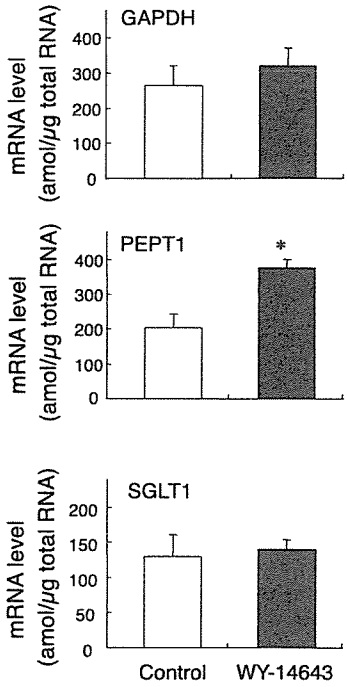

Fig. 5. mRNA levels of PEPT1, Na^+-glucose cotransporter (SGLT)1, and GAPDH in the small intestine of rats dosed with WY-14643 (50 mg/kg) for 5 days. Total RNA was isolated from the small intestine, transcribed to cDNA, and subjected to real-time PCR analysis. Data are means ± SE for 3 rats. *Significantly different from control ($P < 0.05$).

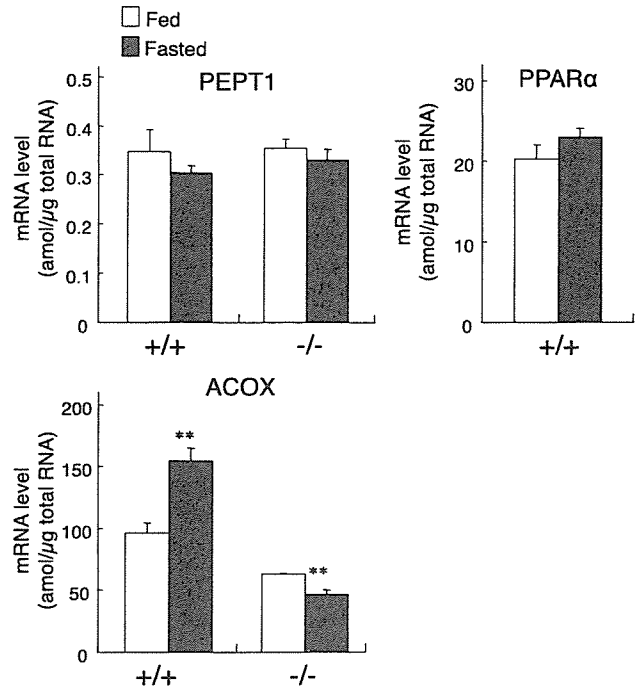

Fig. 7. mRNA levels of PEPT1, PPARα, and PPARα target gene acyl-CoA oxidase (ACOX) in the kidney of fasted or fed wild-type and PPARα-null mice. Total RNA was isolated from the kidney cortex of 48-h fasted or fed mice, transcribed to cDNA, and subjected to real-time PCR analysis. Data corrected by GAPDH levels are means ± SE for 6 mice. **Significantly different from fed mice of the same genotype ($P < 0.01$).

DISCUSSION

In the present study, we investigated the regulatory mechanism underlying the augmentation of intestinal PEPT1 in fasting. In agreement with previous results (4, 8), it was confirmed that the serum concentration of free fatty acids and the expression level of intestinal PPARα mRNA were increased under our experimental condition. We showed that PPARα played a critical role in the augmentation of intestinal PEPT1 as a fasting response mainly through PPARα-null mice. In support of the knockout mouse data, we used Caco-2 cells to show that the PPARα ligand WY-14643 upregulated PEPT1, because Caco-2 cells expressed endogenous PPARα (data not shown). Several studies have used Caco-2 cells as an intestinal model for activating PPAR target genes by synthetic PPARα ligands (19, 32, 37). Furthermore, the effect of WY-14643 on PEPT1 expression was also observed in vivo in rats. These findings led us to speculate that elevated free fatty acids serve as a metabolic signal and activate intestinal PPARα, resulting in the augmentation of PEPT1 in an adaptive response to fasting.

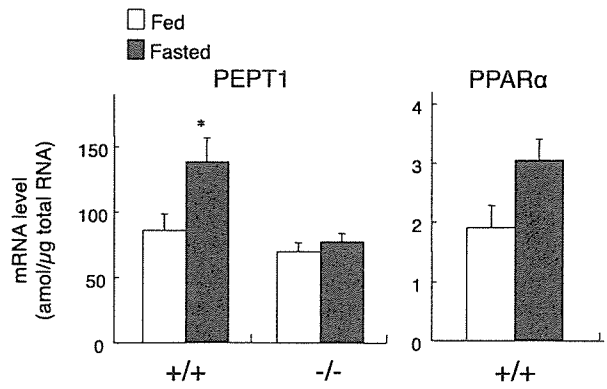

Fig. 6. mRNA levels of PEPT1 and PPARα in the small intestine of fasted or fed wild-type (+/+) and PPARα-null (−/−) mice. Total RNA was isolated from the small intestine of 48-h fasted or fed mice, transcribed to cDNA, and subjected to real-time PCR analysis. Data corrected by GAPDH levels are means ± SE for 6 mice. *Significantly different from fed mice of the same genotype ($P < 0.05$).

With regard to the roles of PPARα in the small intestine, there are several studies. PPARα is reported to be involved in lipid absorption through regulation of fatty acid binding protein (7). In addition, PPARα influences cholesterol absorption through modulation of ATP binding cassette transporter A1 activity in the intestine (18). PPARα also regulates other intestinal genes such as CYP1A1 (32), bile acid binding protein (19), retinol-binding protein (37), and 17β-hydroxysterol dehydrogenase type 11 (25). The presented function of PPARα, i.e., augmentation of PEPT1, is quite different from those previously demonstrated from the point of view that peptide absorption was implicated as a target. In fasting, sloughing of mucosal cells into the intestinal lumen is observed (23), resulting in atrophy of mucosa and decreased mucosal weight. It may be possible to speculate that increased PEPT1 minimizes the loss of nitrogen by efficient absorption of small peptides derived from sloughing cells or secreted hormones. Furthermore, induced PEPT1 will lead to efficient absorption of peptides immediately after food is given again. It has also

been reported that PPARα downregulates the hepatic genes involved in amino acid metabolism, leading to an overall decrease of amino acid degradation (17). It seems reasonable to suppose that PPARα functions to minimize the loss of body amino acids both by suppressing amino acid degradation and by increasing peptide absorption during fasting.

In contrast to the small intestine, no PEPT1 induction was observed in the kidney, suggesting different regulatory mechanisms for PEPT1 between them. This cannot be explained solely by the lack of activation of renal PPARα, because the expression of the representative PPARα target gene, ACOX, was markedly increased. PPARα and free fatty acids may be necessary but not sufficient for the induction of renal PEPT1.

In our previous and present study, the fasting response of PEPT1 was discussed mainly from the findings obtained in rats or mice. In humans, WY-14643 induced the expression of PEPT1 in a human intestinal cell line, Caco-2. This result, together with the fact that plasma free fatty acids are also increased during fasting in humans (36), suggested that a similar regulation of PEPT1 by PPARα might exist also in humans. To test the possibility that PPARα directly regulates the human PEPT1 promoter, we searched for the potential PPAR response element (PPRE) on the promoter as far as 10 kb upstream of the transcription start site and found several proposed PPREs. However, none of these sites, which were subcloned upstream of the PEPT1 proximal promoter, enhanced basal promoter activity in response to WY-14643 treatment in Caco-2 cells (data not shown). Alternatively, it is possible that PPARα stimulates the expression of other transcription factors that regulate PEPT1. mRNA levels of Sp1 and Cdx2 were increased in fasted rats. However, these factors may not be responsible for the augmentation of PEPT1 by PPARα because the PEPT1 promoter activity responsive to Sp1 or Cdx2 was not altered by WY-14643 treatment (data not shown). The functional PPRE and/or other regulatory region related to PPARα may be located in more distal regions or intronic regions.

The observation that PPARα regulates PEPT1 expression raises the question of whether the administration of PPARα agonist increases PEPT1 function in the clinical situation. This issue is important from the viewpoint of clinical drug-drug interaction because PPARα ligands belonging to the fibrate class have been used widely as hypolipidemic drugs. Oral dosing of WY-14643 in rats resulted in increase of PEPT1 expression. It is unclear whether clinically used PPARα ligands affect the expression of human intestinal PEPT1 in vivo. Further studies will be needed to estimate the change in the clinical pharmacokinetics of PEPT1 substrates and the impact on its therapeutic effects.

In conclusion, we demonstrated that PPARα plays a critical role in the augmentation of PEPT1 during fasting. We propose a novel function of PPARα in the small intestine, that is, the regulation of nitrogen absorption through PEPT1 as an adaptive response to fasting.

GRANTS

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Cisplatin and Oxaliplatin, but Not Carboplatin and Nedaplatin, Are Substrates for Human Organic Cation Transporters (SLC22A1–3 and Multidrug and Toxin Extrusion Family)

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ABSTRACT

We have examined the role of the human organic cation transporters [hOCTs and human novel organic cation transporter (hOCTN); SLC22A1–5] and apical multidrug and toxin extrusion (hMATE) in the cellular accumulation and cytotoxicity of platinum agents using the human embryonic kidney (HEK) 293 cells transiently transfected with the transporter cDNAs. Both the cytotoxicity and accumulation of cisplatin were enhanced by the expression of hOCT2 and weakly by hOCT1, and those of oxaliplatin were also enhanced by the expression of hOCT2 and weakly by hOCT3. The hOCT-mediated uptake of tetraethylammonium (TEA) was markedly decreased in the presence of cisplatin in a concentration-dependent manner. However, oxaliplatin showed almost no influence on the TEA uptakes in the HEK293 cells expressing hOCT1, hOCT2, and hOCT3. The hMATE1 and hMATE2-K, but not hOCTN1 and OCTN2, mediated the cellular accumulation of cisplatin and oxaliplatin without a marked release of lactate dehydrogenase. Oxaliplatin, but not cisplatin, markedly decreased the hMATE2-K-mediated TEA uptake. However, the inhibitory effect of cisplatin and oxaliplatin against the hMATE1-mediated TEA uptake was similar. The release of lactate dehydrogenase and the cellular accumulation of carboplatin and nedaplatin were not found in the HEK293 cells transiently expressing these seven organic cation transporters. These results indicate that cisplatin is a relatively good substrate of hOCT1, hOCT2, and hMATE1, and oxaliplatin is of hOCT2, hOCT3, hMATE1, and hMATE2-K. These transporters could play predominant roles in the tissue distribution and anticancer effects and/or adverse effects of platinum agent-based chemotherapy.

Four platinum-based anticancer drugs are currently registered for clinical use. *cis*-Diamminedichloroplatinum II (cisplatin) has been clinically used for over 30 years and continues to play an essential role in cancer chemotherapy against solid tumors of the prostate, bladder, colon, lung, testis, liver, and brain (Ho et al., 2003). However, severe nephrotoxicity limits its clinical application because it was reported that an increase in the serum creatinine concentration was observed in 41% of patients treated with a high dose of cisplatin (de Jongh et al., 2003). *cis*-Diammine(1,1-cyclobutanedicarboxylato)platinum II (carboplatin), *trans*-L-1,2-diaminocyclohexaneoxalatoplatinum II (oxaliplatin), and *cis*-diammineglycolatoplatinum (nedaplatin) are analogs of cisplatin and show a lowered nephrotoxicity compared with cisplatin. However, it is not clear why the nephrotoxicity of these analogs is low, although cisplatin induces severe nephrotoxicity. The chemical structures of platinum agents are shown in Fig. 1.

The mechanisms of cellular uptake and efflux of platinum agents are not fully understood. In our laboratory, the cisplatin uptake was reported to be mediated by specific transporter(s) in the renal tubular cells (Okuda et al., 1999). Recently, we reported that rat kidney-specific organic cation transporter 2 (rOCT2/Slc22a2) transported cisplatin and was responsible for cisplatin-induced nephrotoxicity (Yonezawa et al., 2005). Just after our report, hOCT2 was reported to transport cisplatin (Ciarimboli et al., 2005). However, it is not clear whether these analogs were transported by OCT2.

The organic ion transporter family (SLC22) consists of the organic anion transporter (OAT), OCT, and novel organic cation transporter (OCTN), which transport drugs,

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ABBREVIATIONS: r, rat; OCT, organic cation transporter; h, human; OAT, organic anion transporter; OCTN, novel organic cation transporter; MATE, multidrug and toxin extrusion; HEK, human embryonic kidney; TEA, tetraethylammonium; MPP, 1-methyl-4-phenylpyridium; PAH, *p*-aminohippurate; ES, [6,7-(*N*)]esterone-3-sulfate; ICP-MS, inductively coupled plasma-mass spectrometry; LDH, lactate dehydrogenase.

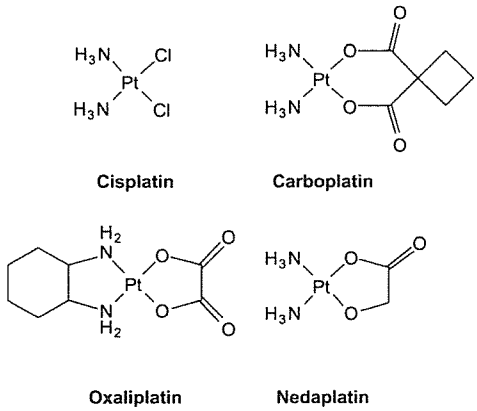

Fig. 1. Chemical structures of four platinum agents.

toxins and endogenous metabolites (Inui et al., 2000; Jonker and Schinkel, 2004). hOCT1 is expressed mainly in the liver, hOCT2 in the kidney (Gorboulev et al., 1997), and hOCT3 is predominantly expressed in the placenta and weakly in the intestine, heart, brain, and kidney (Kekuda et al., 1998). The substrate specificities of hOCT1, hOCT2, and hOCT3 have been considered to not be the same. hOCTN1 and hOCTN2 were expressed not only in the kidney but also in other tissues. They were expressed in the apical membrane of renal proximal tubules. Human multidrug and toxin extrusion 1 (hMATE1) was recently cloned and characterized (Otsuka et al., 2005). It was expressed in the liver, kidney, and skeletal muscle. Moreover, we cloned hMATE2-K, which is specifically expressed in the apical membrane of renal epithelial cells and transports cationic drugs (Masuda et al., 2006).

Based on these backgrounds, we hypothesized that organic cation transporters could transport four platinum drugs and play an important role in the cellular accumulation and toxicity of platinum agents. In the present study, we examined whether the basolateral cation transporters hOCT1, hOCT2, and hOCT3, and the apical cation transporters, hMATE1, hMATE2-K, hOCTN1, and hOCTN2, transported platinum agents and affected the cytotoxicity induced by these drugs.

Materials and Methods

Cell Culture and Transfection. HEK293 cells (CRL-1573; American Type Culture Collection, Manassas, VA) were cultured in complete medium consisting of Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (Whittaker Bioproducts Inc., St. Louis, MO) in an atmosphere of 5% CO_2-95% air at 37°C.

For a transient expression system, pCMV6-XL4 plasmid vector DNA (OriGene Technologies, Rockville, MO), pcDNA3.1(+) plasmid vector DNA (Invitrogen, Carlsbad, CA), or pBK-CMV plasmid vector DNA (Stratagene, La Jolla, CA) containing hOCT1, hOCT2, hOCT3, hMATE1, hMATE2-K, hOCTN1, hOCTN2, hOAT1, and hOAT3 cDNA was purified using Midi-V100 Ultrapure Plasmid Extraction Systems (Viogene, Sunnyvale, CA). The day before transfection, HEK293 cells were seeded onto poly-D-lysine-coated 24-well plates at a density of 2.0×10^5 cells per well. The cells were transfected with 800 ng of plasmid DNA per well in a combination of empty vector and transporter cDNA using 2 μl of Lipofectamine 2000 (Invitrogen) per well according to the manufacturer's instructions. The amount of transporter cDNA was 100 ng except in the experiment examining the transporter cDNA-dependence. Forty-eight hours after the transfection, the cells were used for the experiments.

Uptake Experiment. Cellular uptake of tetraethylammonium (TEA; 88.8 MBq/mmol; PerkinElmer Life Analytical Sciences, Boston, MA), [^{3}H]1-methyl-4-phenylpyridium acetate ([^{3}H]MPP; 2.7 TBq/mmol; PerkinElmer), *p*-[^{14}C]aminohippurate ([^{14}C]PAH; 1.9 GBq/mmol; PerkinElmer), and [6,7-^{3}H(N)]esterone-3-sulfate, ammonium salt ([^{3}H]ES; 2.1 TBq/mmol, PerkinElmer) was measured with HEK293 cells grown on poly-D-lysine-coated 24-well plates. The composition of the incubation buffer was as follows: 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 or pH 8.4 adjusted with NaOH. Experimental procedures were performed as described previously (Urakami et al., 2004).

To measure the cellular accumulation of the platinum agents, HEK293 cells were seeded on poly-D-lysine-coated 24-well plates. Cells were incubated in Dulbecco's modified Eagle's medium with 10% fetal bovine serum containing cisplatin (Sigma), carboplatin (Sigma), oxaliplatin (a gift from Yakult Co. Ltd., Tokyo, Japan), and nedaplatin (LKT Laboratories, Inc., St. Paul, MN). After the incubation, the monolayers were rapidly washed twice with ice-cold incubation buffer containing 3% bovine serum albumin (Nacalai Tesque Inc., Kyoto, Japan) and then washed three times with ice-cold incubation buffer. The cells were solubilized in 0.5 N NaOH, and the amount of platinum was determined using inductively coupled plasma-mass spectrometry (ICP-MS) by the Pharmacokinetics and Bioanalysis Center, Shin Nippon Biomedical Laboratories, Ltd. (Wakayama, Japan). The protein content of the cell monolayers solubilized in 0.5 N NaOH was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Richmond, CA).

Measurement of Released Lactate Dehydrogenase Activity. The cytotoxicity of the platinum agents was measured with HEK293 cells seeded on poly-D-lysine-coated 24-well plates. Cells were incubated with the medium containing platinum agent with or without cimetidine (Nacalai) for 2 h. After the removal of the medium, a drug-free medium was added. After incubation for 24 h, the medium was collected, and the lactate dehydrogenase (LDH) activity in the medium was measured using an LDH cytotoxicity detection kit (Takara Bio Inc., Shiga, Japan), according to the manufacturer's instructions. Cytotoxicity was evaluated by measuring the LDH activity in the medium. The total LDH activity was defined as the LDH activity in the medium containing 1% Triton X-100. LDH release (percentage) represents (LDH activity − LDH activity of control)/(total LDH activity − LDH activity of control) × 100.

Statistical Analysis. Data are expressed as means ± S.E.M. Data were analyzed statistically using the unpaired Student's *t* test. Multiple comparisons were performed with Dunnett's two-tailed test after a one-way ANOVA. Probability values of less than 0.05 were considered statistically significant.

Results

Cytotoxicity of Cisplatin in HEK293 Cells Transiently Expressing hOCT2. We examined the expression level-dependent effect of hOCT2 on the cisplatin-induced cytotoxicity. When HEK293 cells transfected with 50 to 800 ng of hOCT2 cDNA per well were treated with 500 μM cisplatin for 2 h and subsequently cultured in normal medium for 24 h, the release of LDH into the culture medium was increased in a hOCT2 expression-dependent manner (Fig. 2A). Based on these results, we decided that cells were transfected with 100 ng of transporter cDNA. Moreover, cimetidine inhibited the cytotoxicity induced by 500 μM cisplatin in a concentration-dependent manner with an IC_{50} value of 109.3 ± 4.4 μM (mean ± S.E.M. of three separate experiments) in hOCT2-expressing cells (Fig. 2B).

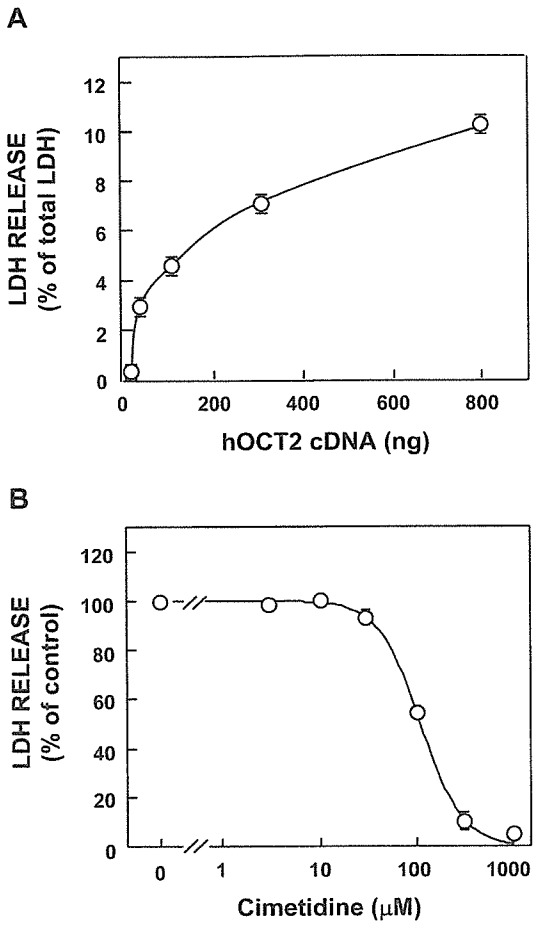

Fig. 2. Role of hOCT2 expression in the cisplatin-induced cytotoxicity. A, HEK293 cells were transfected with each amount of hOCT2 cDNA and vector plasmid added to 800 ng using 2 μl of Lipofectamine 2000. The cells were exposed to 500 μM cisplatin in the medium for 2 h and then incubated in normal medium for 24 h. LDH released into the medium was measured. B, HEK293 cells were transfected with each amount of 100 ng of hOCT2 cDNA and 700 ng of vector plasmid. The cells were incubated in the medium containing 500 μM cisplatin with 0 to 1000 μM cimetidine for 2 h and then incubated in normal medium for 24 h. LDH released into the medium was measured. Each point represents the mean ± S.E.M. of three wells.

Effects of Expression of Basolateral Organic Ion Transporters, hOCT1, hOCT2, hOCT3, hOAT1, and hOAT3, on the Cisplatin-Induced Cytotoxicity in HEK293 Cells. We examined the effect of the basolateral organic ion transporter expression on cisplatin-induced cytotoxicity. When the cells transiently expressing hOCT1, hOCT2, and hOCT3 were treated with 50 to 1000 μM cisplatin for 2 h, the cisplatin-induced cytotoxicity was strongly enhanced by the expression of hOCT2, weakly by the expression of hOCT1, but not by hOCT3 (Fig. 3A). When the cells were transfected with 100 or 800 ng of hOAT1 or hOAT3 cDNA, the cytotoxicity of hOAT-expressing cells was not induced by 500 μM cisplatin (Fig. 3D). In addition, these transporter activities were confirmed by the uptake of their typical substrates, TEA, MPP, PAH, and ES, as shown in Fig. 3, B, C, E, and F.

Effects of hOCT1, hOCT2, and hOCT3 Expression on Platinum Agent-Induced Cytotoxicity in HEK293 Cells. The effects of hOCT1, hOCT2, and hOCT3 expression on the platinum agent-induced cytotoxicity were simultaneously examined using the same passage cells in Fig. 4. When the cells transfected with 100 ng of hOCT1, hOCT2, and hOCT3 were treated with 50 to 1000 μM cisplatin, the highest LDH release in hOCT2-expressing cells among these OCT-expressing cells was observed. Moreover, hOCT1 also enhanced the cisplatin-induced cytotoxicity at higher concentrations, but not hOCT3 (Fig. 4A). The reproducibility (Figs. 3A and 4A) was confirmed in these transfectants, and then we compared the susceptibility of these transfectants to analogs of cisplatin. Oxaliplatin also induced cytotoxicity in HEK293 cells expressing hOCT2 and hOCT3 (Fig. 4C). The treatment with carboplatin and nedaplatin (50–1000 μM) did not affect the LDH release in hOCT1-, hOCT2-, and hOCT3-expressing cells (Fig. 4, B and D).

Transport of Platinum Agents by HEK293 Cells Transiently Expressing Basolateral Organic Cation Transporters, hOCT1, hOCT2, and hOCT3. We compared the accumulations of platinum when HEK293 cells transiently expressing hOCT1, hOCT2, and hOCT3 were treated with the medium containing platinum agents for 1 h. The accumulation of platinum after the treatment with cisplatin was increased in a dose-dependent manner and was much higher in hOCT2-expressing cells and weakly higher in hOCT1-expressing cells (Fig. 5A). When cells were treated with oxaliplatin, it was much higher in hOCT2-expressing cells and slightly in hOCT3-expressing cells (Fig. 5C). The accumulation of carboplatin and nedaplatin was not increased by the expression of hOCT1–3 (Fig. 5, B and D).

Inhibitory Effects of Cisplatin and Oxaliplatin on the [^{14}C]TEA Uptake by HEK293 Cells Expressing Basolateral Organic Cation Transporters, hOCT1, hOCT2, and hOCT3. We compared the affinities of cisplatin and oxaliplatin with hOCT1, hOCT2, and hOCT3 at a concentration range between 50 and 5000 μM because of their solubility. The uptake of [^{14}C]TEA by hOCT1- and hOCT2-expressing cells was partially inhibited by cisplatin but not oxaliplatin at a concentration range between 50 and 5000 μM (Fig. 6, A and B). The inhibitory effect of cisplatin on the uptake of [^{14}C]TEA by hOCT3-expressing cells was stronger than that of oxaliplatin (Fig. 6C).

Effects of the Expression of Apical Organic Cation Transporters, hMATE1, hMATE2-K, hOCTN1, and hOCTN2, on the Cisplatin-Induced Cytotoxicity in HEK293 Cells. When the cells transiently expressing hMATE1, hMATE2-K, hOCTN1, and hOCTN2 were treated with 50 to 1000 μM cisplatin for 2 h, the expression of hMATE1, hMATE2-K, hOCTN1, or hOCTN2 did not affect the cisplatin-induced cytotoxicity (Fig. 7, A and B). In addition, these transporter activities were confirmed by the uptake of [^{14}C]TEA as shown in Fig. 7, C and D.

Transport of Platinum Agents by HEK293 Cells Transiently Expressing Apical Organic Cation Transporters, hMATE1, hMATE2-K, hOCTN1, and hOCTN2. The cellular accumulation of platinum was determined in HEK293 cells expressing hMATE or hOCTN when the cells were treated with 500 μM platinum agents for 1 h. The accumulation of cisplatin was enhanced by hMATE1 more than hMATE2-K. On the other hand, the accumulation of oxaliplatin was enhanced by hMATE2-K more than

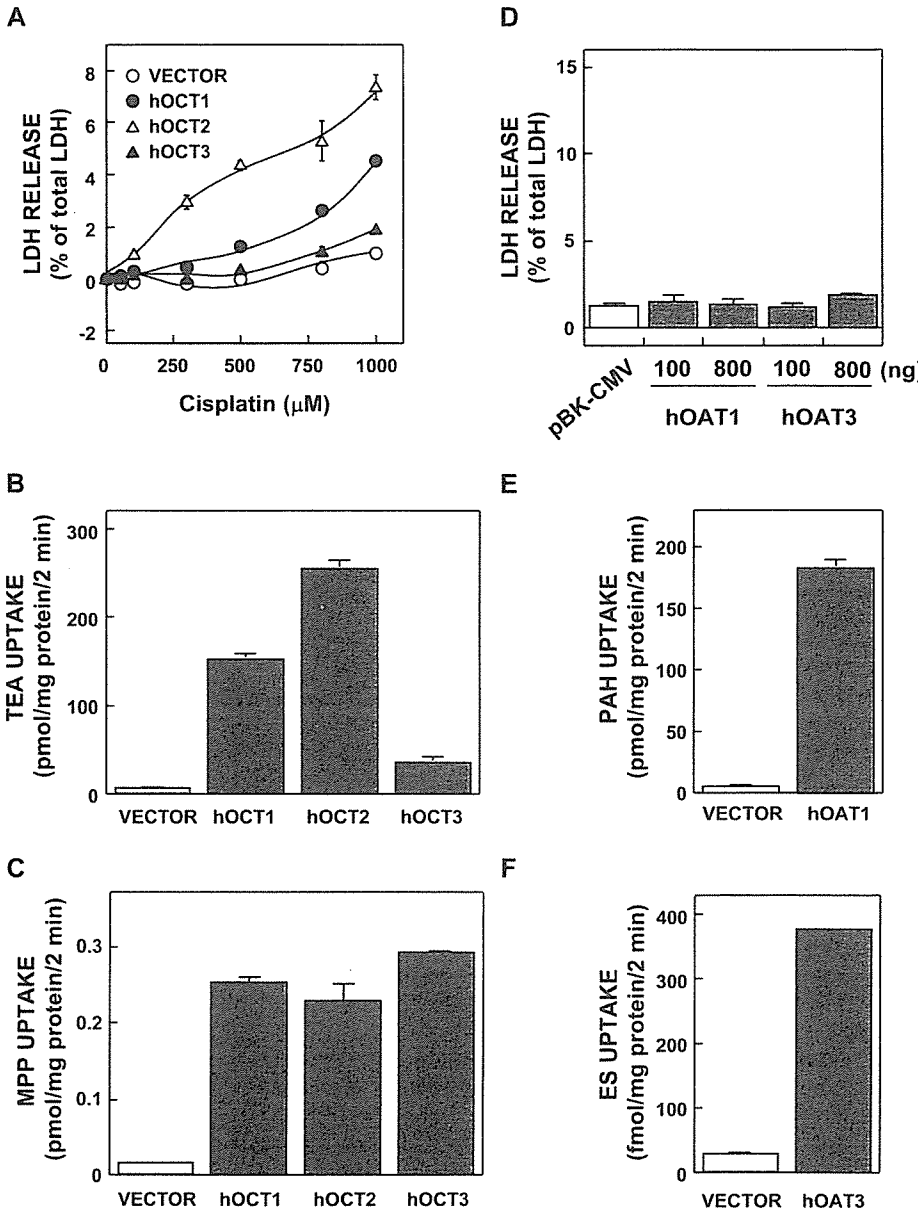

Fig. 3. Effect of basolateral organic ion transporters expression on the cisplatin-induced cytotoxicity. A, HEK293 cells were transfected with empty vector (open circle), hOCT1 (closed circle), hOCT2 (open triangle), or hOCT3 (closed triangle). The cells were treated with the medium containing 50 to 1000 μM cisplatin for 2 h and then incubated in normal medium for 24 h. D, HEK293 cells were transfected with empty vector, hOAT1, and hOAT3 (100 or 800 ng/well). The cells were treated with the medium containing 500 μM cisplatin for 2 h and then incubated in normal medium for 24 h. LDH released into the medium was measured. Uptake of typical substrates, TEA, MPP, PAH, and ES, by HEK293 cells expressing hOCT1, hOCT2, hOCT3, hOAT1, and hOAT3 is shown. HEK293 cells were transfected with empty vector, hOCT1–3 (B and C), hOAT1 (E), or hOAT3 (F). The cells were incubated with the incubation buffer containing 50 μM [^{14}C]TEA (B), 13.7 nM [^{3}H]MPP (C), 5 μM [^{14}C]PAH (E), or 20 nM [^{3}H]ES (F) at pH 7.4 for various periods at 37°C. The amount of substrates in the cells was determined by measuring the radioactivity of solubilized cells. Each point and each bar represent the mean ± S.E.M. of three wells.

hMATE1. These transporters could transport cisplatin and oxaliplatin in culture medium without an artificial pH gradient (Fig. 8A). hOCTN1 or hOCTN2 did not stimulate the transport of platinum agents (Fig. 8B).

Inhibitory Effects of Cisplatin and Oxaliplatin on [^{14}C]TEA Uptake by HEK293 Cells Expressing hMATE1 and hMATE2-K. To examine the uptake of [^{14}C]TEA by hMATE1- and hMATE2-K-expressing cells, the cells were preincubated in the buffer containing ammonium chloride (30 mM) to make an artificial pH-gradient and then were incubated in the buffer, pH 7.4, containing 50 μM [^{14}C]TEA with or without cisplatin or oxaliplatin for 2 min. Cisplatin and oxaliplatin similarly inhibited the uptake of [^{14}C]TEA by hMATE1-expressing cells (Fig. 9A). On the other hand, cisplatin did not inhibit the uptake of [^{14}C]TEA by HEK293 cells expressing hMATE2-K, although oxaliplatin did (Fig. 9B).

Discussion

We previously reported that rOCT2 was responsible for the cisplatin-induced renal tubular toxicity (Yonezawa et al., 2005). In that report, the expression of rOCT2 enhanced the cisplatin-induced cytotoxicity in HEK293 cells, and rOCT2 played a role in the tissue distribution of cisplatin and the cisplatin-induced renal tubular toxicity in the rat. In the present study, we progressively indicated that hOCT2 also abundantly transported oxaliplatin as well as cisplatin, but not carboplatin and nedaplatin (Figs. 4 and 5). In addition, cisplatin and oxaliplatin were transported by hOCT1 and hOCT3, respectively. These results suggested that the basolateral hOCTs are the determinants for the tissue distribution of platinum agents from the circulation. Moreover, apical transporter hMATE1 and hMATE2-K also mediated the transport of cisplatin and oxaliplatin (Fig. 8A). This is the

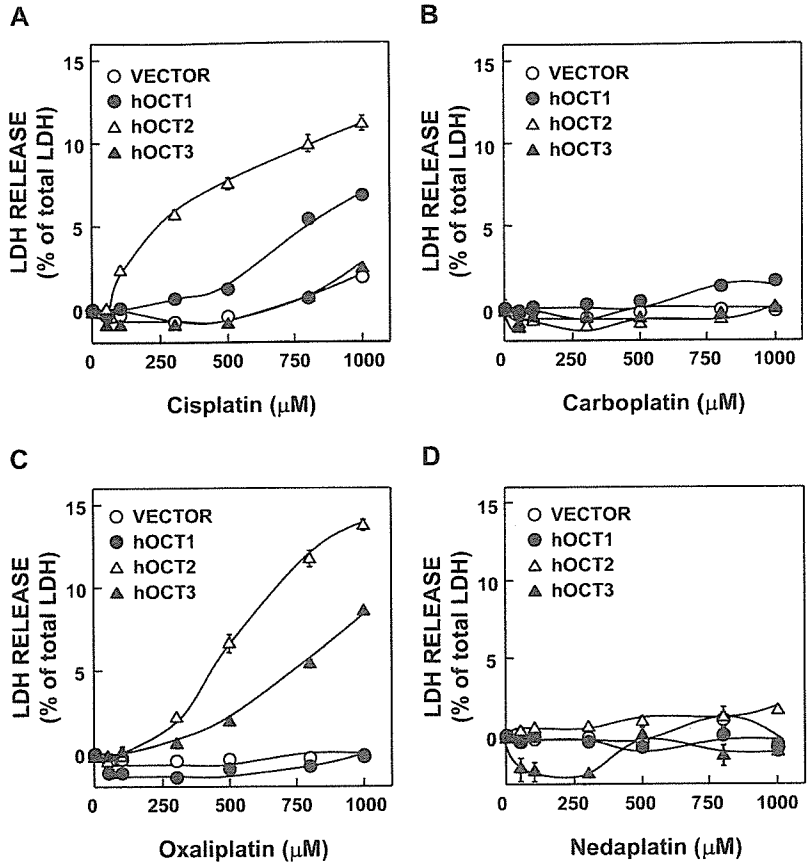

Fig. 4. Effect of hOCT1–3 expression on the platinum agent-induced cytotoxicity. HEK293 cells were transfected with empty vector (open circle), hOCT1 (closed circle), hOCT2 (open triangle), or hOCT3 (closed triangle). The cells were treated with the medium containing 50 to 1000 μM cisplatin (A), carboplatin (B), oxaliplatin (C), and nedaplatin (D) for 2 h and then incubated in normal medium for 24 h. LDH released into the medium was measured. Each point represents the mean ± S.E.M. of three wells.

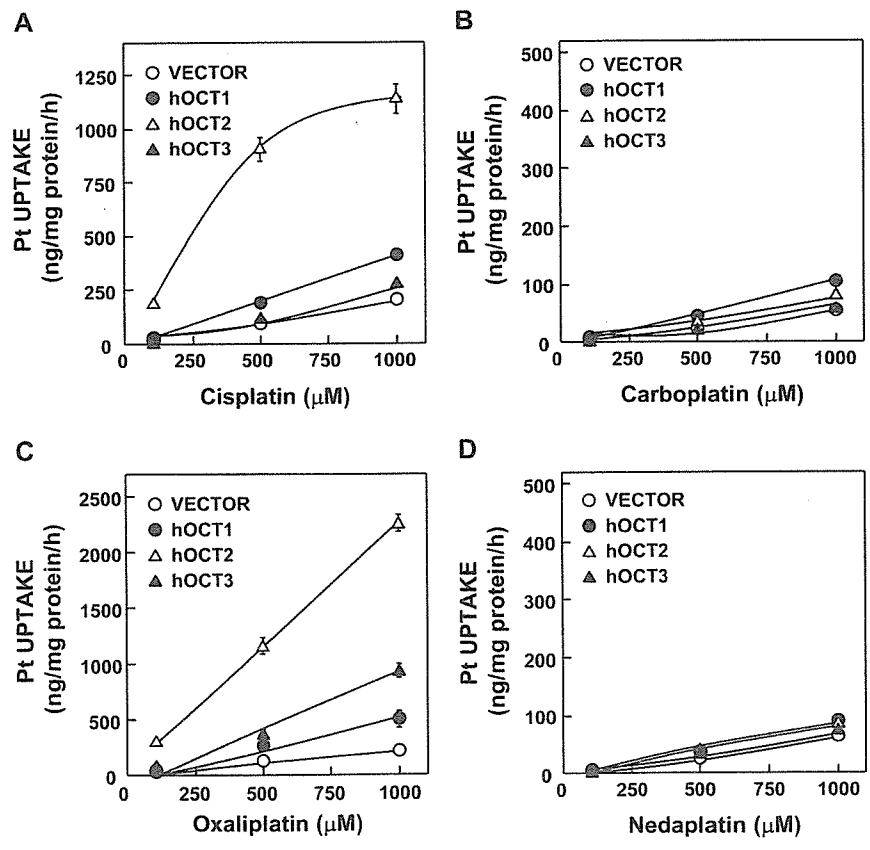

Fig. 5. Uptake of platinum agents by HEK293 cells expressing hOCT1, hOCT2, and hOCT3. HEK293 cells were transfected with empty vector (open circle), hOCT1 (closed circle), hOCT2 (open triangle), or hOCT3 (closed triangle). The cells were treated with the medium containing 100, 500, and 1000 μM cisplatin (A), carboplatin (B), oxaliplatin (C), and nedaplatin (D) for 1 h. After washing, the cells were solubilized in 0.5 N NaOH, and the amount of platinum was determined by ICP-MS. Each point represents the mean ± S.E.M. of four wells.

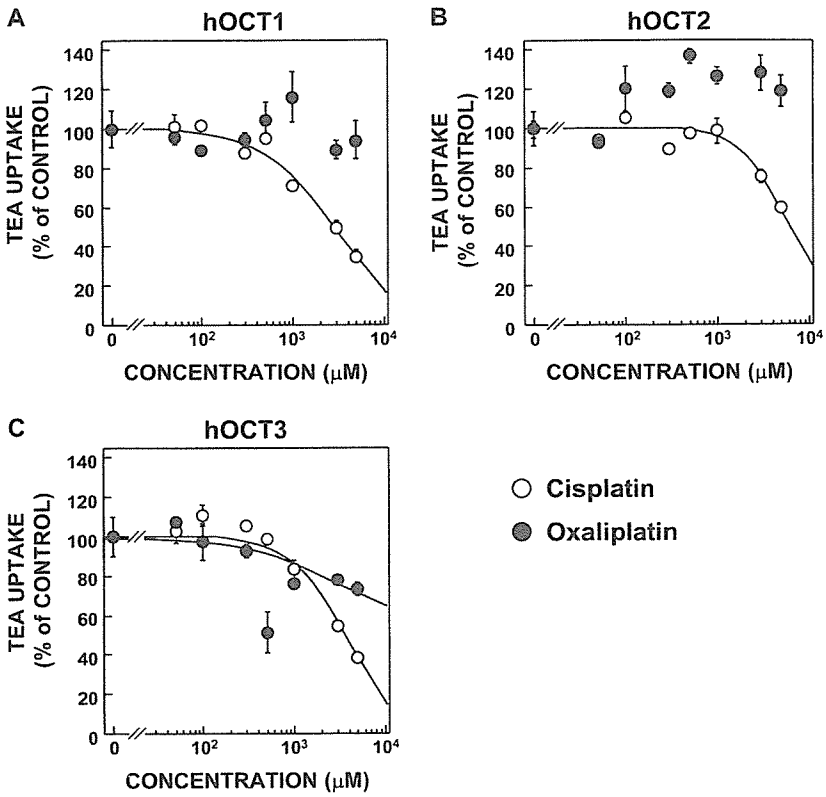

Fig. 6. Inhibitory effects of cisplatin and oxaliplatin on the uptake of [^{14}C]TEA by HEK293 cells expressing hOCT1 (A), hOCT2 (B), and hOCT3 (C). The cells were preincubated in the incubation buffer for 10 min and then were incubated in the buffer containing 50 μM [^{14}C]TEA for 2 min with or without cisplatin and oxaliplatin at various concentrations. The amount of [^{14}C]TEA in the cells was determined by measuring the radioactivity of solubilized cells. Each point represents the mean ± S.E.M. of three wells.

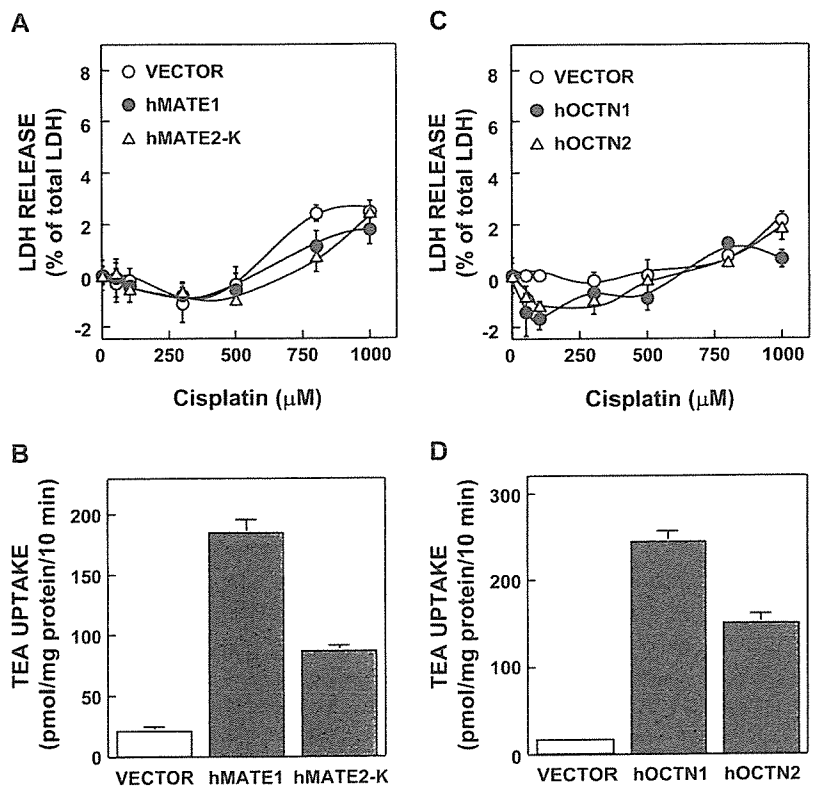

Fig. 7. Effect of apical organic cation transporters expression on cisplatin-induced cytotoxicity. A, HEK293 cells were transfected with empty vector (open circle), hMATE1 (closed circle), or hMATE2-K (open triangle). B, HEK293 cells were transfected with empty vector (open circle), hOCTN1 (closed circle), and hOCTN2 (open triangle). The cells were treated with the medium containing 50 to 1000 μM cisplatin for 2 h and then incubated in normal medium for 24 h. LDH released into the medium was measured. The uptake of typical substrates by HEK293 cells expressing hMATE1, hMATE2-K, hOCTN1, and hOCTN2. HEK293 cells were transfected with empty vector, hMATE1, or hMATE2-K (C) and hOCTN1 or hOCTN2 (D). The cells were incubated with the incubation buffer at pH 8.4 (C) or 7.4 (D) containing 50 μM [^{14}C]TEA for 10 min at 37°C. The amount of substrates in the cells was determined by measuring the radioactivity of solubilized cells. Each point and each bar represent the mean ± S.E.M. of three wells.

first report indicating that cisplatin was transported by hOCT1, hMATE1 and hMATE2-K, as well as hOCT2, and oxaliplatin was transported by hOCT2, hOCT3, hMATE1, and hMATE2-K.

Cisplatin is commonly used against hepatocellular carcinoma, administered from a catheter in the hepatic artery. The systemic blood concentration of cisplatin after its administration from the hepatic artery was not different from that

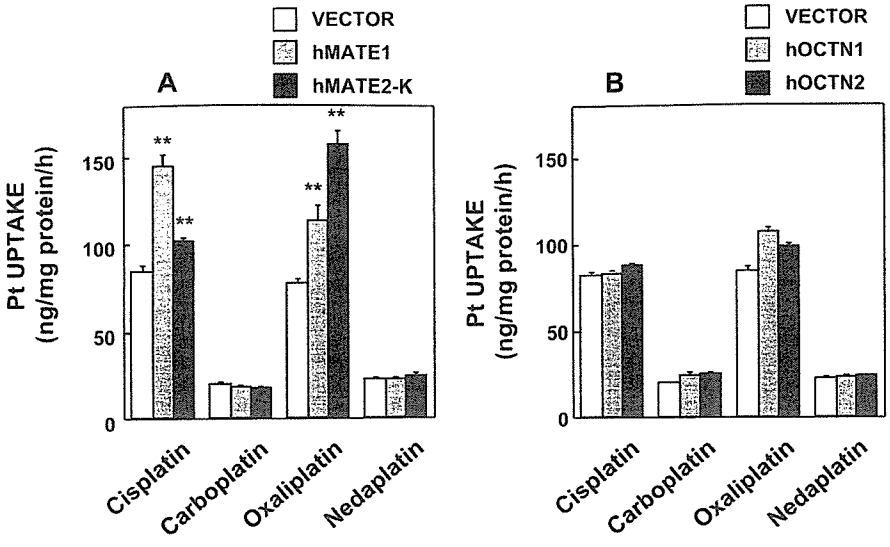

Fig. 8. Uptake of platinum agents by HEK293 cells expressing hMATE1, hMATE2-K, hOCTN1, and hOCTN2. A, HEK293 cells were transfected with empty vector (open bar), hMATE1 (gray bar), and hMATE2-K (black bar). B, HEK293 cells were transfected with empty vector (open bar), hOCTN1 (gray bar), and hOCTN2 (black bar). The cells were treated with the medium containing 500 μM platinum agents for 1 h. After washing, the cells were solubilized in 0.5 N NaOH, and the amount of platinum was determined by ICP-MS. Each bar represents the mean ± S.E.M. of four wells. **, $P < 0.01$, significantly different from vector-transfected cells.

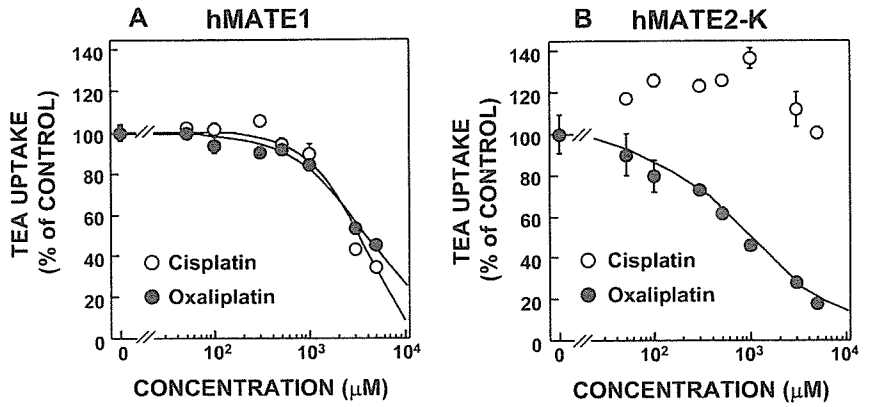

Fig. 9. Inhibitory effects of cisplatin and oxaliplatin on the uptake of [^{14}C]TEA by HEK293 cells expressing hMATE1 (A) and hMATE2-K (B). The cells were preincubated in the incubation buffer containing 30 mM ammonium chloride for 20 min and then were incubated in the buffer containing 50 μM [^{14}C]TEA for 2 min with or without cisplatin and oxaliplatin at various concentrations. The amount of [^{14}C]TEA in the cells was determined by measuring the radioactivity of solubilized cells. Each point represents the mean ± S.E.M. of three wells.

after i.v. administration. Thus, the probability of the adverse effect was not different despite the advantages regarding antitumor efficacy (Lo et al., 2002). It was reported that hepatic OCT1 was expressed in rat diethylnitrosamine-induced hepatocarcinoma cells and differentiated Fao cells, a hepatoma cell line (Lecureur et al., 1998). Interestingly, the cytotoxicity of cisplatin was also enhanced by hOCT1 at a relatively higher concentration (Figs. 3A and 4A). Therefore, hOCT1 was suggested to play an important role in the hepatic uptake of cisplatin.

Oxaliplatin has a different spectrum of activity and the different mechanisms of action and resistance from those of cisplatin and carboplatin (Raymond et al., 2002; Fuertes et al., 2003; Wang and Lippard, 2005). Carboplatin, oxaliplatin, and nedaplatin are low nephrotoxic agents despite their good efficacy. Therefore, it had been assumed that they would not be transported by hOCT2. Expectedly, carboplatin and nedaplatin were not transported by hOCT2 (Figs. 4, B and D, and 5, B and D). Surprisingly, oxaliplatin was transported by hOCT2 (Figs. 4C and 5C), although oxaliplatin did not induce nephrotoxicity (Raymond et al., 2002). In addition, oxaliplatin was transported by hMATE1 and hMATE2-K (Fig. 8A). The nephrotoxic pattern of oxaliplatin observed in the renal slice with collapsed and nonperfused lumens resembled to that of cisplatin (Kanou et al., 2004). It is likely that oxaliplatin would be accumulated in the renal slice without efflux systems, thereby exacerbating its toxicity. Based on these findings and the present results, it is presumed that the basolateral hOCT2 is an influx transporter as the inducer of oxaliplatin-induced toxicity, and the apical hMATE1 and hMATE2-K are efflux transporters as a means to protect cells. Therefore, transcellular transport and cellular toxicity of oxaliplatin should be further examined using polarized double transfectants with both basolateral OCT and apical MATE transporters.

hOCT3 is the organic cation transporter predominantly expressed in the placenta and widely expressed in tissues, including the kidney and intestine (Kekuda et al., 1998). The substrate specificity and pharmacokinetic role of hOCT3 are not fully understood. We identified oxaliplatin as a hOCT3 substrate, but not cisplatin (Fig. 5, A and C). In addition, there is a previous report that oxaliplatin had a more potent cytotoxicity than cisplatin against colon cancer cells, although the molecular mechanism was not clear (Ducreux et al., 1998). Because hOCT3 was reported to be expressed in the intestine (Kekuda et al., 1998), it was supposed that hOCT3 expression would contribute at least in part to the oxaliplatin sensitivity in colon cancer cells.

hMATE1, hMATE2-K, hOCTN1, and hOCTN2 are expressed in the apical membrane of renal proximal tubules. In this study, cisplatin and oxaliplatin were weakly but significantly transported by hMATE1 and hMATE2-K, but not

carboplatin and nedaplatin (Fig. 8A). In contrast, hOCTN1 or hOCTN2 did not transport four platinum agents (Fig. 8B). Because the H^+-gradient stimulated the hMATE1- (Otsuka et al., 2005), hMATE2-K- (Masuda et al., 2006), and rMATE1- (Terada et al., 2006) mediated transport of cationic compounds, the artificial H^+-gradient is necessary as the driving force for the substrate transport. Intracellular acidification by ammonium chloride markedly stimulated the hMATE1-, hMATE2-K-, and rMATE1-mediated uptake of [^{14}C]TEA. As shown in Fig. 8A, the cells were incubated in the culture medium containing platinum agents without an artificial H^+-gradient because of the stability of platinum agents, as described under *Materials and Methods*. It is possible that the transport activities of hMATE1 and hMATE2-K for cisplatin and oxaliplatin are much higher at the renal apical membrane of proximal tubules with an oppositely directed H^+-gradient in the physiological condition.

The clearance of cisplatin was reported to exceed the glomerular filtration rate, suggesting that cisplatin was secreted across the renal tubular cells (Jacobs et al., 1980). In addition, it was reported that cisplatin competitively inhibited the TEA uptake in the basolateral and brush border membrane vesicles (Williams and Hottendorf, 1985), implying that cisplatin was also transported by the organic cation transport system in the basolateral and brush border membrane. In the present study, cisplatin was transported by hOCT2, hMATE1, and hMATE2-K (Figs. 5A and 8A). In addition, the IC_{50} of cisplatin against [^{14}C]TEA uptake by hMATE1-expressing cells was lower than by hMATE2-K-expressing cells (Fig. 9, A and B). It was indicated that cisplatin was a good substrate for hOCT2 and hMATE1 but a low-affinity substrate for hMATE2-K. Therefore, it was suggested that the tubular secretion of cisplatin was explained as follows. Basolateral uptake of cisplatin was mediated by hOCT2 and apical secretion was mediated mainly by hMATE1 and slightly by hMATE2-K. Moreover, carboplatin was reported to not be excreted by the tubular secretion (Sorensen et al., 1992). This report corresponded to our results that carboplatin was not transported by organic cation transporters (Figs. 5B and 8, A and B). Oxaliplatin was transported by hOCT2, hMATE1, and hMATE2-K, but not nedaplatin (Figs. 5C and 8A). The inhibitory effects of oxaliplatin on the hMATE1- and hMATE2-K-mediated transport of [^{14}C]TEA were similar (Fig. 9, A and B). These results suggested that oxaliplatin was secreted across the renal epithelial cells via hOCT2, hMATE1, and hMATE2-K, although nedaplatin was not.

In conclusion, we clearly indicated that cisplatin was transported greatly by hOCT2 and weakly by hOCT1 and that oxaliplatin was transported potently by hOCT2 and weakly by hOCT3. Moreover, cisplatin and oxaliplatin were revealed to be preferentially transported by hMATE1 and hMATE2-K, respectively. These basolateral and apical transporters could play a predominant role in the tissue distribution and tubular secretion of platinum agents, being responsible for anticancer effects and/or adverse effects. Therefore, these transporters may be the molecular targets for the prevention from the resistant and adverse effects in platinum-based chemotherapy.

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Gene expression and regulation of drug transporters in the intestine and kidney☆

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ABSTRACT

Intestinal absorption and renal secretion of ionic drugs are controlled by a number of drug transporters expressed at the brush-border and basolateral membranes of epithelial cells. Over the last several years, considerable progress has been made regarding the molecular identification and functional characterization of drug transporters. Under some physiological and pathophysiological conditions, the expression and transport activity of drug transporters are changed, affecting the pharmacokinetics of substrate drugs. The regulation of transport activity in response to endogenous and exogenous signals can occur at various levels such as transcription, mRNA stability, translation, and posttranslational modification. Transcriptional regulation is of particular interest, because changes in transport activity are dynamically regulated by increases or decreases in levels of mRNA expression. The tissue-specific expression of drug transporters is also under transcriptional control, and recent studies using clinical samples from human tissues have revealed the expression profiles of drug transporters in the human body. The purpose of this research updates is to review the recent progress in the study of the gene expression and regulation of intestinal and renal drug transporters.

1. Introduction

Mucosal surfaces of tissues such as the intestine and kidney are lined by a single layer of epithelial cells. Epithelial cells function as a barrier to select essential (such as nutrients) and waste (such as toxic xenobiotics) compounds, being equipped with uptake and efflux transport systems. During the last decade, many kinds of nutrient and drug transporters in the intestine and kidney have been identified as uptake and efflux transport systems. Currently, various transporters have been classified as ATP-binding cassette (ABC) transporters and solute carriers (SLCs) based on sequence similarity by the Human Gene Nomenclature Committee.

In general, nutrient transporters in the intestine are tightly regulated by nutrient load [1]. Observed patterns of response for essential nutrients and/or nutrients that are toxic in excess, such as zinc and iron, are generally consistent with the maintenance of the body's nutrient status under conditions of

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Abbreviations: ABC, ATP-binding cassette; ATF, activating transcription factor; BCRP, breast cancer resistance protein; Cdx2, caudal-related homeobox protein; CRE, cAMP responsive element; CREB, CRE binding protein; EMSA, electrophoretic mobility shift assay; HNF-4α, hepatocyte nuclear factor-4α; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; OAT, organic anion transporter; OCT, organic cation transporter; OCTN, novel organic cation transporter; PEPT, peptide transporter; Pgp, P-glycoprotein; PKA, protein kinase A; PPARα, peroxisome proliferator-activated receptor α; SLC, solute carrier

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variable intake. For example, expression of the divalent metal ion transporter (DMT1/SLC11A2), involved in iron absorption, is increased in the intestine by an iron-deficient diet [2]. Drug transporters are also regulated by many biochemical signalling pathways, and such regulation may influence the pharmacokinetics of substrate drugs. The regulation of transport activity in response to endogenous and exogenous signals may occur at various levels such as transcription, mRNA stability, translation, and posttranslational modification (Fig. 1). This diversity of regulatory mechanisms may be advantageous to correspond to various biological signals. In general, transcriptional regulation and posttranslational modification are believed to be responsible for long-term and short-term regulation, respectively. We are interested in the transcriptional regulation of drug transporters, because changes in transport activity are dynamically regulated by increases or decreases in levels of mRNA expression. The tissue-specific expression of drug transporters is also under transcriptional control, although there is little information about the mechanisms behind intestinal and renal-specific expression.

This research updates will focus on our current understanding of the expression and gene regulation of drug transporters in the intestine and kidney, concentrating on the control mechanisms governing the expression of each transporter. For SLC drug transporters, H^+/peptide transporters (PEPT) and organic ion transporters (OCT/OCTN/OAT) were selected as representative of transporters expressed in the intestine and kidney. The transporters mainly referred to here are listed in Table 1. On the other hand, only the expression profiles of ABC transporters are covered in this article. We do not refer to the gene regulation of ABC transporters, because several excellent reviews about gene regulation of ABC transporters have been already published [3–6].

2. Function and regulation of drug transporters

2.1. PEPT1 (SLC15A1)

2.1.1. General function and pharmacokinetic roles

H^+/peptide cotransporter 1 (PEPT1, SLC15A) is localized at the brush-border membranes of intestinal epithelial cells and plays an important role for protein absorption to mediate the cellular uptake of di- and tripeptides digested from ingested food [7]. Because of its broad substrate specificity, PEPT1 recognizes various peptide-like drugs such as oral β-lactam antibiotics, which are structurally resemble to small peptides [8]. Intestinal PEPT1 can be utilized as a target for improving the intestinal absorption of poorly absorbed drugs such as nucleoside analogues [9,10]. Recently, mathematical models of H^+-coupled substrate transport mediated by PEPT1 were proposed to elucidate the transport characteristics of differently charged substrates [11,12].

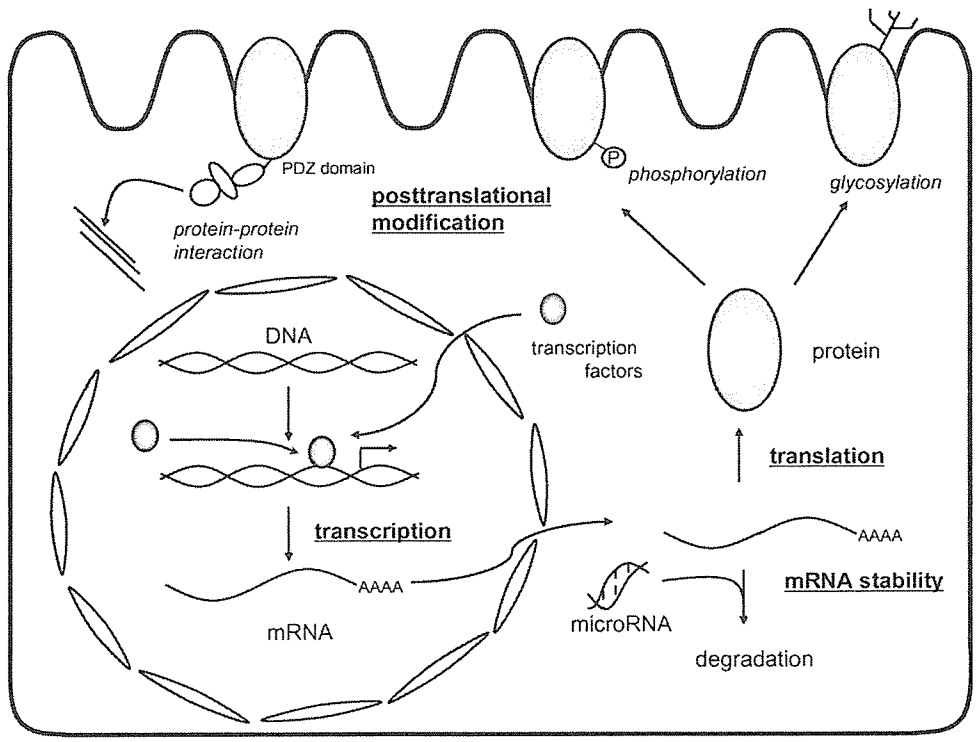

Fig. 1 – Various factors influencing the expression of drug transporters. The activity of drug transporters may be regulated at various levels including transcription, mRNA stability, translation, and posttranslational modification. Posttranslational modification may involve glycosylation, phosphorylation, and protein–protein interaction. Moreover, transcriptional regulation is of particular interest, because many extra- and intracellular signals eventually alter the activity of transcription factors. In addition to the regulation of various signals, the tissue-specific expression of drug transporters is also under transcriptional control.