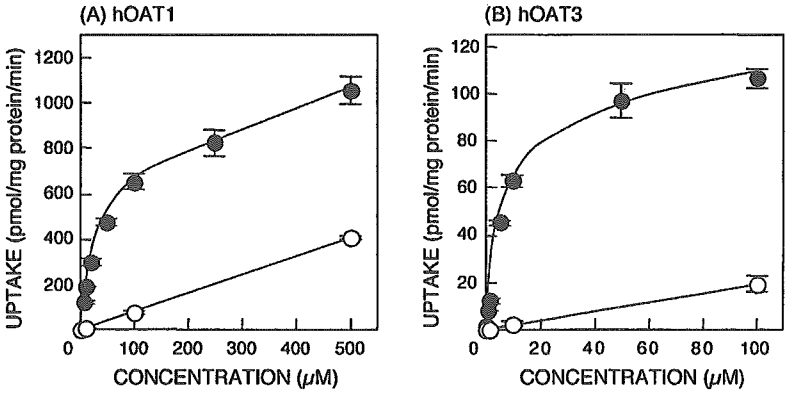

Fig. 2. Concentration dependence of *p*-[^{14}C]aminohippurate (A) and [^{3}H]estrone sulfate (B) accumulation in HEK-hOAT1 and HEK-hOAT3, respectively. HEK-hOAT1 and HEK-hOAT3 were incubated with various concentrations of *p*-[^{14}C]aminohippurate or [^{3}H]estrone sulfate in the absence (●) or presence (○) of unlabeled 5 mM *p*-aminohippurate (A) or 1 mM estrone sulfate (B) for 1 min at 37 °C. After the incubation, the radioactivity of solubilized cells was measured. Each point represents the mean ± S.E. of three monolayers from a typical experiment.

28.0 ± 1.9 and 6.3 ± 2.2 μM ($n = 3$, mean ± S.E.), respectively, which were consistent with previous reports [17–19]. Maximal uptake rate (V_{max}) values for HEK-hOAT1 and HEK-hOAT3 were 553.2 ± 70.9 and 102.7 ± 19.7 pmol/mg protein/min ($n = 3$, mean ± S.E.), S.E.), respectively.

In addition, we measured the uptake of various compounds by these transfectants. Results of these uptake experiments are shown in Fig. 3. A remarkable increase in the uptake of *p*-[^{14}C]aminohippurate, [^{14}C]captopril, [^{3}H]ochratoxin A and [^{3}H]leucovorin, and slight increase (within two-fold) in the uptake of [^{3}H]estrone sulfate, [^{3}H]methotrexate and [^{3}H]cimetidine were observed in HEK-hOAT1 compared to control cells. A remarkable increase in the uptake of *p*-[^{14}C]aminohippurate, [^{3}H]estrone sulfate, [^{14}C]captopril, [^{3}H]methotrexate, [^{3}H]ochratoxin A, [^{3}H]leucovorin, [^{3}H]cimetidine and [^{3}H]dehydroepiandrosterone sulfate was observed in HEK-hOAT3 compared to control cells.

3.2. Inhibitory effects of cephalosporin antibiotics on hOAT1 and hOAT3

To determine the affinity of cephalosporins for hOAT1 and hOAT3, we examined the inhibitory effects of these drugs on the uptake of *p*-[^{14}C]aminohippurate and [^{3}H]estrone sulfate by HEK-hOAT1 and HEK-hOAT3, respectively. As shown in Fig. 4, cephalosporin anitibiotics inhibited the uptake of organic anions by HEK-hOAT1 and HEK-hOAT3 in a dose-dependent manner. The IC_{50} values were estimated by nonlinear regression analysis of the competition curves with a one-compartment model with the following equation: $V = 100 \times IC_{50}/(IC_{50} + [I]) + A$, where V the uptake amount (% of control), [I] the concentration of cephalosporin antibiotic and A is the non-specific organic anion uptake (% of control). The findings are summarized in Table 1.

3.3. Characterization of the uptake of cephalosporin antibiotics by hOAT1 and hOAT3

To investigate whether hOAT1 and hOAT3 transport cephalosporin antibiotics, we measured the accumulations of these drugs in HEK-hOAT1 and HEK-hOAT3. As shown in Fig. 5, the uptake of cephaloridine, cefdinir and cefotiam by HEK-hOAT3 was 35–50-fold higher than that by control cells. Moreover, the accumulation of ceftibuten, cefaclor, ceftizoxime, cefoselis and cefazolin was significantly greater in HEK-hOAT3 than in control cells. Those cephalosporin antibiotics whose accumulation was significantly greater in HEK-hOAT1 than control cells were cephaloridine, cefdinir, ceftibuten and ceftizoxime. The uptake of these antibiotics by HEK-hOAT1 was within two-fold of that by control cells.

Fig. 6 shows the time course of the uptake of cephaloridine, cefotiam or cefazolin by HEK-hOAT1 or HEK-hOAT3. The accumulation of cephaloridine, cefotiam and cefazolin in HEK-hOAT3 increased markedly in a time-dependent manner. The accumulation of these antibiotics in HEK-hOAT1 was comparable to that in control cells.

Table 1
The IC_{50} values of various cephalosporin antibiotics for the uptake of *p*-aminohippurate and estrone sulfate by hOAT1 and hOAT3, respectively

Cephalosporin	IC_{50} (μM)	
	hOAT1	hOAT3
Cephaloridine	2470.0 ± 339.3	626.4 ± 66.7
Cefdinir	691.8 ± 222.9	271.5 ± 46.5
Cefotiam	639.7 ± 63.0	212.6 ± 26.9
Ceftibuten	563.1 ± 50.1	247.3 ± 74.0
Cefaclor	1095.6 ± 95.9	120.2 ± 7.2
Ceftizoxime	3598.6 ± 368.6	956.7 ± 29.7
Cefoselis	2600.5 ± 439.3	2925.1 ± 27.1
Cefazolin[a]	100.6 ± 25.3	116.6 ± 13.0

The values represent the means ± S.E. of three separate experiments.
[a] Values are from Ref. [14].

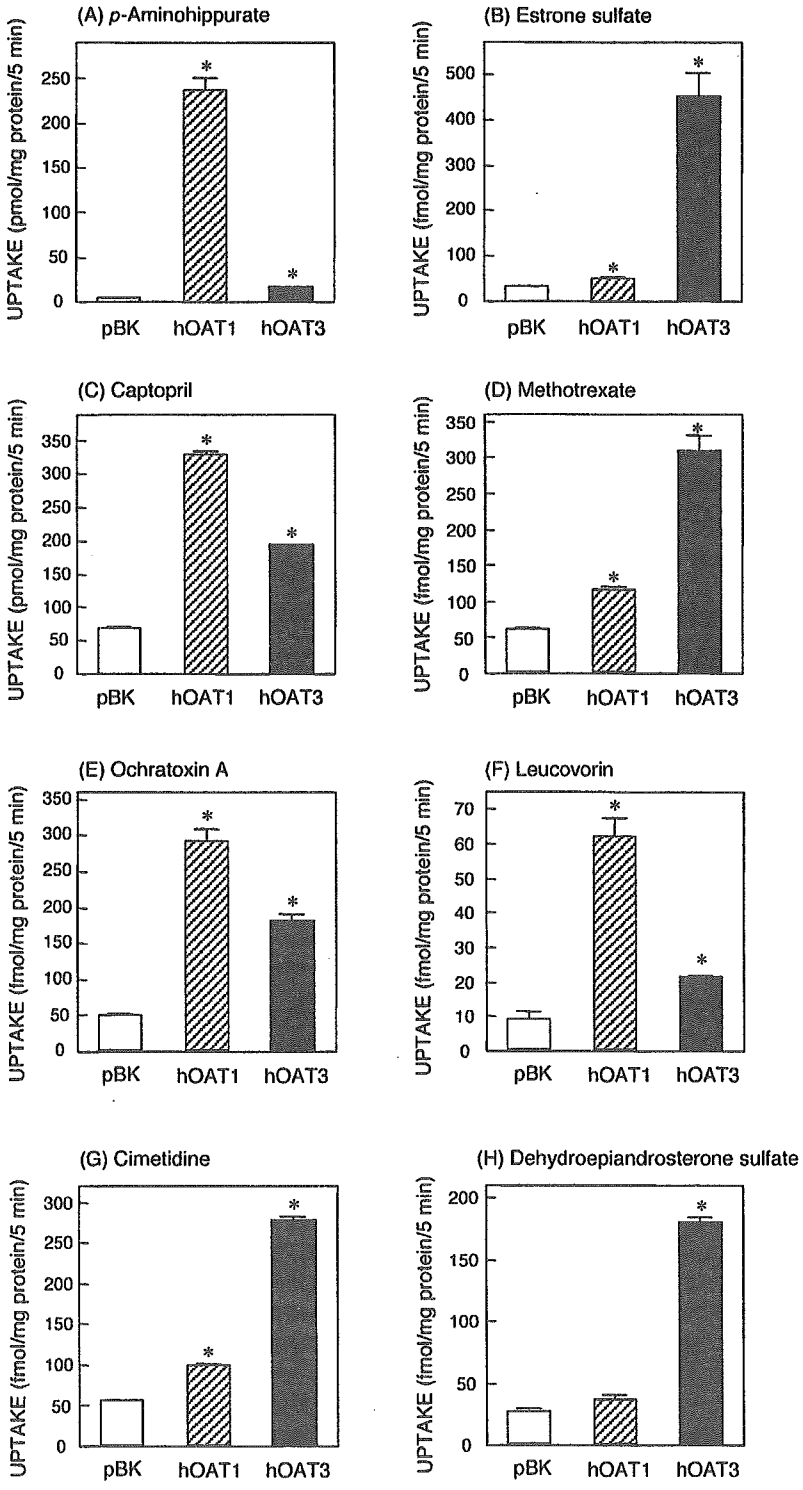

Fig. 3. Uptake of various anionic compounds by HEK-hOAT1 or HEK-hOAT3. HEK-pBK, HEK-hOAT1 and HEK-hOAT3 were incubated with 5 μM *p*-[^{14}C]aminohippurate (A), 20 nM [^{3}H]estrone sulfate (B), 160 μM [^{14}C]captopril (C), 40 nM [^{3}H]methotrexate (D), 20 nM [^{3}H]ochratoxin A (E), 40 nM [^{3}H]leucovorin (F), 80 nM [^{3}H]cimetidine (G) or 20 nM [^{3}H]dehydroepiandrosterone sulfate (H) for 5 min at 37 °C. After the incubation, the radioactivity of solubilized cells was measured. Each column represents the mean ± S.E. of three monolayers from a typical experiment. $^{*}p < 0.05$, significant differences from control.

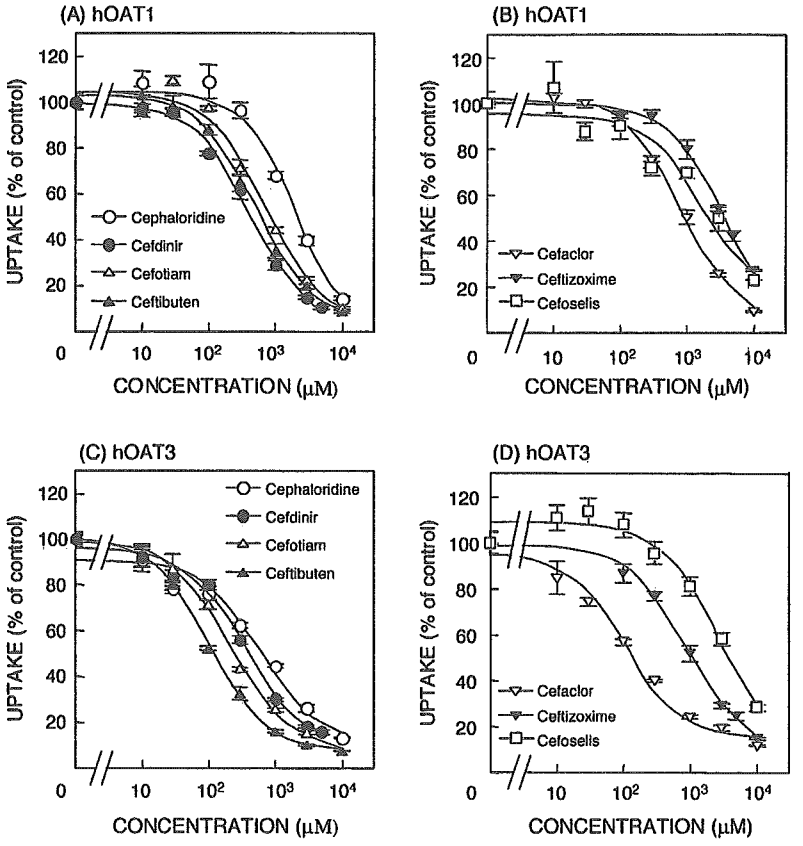

Fig. 4. Effects of cephalosporin antibiotics on the uptake of *p*-[^{14}C]aminohippurate (A and B) and [^{3}H]estrone sulfate (C and D) by HEK-hOAT1 and HEK-hOAT3, repectively. HEK-hOAT1 and HEK-hOAT3 was incubated with 5 μM *p*-[^{14}C]aminohippurate (A and B) and 20 nM [^{3}H]estrone sulfate (C and D), respectively, for 1 min at 37 °C in the presence of various concentrations of cephaloridine (○), cefdinir (●), cefotiam (△), ceftibuten (▲), cefaclor (▽), ceftizoxime (▼) or cefoselis (□). After the incubation, the radioactivity of solubilized cells was measured. Each point represents the mean ± S.E. of three monolayers from a typical experiment.

4. Discussion

Renal organic anion transporters, hOAT1 and hOAT3, mediate the basolateral uptake of various drugs in proximal tubules. In our previous studies, Uwai et al. [12] reported that rOAT1 transported cephalosporin antibiotics, cefazolin, cefotiam and cefalexin, and Sakurai et al. [14] reported that hOAT3 plays an important role in the renal secretion of cefazolin in patients with renal diseases. The present study examined the transport of various cephalosporin antibiotics via hOAT1 and hOAT3. In this study, for all cephalosporin antibiotics tested, particularly, cephaloridine, cefdinir and cefotiam, the uptake by HEK-hOAT3 was greater than that by HEK-hOAT1. The mRNA level of hOAT3 in HEK-hOAT3 was about three-fold higher than that of hOAT1 in HEK-hOAT1, and we have previously reported that the level of hOAT3 mRNA is about three-fold higher than that of hOAT1 mRNA in human kidney [7]. Therefore, we suggest that the uptake by HEK-hOAT1 and HEK-hOAT3 reflects to some extent the basolateral uptake via hOAT1 and hOAT3 in renal epithelial cells. The present results showed that hOAT3 plays a major role in the basolateral uptake of various cephalosporin antibiotics, as well as cefazolin [14], into epithelial cells from the blood.

We examined the transport of various compounds via hOAT1 and hOAT3. It has been suggested that hOAT3 can mediate the transport of organic anions with bulky side groups, compared with hOAT1 [18], and in the current study, the remarkable increase in the uptake of [^{3}H]estrone sulfate and [^{3}H]dehydroepiandrosterone sulfate was observed in HEK-hOAT3 compared to control cells, while little or no increase was observed in HEK-hOAT1. Nevertheless, the difference in substrate specificity between hOAT1 and hOAT3 remains to be properly elucidated. We previously reported that hOAT1 mRNA levels are significantly lower in the kidneys of patients with renal diseases than in the normal kidney cortex, whereas hOAT3 mRNA levels are not significantly reduced, and suggested that each transporter undergoes a different effect in the impaired kidney [14]. By combining information about the alteration in the expression level and substrate specificity of a transporter, we could at least in part contribute to establishment of the administration schedule in cases of renal disease.

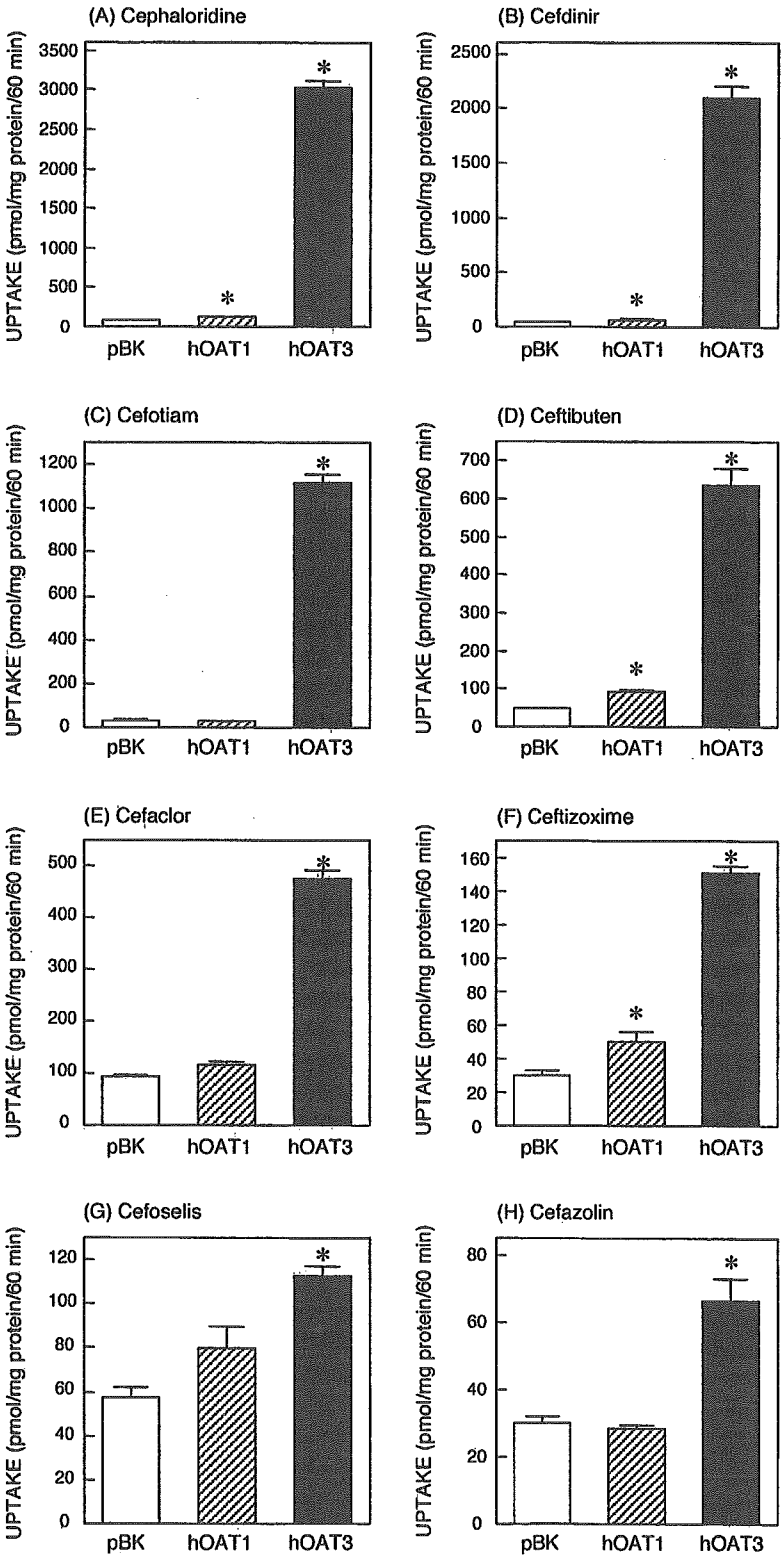

Fig. 5. Uptake of cephalosporin antibiotics by HEK-hOAT1 and HEK-hOAT3. HEK-pBK, HEK-hOAT1 and HEK-hOAT3 were incubated for 60 min at 37 °C with 500 μM cephaloridine (A), cefdinir (B), cefotiam (C), ceftibuten (D), cefaclor (E), ceftizoxime (F), cefoselis (G) or cefazolin (H). After the incubation, the accumulation of these antibiotics in the cells was measured by use of a high performance liquid chromatograph. Each column represents the mean ± S.E. of three monolayers from a typical experiment. $^{*}p < 0.05$, significant differences from control.

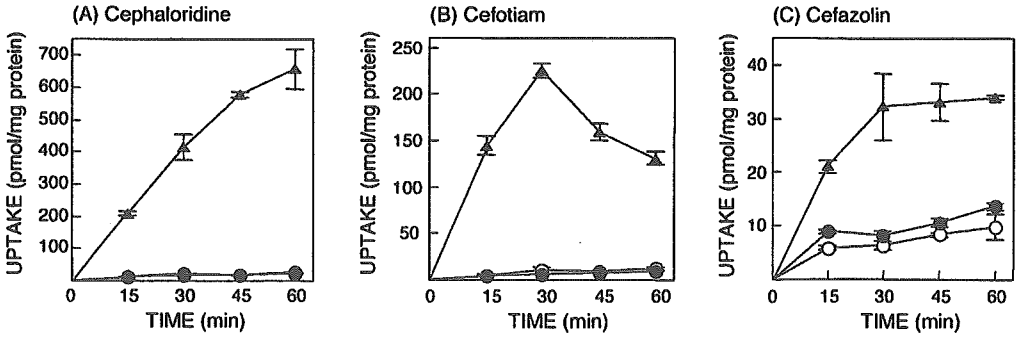

Fig. 6. Time course of cephaloridine (A), cefotiam (B) and cefazolin (C) accumulation in HEK-hOAT1 and HEK-hOAT3. HEK-pBK (○), HEK-hOAT1 (●) and HEK-hOAT3 (▲) were incubated for the indicated periods at 37 °C with 100 μM cephaloridine (A), 200 μM cefotiam (B) or 200 μM cefazolin (C). After the incubation, the accumulation of these antibiotics in the cells was measured by use of a high performance liquid chromatograph. Each point represents the mean ± S.E. of three monolayers from a typical experiment.

Uwai et al. [20] reported that probenecid markedly inhibited the transport of *p*-aminohippurate via rOAT1, although was not transported via rOAT1. Zhang et al. [21] showed that HIV protease inhibitors including indinavir, nelfinavir, ritonavir and saquinavir are potent inhibitors of hOCT1; however, they are poor substrates for hOCT1-mediated transport. In the current study, the IC_{50} values of cephaloridine, cefdinir and cefotiam for the hOAT1-mediated uptake of *p*-[^{14}C]aminohippurate were within four-fold of those for the hOAT3-mediated uptake of [^{3}H]estrone sulfate. On the other hand, the uptake of these antibiotics by HEK-hOAT3 (35–50-fold higher than that by control cells) was remarkably greater than that by HEK-hOAT1 (within 1.6-fold of that by control cells). In addition, although the IC_{50} value of cephaloridine was six-fold higher than that of cefazolin for hOAT3-mediated organic anion uptake, cephaloridine uptake was 46-fold higher than cefazolin uptake in HEK-hOAT3. The results suggested that these marked differences in transport activity are due to the efficacy of the translocation process. These previous findings and the current study have indicated that it is necessary to investigate not only substrate affinity but also substrate transport when characterizing the substrate specificity of a transporter.

Cephaloridine produces acute renal failure in humans and animals [22]. In the kidney, cephaloridine is rapidly transported into the epithelial cells, but undergoes minimal subsequent movement into the lumen [23], and the accumulation of drug in the tubular cells attributable to this process plays a role in the mechanism of renal failure [24]. It has been reported that rOAT1 and rOAT3 are, at least in part, responsible for the basolateral uptake of cephaloridine and therefore cephaloridine-induced renal failure [25,26]. In a previous study, the K_i values of cephaloridine for the rOAT1 and rOAT3-mediated uptake of organic anions were similar (1.32 mM for rOAT1 and 1.14 mM for rOAT3) and it was suggested that both rOAT1 and rOAT3 participate in the transport of cephaloridine and in cephaloridine-induced renal failure, although the uptake of cephaloridine by these transporters was not compared [26]. In the current study, the uptake of cephaloridine by HEK-hOAT3 was remarkably higher than that by HEK-hOAT1, demonstrating that hOAT3 plays a more important role in cephaloridine-induced renal failure than hOAT1.

Lu et al. [27] reported that the *p*-aminohippurate transporter PAHT (synonym: hOAT1) [28], displayed an overshoot characterized by a time-dependent, saturable accumulation of substrate, followed by a gradual return to the baseline. In the current study, the uptake of *p*-[^{14}C]aminohippurate by HEK-hOAT1 also displayed this phenomenon (Fig. 1). OAT1 has been known as an organic anion/dicarboxylate exchanger [29], and it has been suggested that the overshoot is consistent with exchange-mediated secondary active transport in which an outwardly directed gradient for a cytosolic exchange partner (likely α-ketoglutarate) is depleted during the uptake experiment because of an abundance of the external exchange partner [27]. Recently, it was revealed that OAT3 is also an anion/dicarboxylate exchanger [30], and in the current study, the uptake of cefotiam by HEK-hOAT3 also displayed an overshoot. This is the first example of this phenomenon in hOAT3-mediated uptake after hOAT3 was recognized as an exchanger. In the uptake of cephaloridine by HEK-hOAT3, no overshoot was observed, although the uptake rate of cephaloridine was higher than that of cefotiam (2.08 μl/mg protein/15 min versus 0.72 μl/mg protein/15 min). Tune and Hsu [31] reported that cephaloridine reduces mitochondrial carnitine transport through competitive inhibition, and the zwitterionic region of this drug, like carnitine, is responsible for this ability. Given their study, it is suggested that cephaloridine was accumulated in mitochondria following its transport into the cytoplasm. Cytoplasmic concentration of cephaloridine may be low because intracellular cephaloridine was mainly in mitochondria. Therefore, the efflux of the antibiotic via hOAT3 was not occurred. We assume that this phenomenon also occurs in the proximal tubular cells, and participates in cephaloridine-induced renal failure.

We previously speculated that hOAT3 should play an important role in the secretion of cefazolin in patients with

renal diseases [14]. The current study demonstrated that other cephalosporin antibiotics are also secreted mainly via hOAT3. The uptake of cefoselis by HEK-hOAT3 was lower than the uptake of the other antibiotics, except for cefazolin, by HEK-hOAT3, and Sakamoto et al. [32] showed that cefoselis is mainly excreted by glomerular filtration. Accordingly, it should be that transport via hOAT3 is important for secretion in renal epithelial cells. It was reported that tubular secretion accounted for 50–80% of all the cefazolin excreted in patients with a glomerular filtration rate above 25 ml/min [33]. However, in the current study, the uptake of cefazolin by HEK-hOAT3 was lower than the uptake of the other antibiotics by HEK-hOAT3. The IC_{50} value of cefazolin for hOAT3 is remarkably lower than that of cefoselis for hOAT3 (116.6 μM versus 2925.1 μM). The protein-binding ratio of cefazolin (87%) is remarkably higher than that of cefoselis (8.8%) [32,33]. To elucidate the renal handling of drugs, it may be necessary to take such factors into consideration. We propose that information on transport via hOAT3 is, at least in part, useful for determining whether drugs can be secreted or not in renal epithelial cells.

In conclusion, hOAT3 plays a more important role than hOAT1 in the renal secretion of cephalosporin antibiotics. Furthermore, our findings provide useful information about the difference in substrate specificity between hOAT1 and hOAT3, and will contribute to further investigation of the renal handling of various drugs.

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Regular Article

Metformin is a Superior Substrate for Renal Organic Cation Transporter OCT2 rather than Hepatic OCT1

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Full text of this paper is available at http://www.jstage.jst.go.jp/browse/dmpk

Summary: Although metformin, a cationic agent for type II diabetes, shows its pharmacological effect in the liver, the drug is mainly eliminated into urine. The tissue selectivity based on the function of drug transporters is unclear. In the present study, the transport of metformin was examined using HEK293 cells transiently transfected with five human renal organic ion transporter cDNAs. Human OCT1 and OCT2, but not OAT1, OAT3 or OCT2-A, stimulated the uptake. A kinetic analysis of metformin transport demonstrated that the amount of plasmid cDNA for transfection was also important parameter to the quantitative elucidation of functional characteristics of transporters, and both human and rat OCT2 had about a 10- and 100-fold greater capacity to transport metformin than did OCT1, respectively. In male rats, the mRNA expression level of rOCT2 in the whole kidneys was 8-fold greater than that of rOCT1 in the whole liver. The *in vivo* distribution of metformin in rats revealed that the expression level of renal OCT2 was a key factor in the control of the concentrative accumulation of metformin in the kidney. These findings suggest that metformin is a superior substrate for renal OCT2 rather than hepatic OCT1, and renal OCT2 plays a dominant role for metformin pharmacokinetics.

Key words: organic cation transporter; kidney; liver; diabetes; biguanide; TEA

Introduction

Tissue-specific organic cation transporters contribute to the hepatic- or renal-selective distribution of cationic compounds including endogenous substrates, drugs, and their metabolites. Gründemann *et al.*[1] isolated a rat organic cation transporter, rOCT1 (slc22a1), which was preferentially expressed in the liver and kidney. We isolated rOCT2 (slc22a2) which was homologous to rOCT1, and found that its mRNA was solely expressed in the kidney.[2] In humans, OCT1 (SLC22A1) is primarily expressed in the liver,[3] and the human (h)OCT1 could not be detected in the human kidney.[4] In addition, hOCT2 (SLC22A2) was found to be the most abundant organic cation transporter in the basolateral membranes of human kidney.[4] Because OCT1 and OCT2 were similar in substrate specificity, it had been difficult to explain the difference in the tissue distribution of cationic drugs between liver and kidney.[5]

One of the biguanide agents, metformin, is extensively excreted into urine, mostly via the tubular secretion.[6] However, it has been considered that the phrmaclogical target organ is the liver, and the lactic acidosis is one of the severe side effects of the drug. Wang *et al.*[7] reported that the OCT1 mediated the intestinal and hepatic distribution of metformin in rats and mice. The relation between the OCT1 expression and lactic acidosis was reported by use of OCT1 null mice.[8] Using the electrophysiological technique in *Xenopus* oocytes, Dresser *et al.*[9] suggested that metformin and phenformin interacted with hOCT1 and hOCT2. Recently, we demonstrated that metformin was a substrate for the renal OCT2.[10] However, there is no

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information about the contribution of OCT1 and OCT2 on the *in vivo* pharmacokinetics of metformin. The quantitative characteristics of metformin transport by OCT1 or OCT2 are needed to understand the hepatic or renal selectivity of metformin.

In the present study, the quantitative analyses for metformin transport activity, using the limited amount of transfected cDNA, have been performed to clarify the difference in the transport capacity as well as the substrate affinity between OCT1 and OCT2 using both the human and rat clones. The results clearly revealed that metformin was a superior substrate for the renal OCT2 rather than hepatic OCT1.

Methods

Materials: [Biguanidine-^{14}C]metformin hydrochloride (26 mCi/mmol) was purchased from Moravek Biochemicals, Inc. (Brea, CA). Metformin and 1-methyl-4-phenylpyridinium iodide were obtained from Sigma-Aldrich Co. (St. Louis, MO). All other compounds used were of the highest purity available.

Cell culture and transfection: HEK 293 cells (ATCC CRL-1573, American Type Culture Collection, Manassas, VA) were cultured in complete medium consisting of Dulbecco's modified Eagle's medium with 10% fetal bovine serum in an atmosphere of 5% CO_2/95% air at 37°C, and used as host cells. pCMV6-XL4 plasmid vector (OriGene Technologies, Rockville, MD) DNA (800 ng) containing hOCT1, hOCT2, and hOCT2-A cDNA, and pBK-CMV vector (Stratagene, La Jolla, CA) DNA (800 ng) containing hOAT1, hOAT3, rOCT1, and rOCT2 cDNA were used to conduct the transient expression analysis, as described.[11] The transfectant stably expressing hOCT1 was established as described previously.[10,12] The cell monolayers were used at day 3 of culture for uptake experiments.

Uptake experiments: Cellular uptake of cationic compounds was measured with monolayer cultures of HEK293 cells grown on poly-D-lysine-coated 24-well plates.[10,12] The incubation medium for uptake experiments contained: 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose, and 5 mM HEPES (pH 7.4). The pH of the medium was adjusted with NaOH or HCl. The cells were preincubated with 0.2 mL of incubation medium for 10 min at 37°C. The medium was then removed, and 0.2 mL of incubation medium containing [^{14}C]metformin or [ethyl-1-^{14}C]tetraethylammonium (TEA) bromide was added. The medium was aspirated off at the end of the incubation, and the monolayers were rapidly rinsed twice with 1 mL of ice-cold incubation medium. The cells were solubilized in 0.5 mL of 0.5N NaOH, and then the radioactivity in aliquots was determined by liquid scintillation counting. The protein content of the solubilized cells was determined by the method of Bradford,[13] using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) with bovine γ-globulin as a standard.

mRNA expression of organic cation transporters: The expression levels of hOCT1, hOCT2, rOCT1, and rOCT2 in HEK293 transfectants were quantified as described previously with some modifications.[4] Briefly, total cellular RNA was isolated from specimens using a MagNA Pure LC RNA isolation Kit II (Roche Diagnostic GmbH, Mannheim, Germany) and was reverse-transcribed to cDNA. Real-time polymerase chain reaction (PCR) was performed using the ABI prism 7700 sequence detector (Applied Biosystems, Foster, CA). Glyceraldehyde-3-phosphate dehydrogenase mRNA was also measured as an internal control with glyceraldehyde-3-phosphate dehydrogenase Control Reagent (Applied Biosystems).

For quantification of the organic cation transporter mRNAs in the whole liver or kidneys in rats, we harvested each tissue from Wistar male rats weighing between 300 g and 330 g (12 weeks old, n=4), and the isolated whole liver or kidneys were minced and incubated in the RNA Later® (QIAGEN GmbH, Hilden, Germany) for 10 hours at 4°C. After re-mincing the tissues, a part of the mixed tissue samples were subjected to total RNA extraction. The total RNA extraction, reverse-transcription and real-time PCR were performed as described above.

***In Vivo* intravenous administration study:** The animal experiments were performed in accordance with the *Guidelines for Animal Experiments of Kyoto University*. Male and Female Wistar/ST rats weighing 210–240 g were anesthetized with sodium pentobarbital and the femoral artery and vein were cannulated with polyethylene tubing. Tracer amounts of metformin (1 mg/kg) dissolved in saline were administered as a bolus *via* the catheterized right femoral vein. Blood samples were collected at 0.5, 1, 1.5, 2, 2.5, and 3 min from the left femoral artery. Three minutes after the injection, the kidneys and livers were collected immediately after sacrifice. The excised tissues were gently washed, weighed, and homogenized in 3 volumes of saline.[14] Aliquots (100 μL) of blood and tissue homogenates were deproteinized with methanol (200 μL) and then subjected to HPLC.

HPLC analysis: A high-performance liquid chromatograph LC-10AD (Shimadzu Co., Kyoto, Japan) was equipped with an UV spectrophotometric detector (SPD-100A; Shimadzu Co.) adjusted to 236 nm for metformin and integrator (Chromatopac C-R8A;

The accession numbers of cDNAs used in the present study were as follows: hOCT1, **X98332**; hOCT2, **X98333**; hOCT2-A, **AB075951**; hOAT1, **AB009698**; hOAT3, **AF097491**; rOCT1, **X78855**; rOCT2, **D83044**.

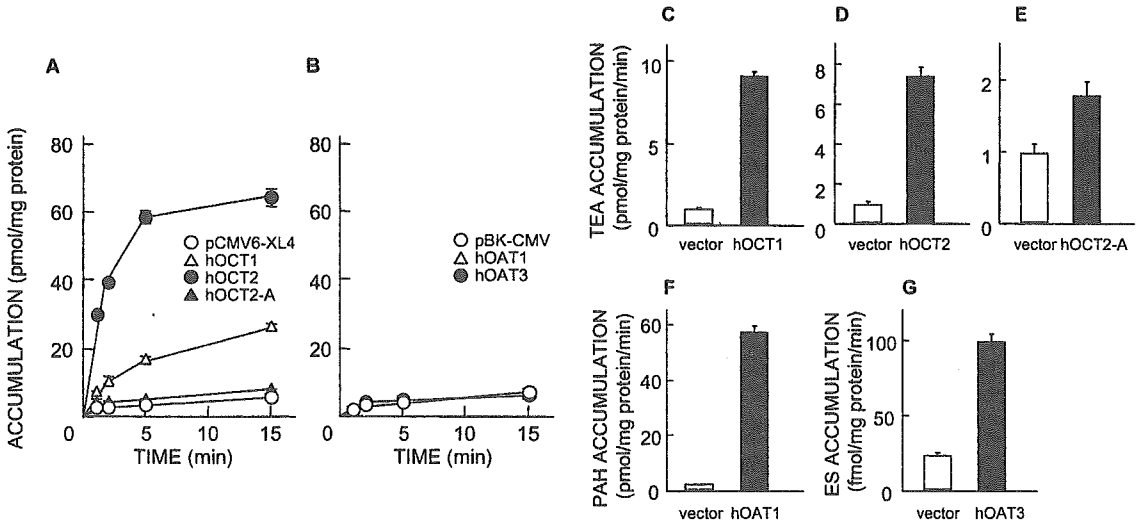

Fig. 1. Transport activity for [^{14}C]Metformin by HEK293 cells transiently expressing human organic ion transporters.

(A) HEK293 cells transfected with pCMV6-XL4 vector (open circle), hOCT1 (open triangle), hOCT2 (closed circle), or hOCT2-A (closed triangle) were incubated for the specified periods at 37°C with 0.2 mL of 10 μM (9.26 kBq/mL) [^{14}C]metformin. (B) HEK293 cells transfected with pBK-CMV vector (open circle), hOAT1 (open triangle), or hOAT3 (closed circle) were incubated for the specified periods at 37°C with 0.2 mL of 10 μM (9.26 kBq/mL) [^{14}C]metformin. Each point represents the mean ± S.E. of three monolayers. (C-E) HEK293 cells transfected with pCMV6-XL4 vector (open column), hOCT1 (closed column) (C), hOCT2 (closed column) (D), or hOCT2-A (closed column) (E) were incubated at 37°C for 1 min with 0.2 mL of 5 μM (10.36 kBq/mL) [ethyl-1-^{14}C]tetraethylammonium (TEA) bromide. (F) HEK293 cells transfected with pBK-CMV vector (open column), and hOAT1 (closed column) were incubated at 37°C for 1 min with 0.2 mL of 5 μM (9.25 kBq/mL) *p*-[glycyl-^{14}C]aminohippuric acid (PAH). (G) HEK293 cells transfected with pBK-CMV vector (open column), and hOAT3 (closed column) were incubated at 37°C for 1 min with 0.2 mL of 20 nM (37 kBq/mL) [6,7-^{3}H(*N*)]estrone sulfate (ES) ammonium salt. Each column represents the mean ± S.E. of three monolayers.

Shimadzu Co.). The stationary phase was a Cosmosil $5C_{18}$-MS-II column (4.6-mm inside diameter × 150 mm, Nacalai Tesque, Kyoto Japan). The flow rate was 1 mL/min, and the column temperature was maintained at 40°C. The mobile phase consisted of 60% phosphate buffer (10 mM, pH 6.5) and 40% methanol.

Analytical methods: The plasma concentration at 0 min was extrapolated assuming that the concentration data could be fitted to the two-compartment model. The area under the plasma concentration-time curve until 3 min ($AUC_{0\text{-}3min}$) was calculated by the trapezoidal rule. The tissue uptake clearance of metformin was calculated as dividing the tissue accumulation at 3 min by the $AUC_{0\text{-}3min}$.[14]

Statistical analysis: Data are expressed as the mean ± S.E. Data were analyzed statistically using a one-way analysis of variance (ANOVA) followed by Fisher's *t* test. Significance was set at $P<0.05$.

Results

[^{14}C]metformin by organic ion transporters: First, we examined the [^{14}C]metformin uptake by HEK293 cells transfected with hOCT1, hOCT2, hOCT2-A, hOAT1, and hOAT3 cDNAs. The uptake was markedly stimulated in the OCT2-transfected cells. On the other hand, much less [^{14}C]metformin was taken up by OCT1-transfected cells than OCT2-transfected cells. Other transfectants did not show an increase in the uptake of metformin (**Figs. 1A and 1B**). The functional expression of each transporter was confirmed using typical substrates (**Figs. 1C-1G**).

Difference in metformin transport capacity between OCT1 and OCT2: Next, we assessed whether the difference in the uptake of [^{14}C]metformin via hOCT1 and hOCT2 was dependent on the expression level in the transfectants. After transfection with several amounts of hOCT1 or hOCT2 cDNA, each mRNA level (**Fig. 2A**) and metformin uptake (**Fig. 2B**) was evaluated. The metformin uptake by each hOCT1- or hOCT2-cDNA transfected HEK293 cells was saturated at the high mRNA level range. **Figure 2C** shows that the uptake of metformin by hOCT2 was much greater than that by hOCT1 at various mRNA levels. We recently reported that the apparent K_m and V_{max} values for the transport of metformin by HEK293 cells stably expressing hOCT2 were 1.38 ± 0.21 mM and 11.9 ± 1.5 nmol/mg protein/min, respectively.[10] In the current study, we also established HEK293 cells stably expressing hOCT1. The apparent K_m and V_{max} values for the uptake of metformin by cells stably expressing hOCT1 were 4.95 ± 1.12 mM and 4.34 ± 0.59 nmol/mg protein/min, respectively. Because the hOCT1 and hOCT2

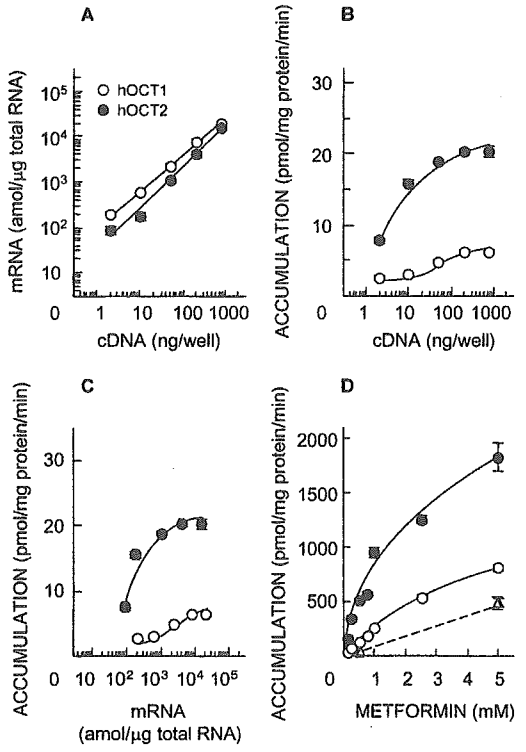

Fig. 2. Effects of mRNA expression levels of hOCT1 and hOCT2 on [^{14}C]metformin transport.

(A) HEK293 cells cultured in 24-well plate were transfected into a well with 2, 10, 50, 200, and 800 ng of plasmid cDNA coding hOCT1 or hOCT2. The pCMV6-XL4 empty vector DNA was added to the DNA solution to give the final volume (800 ng/well) before transfection. Total cellular RNA was extracted from HEK293 cells transfected with several amounts of hOCT1 cDNA or hOCT2 cDNA. The mRNA levels of hOCT1 (open circle) and hOCT2 (closed circle) were determined by real-time PCR. Each point represents the mean ± S.E. of three monolayers. (B) HEK293 cells transfected with several amounts of hOCT1 cDNA (open circle) or hOCT2 (closed circle) were incubated for 2 min at 37°C with 0.2 mL of 10 μM (9.26 kBq/mL) [^{14}C]metformin. Each point represents the mean ± S.E. of three monolayers. (C) An illustration of the correlation between the mRNA level of hOCT1 or hOCT2 (A), and the accumulation of [^{14}C]metformin (B). (D) HEK293 cells transfected with 50 ng/well of hOCT1 cDNA (open circle and open triangle) or hOCT2 (closed circle and closed triangle), and 750 ng/well of pCMV6-XL4 empty vector were incubated for 2 min at 37°C with 0.2 mL of 10 μM (9.26 kBq/mL) [^{14}C]metformin in the absence (circle) or presence (triangle) of 5 mM MPP (pH 7.4). Open and closed triangles are overlapping. Unlabeled metformin was added to [^{14}C]metformin to give the final concentrations indicated. Each point represents the mean ± S.E. of three monolayers.

mRNA were not detected in the cells transfected with the empty vector (data not shown), the present data were suggested to be derived by the transfected transporters.

Kinetic evaluation of metformin transport by OCTs: To establish more of a quantitative difference between hOCT1 and hOCT2 in the transport of metformin, we examined the concentration-dependence of [^{14}C]metformin uptake without saturating the mRNA expression levels using cells transfected with each OCT-cDNA (50 ng/well) and vector-cDNA (750 ng/well) (**Fig. 2D**). **Table 1** shows the apparent K_m and V_{max} values for the uptake of metformin by hOCT1 and hOCT2. The clearance of metformin, V_{max}/K_m, was much higher in hOCT2-transfectants. Concerning the expression level of each transporter mRNA, the intrinsic clearance of the hOCT2-mediated uptake of metformin was about 10-fold that for the hOCT1-mediated uptake (**Table 1**). Prior to examining the tissue distribution of metformin in rats, we ascertained the correspondence between hOCTs and rOCTs. As shown in **Fig. 3**, the expression-level dependent manners of metformin transport by rOCT1 and rOCT2 was similar in comparison with those of human orthologue transporters. The apparent intrinsic clearance of rOCT2-mediated uptake of metformin was about 100-fold greater than rOCT1 (**Table 1**). To confirm the transport activity of these OCTs, we also examined the similar experiments using TEA as a reference substrate (**Fig. 4**). The expression-level dependent manners of TEA uptakes by OCTs were observed. Although hOCT2-mediated uptake of TEA was tended to be similar with hOCT1 (**Fig. 4A**), rOCT2-mediated TEA uptake was much greater than rOCT1 (**Fig. 4B**).

Table 1. Apparent K_m values of [^{14}C]metformin uptake by human or rat OCT1 and OCT2.

	K_m	V_{max}	Intrinsic clearance*
	(mM)	(pmol/mg protein/min)	(μL/min/fmol mRNA)
hOCT1	1.47 ± 0.19	396 ± 42	5.09 ± 0.52
hOCT2	0.99 ± 0.03	1444 ± 81	54.49 ± 4.64
rOCT1	3.73 ± 0.15	145 ± 6	0.39 ± 0.04
rOCT2	0.63 ± 0.09	1446 ± 55	36.98 ± 4.39

*Vaules of intrinsic clearance were calculated as follows: V_{max} (pmol/mg protein)/K_m (mM)/mRNA expression level (amol/mg protein). Experimental conditions are in the legends of **Fig. 2D** and **Fig. 3D**. The apparent K_m and V_{max} values were determined from Eadie-Hofstee plots of metformin uptake after the corrections for nonsaturable components. Data are shown as means ± S.E. of three monolayers.

To estimate the tissue intrinsic clearance of metformin in the liver or kidney focusing on the organic cation transporters, we quantified the mRNA expression amounts of rOCT1 and rOCT2 in the whole liver (12.3 ± 0.3 g/rat, mean ± S.E. of four rats) and kidney (2.1 ± 0.03 g/rat, mean ± S.E. of four rats) of rats. As shown in the **Fig. 5**, the mRNA expression level of rOCT2 in the kidneys was 10.3 and 6.5 fold higher in comparison with the renal rOCT1 and the hepatic rOCT1, respectively. Next, the tissue intrinsic clearance of rOCT-mediated uptake of metformin in each tissue per rat was estimated in combination with the data in **Table 1**. The renal intrinsic clearance of rOCT2-mediated uptake of

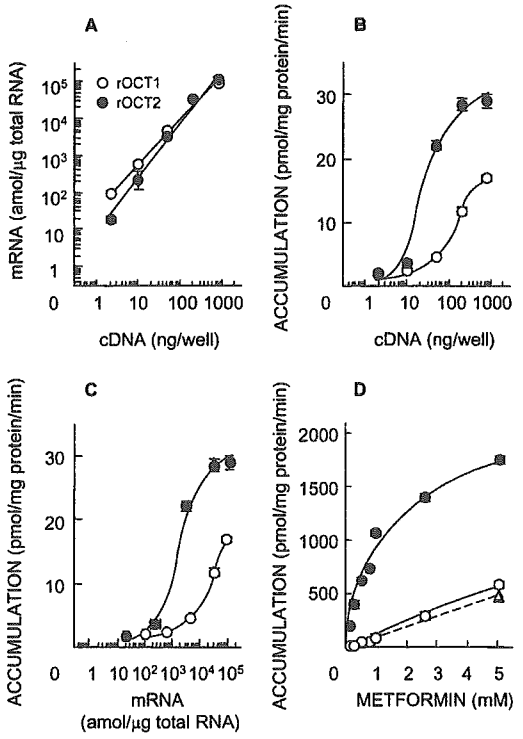

Fig. 3. Effects of expression levels of rOCT1 and rOCT2 on [^{14}C]metformin transport.

The experimental conditions were correspondingly conducted to those of **Fig. 2.** HEK293 cells were transfected with the several amounts of plasmid cDNA coding rOCT1 or rOCT2, and pBK-CMV empty vector DNA instead of hOCT1 or hOCT2, and pCMV6-XL4 empty vector, respectively.

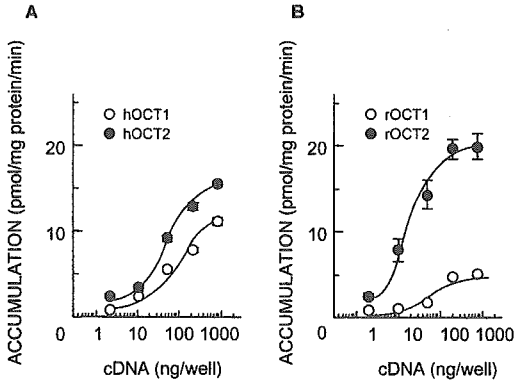

Fig. 4. Effects of expression levels of organic cation transporters on [^{14}C]TEA transport.

The experimental conditions were correspondingly conducted as described for **Fig. 2B.** HEK293 cells were transfected with the several amounts of plasmid cDNA coding hOCT1 (open circle, A), hOCT2 (closed circle, A), pCMV6-XL4 empty vector DNA (A), rOCT1 (open circle, B) or rOCT2 (closed circle, B), and pBK-CMV empty vector DNA (B).

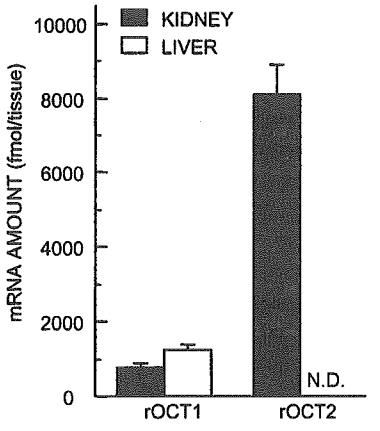

Fig. 5. mRNA expression levels of rOCT1 and rOCT2 in whole liver and kidney.

After harvesting the whole liver and kidneys from the male rats (12 weeks, 300–330 weighing), total RNA was extracted as described in the method section. The mRNA levels of rOCT1 and rOCT2 in the each tissue were determined by real-time PCR. The wet weights of liver and kidneys were 12.3 ± 0.3 and 2.1 ± 0.03 g/rat (mean ± S.E. of four rats), respectively. Each point represents the mean ± S.E. of four rats. N.D., not detected.

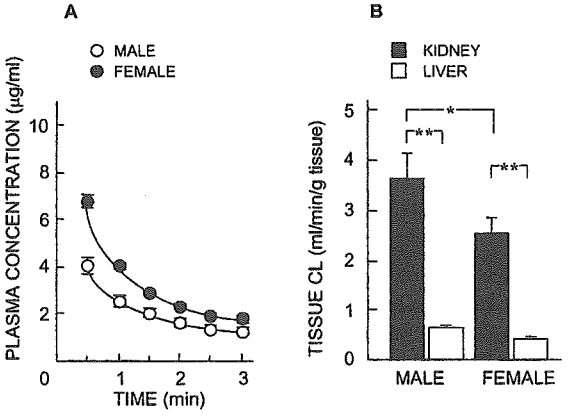

Fig. 6. Plasma concentration curves (A) and tissue uptake clearance (B) of metformin in rats.

Metformin (1 mg/kg) was administered as a bolus *via* the right femoral vein of male (open circle) or female (closed circle) rats. (A) Blood samples were collected from the left femoral artery at 0.5, 1, 1.5, 2, 2.5, and 3 min after the injection. Each point represents the mean ± S.E. of five rats. (B) Three minutes after the administration of metformin, the tissues were harvested. The concentration of metformin in each tissue was measured. The tissue uptake clearance of metformin was calculated as dividing the tissue accumulation at 3 min by the $AUC_{0\text{-}3min}$ from each rat. The wet weights of liver and kidneys in male rats were 8.5 ± 0.3 and 1.94 ± 0.05 g/rat, and those in female rats were 7.9 ± 0.3 and 1.5 ± 0.04 g/rat, respectively (mean ± S.E. of five rats). Each column represents the mean ± S.E. for five rats. $^{*}p<0.05$, $^{**}p<0.01$, significant differences.

metformin was 299.8 mL/min/tissue, which was markedly higher than that by renal rOCT1 (0.31 mL/min/tissue) and hepatic rOCT1 (0.49 mL/min/tissue). The contribution of rOCT1 on the renal distribution of metformin was negligible compared to rOCT2.

***In vivo* pharmacokinetic role of rOCT1 and rOCT2 on metformin tissue distribution:** Furthermore, we examined the tissue distribution of metformin *in vivo* focusing on rOCT1 and rOCT2. We previously reported that the levels of rOCT2 mRNA and protein in the kidney were much higher in males than females, but there was no gender-based difference in the mRNA expression of renal rOCT1.[15] Considering these findings, we examined the contribution of rOCT2 to the tissue distribution of metformin using male and female rats. The plasma concentrations of metformin until 3 min after the intravenous administration and the tissue uptake clearances of metformin were determined (**Fig. 6**). The tissue uptake clearance of metformin in the male kidney was much higher than that in male liver, female liver, and female kidney.

Discussion

In the present study, we have quantitatively elucidated the substrate specificity of OCTs. hOCT2 was identified as a superior transporter mediating the uptake of metformin among several human organic ion transporters examined (**Fig. 1**), and two transporters, OCT1 and OCT2, had distinct substrate affinity for metformin (**Figs. 2 and 3**). Although the expression-level dependent profile of TEA uptake by hOCT1 and hOCT2 was similar, the TEA uptake activity of rOCT2 was much greater in comparison with rOCT1 (**Fig. 4**). By analysing the relation between the expression level and transport activity, some differences on the transport characteristics between rOCT1 and rOCT2 would be clarified. It had been considered that OCT1 and OCT2 possess similar multispecificities for various compounds.[5,16-18] However, recent reports suggest that some chemical compounds such as guanidine and creatinine could be used to determine the molecular selectivity between OCT1 and OCT2.[11,19] In addition, hOCT2-A, a splicing variant of hOCT2, did not transport metformin (**Fig. 1A**). Therefore, metformin may be an useful probe substrate for clarifying the structural determinant(s) to distinguish the substrate specificities between hOCT2 and hOCT2-A. By use of the transfectants with a limit amount of cDNA, we have simultaneously demonstrated that metformin, which is a derivative of guanidine, was dominantly transported by OCT2 compared to OCT1. To our knowledge, this is the first report demonstrating the quantitative difference in the transport activities of metformin between OCT1 and OCT2. Information about the molecular determinants of substrate binding has emerged gradually.[20] The use of guanidine derivatives including metformin, will clarify the chemical structures required for specific transport by OCT2.

In rat kidney, rOCT2 expression levels were higher in male than female rats, and the uptake of TEA in the renal slices was greater in the male rats.[15] In the present study, the renal uptake clearance of metformin was also higher in male than female rats, comparable with previous findings. In the male rats, the mRNA expression level of rOCT2 in the kidneys was markedly higher than the hepatic rOCT1 (**Fig. 5**), and the estimated renal intrinsic clearance of metformin by rOCT2 was about 600 times larger than that of the liver by rOCT1. The *in vivo* tissue uptake clearance of metformin in the male kidney was about 6 times larger than that in the male liver (**Fig. 6**), and therefore, the plasma flow rate in the kidney might be a limiting factor for metformin renal distribution. Considering the expression levels and the intrinsic clearance of transporter-mediated metformin uptake, there is little contribution of rOCT1 on renal uptake of metformin. Because the hOCT1 was not detected in the human kidney, the hOCT2 was considered as a primary organic cation transporter determining the renal distribution of cationic drugs.[4] These results indicate that rat is still useful model animal for pharmacokinetic studies of metformin focusing on the hepatic rOCT1 and renal rOCT2. This is the first report to estimate the expressional amounts of organic cation transporter isoforms in the whole liver and kidneys, and these data will be useful to quantitative evaluation of the tissue selectivity of cationic drugs in rodents.

In the present study, the accumulation of metformin in the liver was much lower than that in the kidney (**Fig. 6**). Because the plasma flow rate could be a limited step, the difference between metformin accumulation into the kidneys and livers was small compared to the *in vitro* estimation (**Table 1, Figs. 5 and 6**). However, the expressional dominancy of the rOCT2 in male kidney reflected the concentrated accumulation of metformin in comparison with that in the female rats. Therefore, the expressional amount as well as substrate specificity of transporters in each tissue was suggested to help understanding the tissue selectivity of cationic drugs. In the humans, the kidney function as well as the age was postulated as the predictors for pharmacokinetics of metformin.[6] Although the OCT1 protein is expressed in the kidney and liver in the rat, the expression of OCT1 in the human kidney is negligible.[4,5] Therefore, considering the present *in vitro* and *in vivo* results, it was suggested that renal OCT2 should be more important for the pharmacokinetics of metformin in comparison with hepatic OCT1.

The mechanisms behind the pharmacological actions of metformin have been described, including decreased hepatic glucose production and increased glycogenesis

and lactate production. The liver had been generally considered to be the primary glucogenic organ, except in acidotic conditions. But several studies have provided considerable evidence that mammalian kidney can make glucose and release it under various conditions.[21] Since 1938, it has been said that animals' kidney is also a producer of glucose.[22,23] Stumvoll *et al.*[24] suggested an important role for the human kidney in glucose homeostasis, using the combined isotopic-net renal balance approach. Furthermore, the role of the kidney in gluconeogenesis during diabetes has been studied.[21] In the present study, the concentrative accumulation of metformin in the kidney by OCT2 has been revealed. These backgrounds, including the present results, suggest that the pharmacological effects of metformin in the kidney as well as liver may be important. Clinical studies of biguanides in diabetic patients should be performed to clarify the pharmacodynamic as well as pharmacokinetic significance of renal OCT2 for the control of blood glucose levels.

In conclusion, the quantitative difference in the metformin transport between OCT1 and OCT2 has been firstly clarified using the limited amount of transfected cDNA, and it has been suggested that the renal OCT2 plays a dominant role for metformin pharmacokinetics.

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Short Communication

MODULATION OF P-GLYCOPROTEIN EXPRESSION IN HYPERTHYROID RAT TISSUES

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ABSTRACT:

P-glycoprotein (Pgp) is expressed in various normal tissues and plays an important role in drug absorption and disposition. In addition, it is supposed that alterations in the expression levels of Pgp are involved in the inter- and intraindividual variability of pharmacokinetics of many drugs. Since pharmacokinetic properties of various drugs are altered in patients with thyroid disorders, we examined the expression of Pgp and mdr1a/1b mRNA in the kidney, liver, jejunum, and ileum from euthyroid and hyperthyroid rats. Western blot analysis revealed that Pgp expression was markedly increased in the kidney and liver of hyperthyroid rats. In contrast, it was slightly increased in the jejunum and ileum. mdr1a/1b mRNA levels were significantly increased in the kidney of hyperthyroid rats. However, they were not increased in the liver as well as in the jejunum and ileum of hyperthyroid rats. Expression levels of bile salt export pump and mdr2 mRNA were also unchanged in hyperthyroid rat liver. Taken together, these findings suggest that thyroid hormone induces Pgp expression in a tissue-selective manner, and that the modulation of mdr1a/1b mRNA expression in the hyperthyroid state varies among tissues.

P-glycoprotein (Pgp) is expressed in various tissues such as brain, liver, kidney, and intestine (Cordon-Cardo et al., 1990; Brady et al., 2002) and plays an important role in defining the pharmacokinetics of many drugs. Pgp functions as a drug efflux pump and exports hydrophobic, bulky drugs such as anticancer agents, cardiac glycosides, β-blockers, calcium channel blockers, and immunosuppressants. Since Pgp has a broad substrate recognition, the concomitant administration of drugs often causes drug interactions by inhibiting Pgp-mediated transport (Yu, 1999). For example, inhibition of digoxin transport in cultured epithelial cell lines expressing Pgp by various drugs such as quinidine (Tanigawara et al., 1992; Fromm et al., 1999), verapamil (Tanigawara et al., 1992), and cyclosporin A (Okamura et al., 1993) has been reported. We have also demonstrated that the renal clearance of digoxin was decreased in patients receiving a concomitant administration of clarithromycin and, accordingly, the plasma concentration of digoxin was increased (Wakasugi et al., 1998). The mechanism of this interaction was explained by the inhibition of Pgp-mediated tubular secretion of digoxin. On the other hand, recent studies have demonstrated that changes in the expression levels of Pgp affect the pharmacokinetic properties of Pgp substrates. Greiner et al. (1999) reported that rifampin administration induced Pgp expression in the small intestine and reduced the plasma concentration of orally administered digoxin, suggesting that alterations in the expression levels of Pgp are closely involved in the inter- and intraindividual variability of pharmacokinetics of Pgp substrates.

Thyroid hormone is secreted from the thyroid gland to maintain normal growth and development, normal body temperature, and normal energy levels. Most of its effects appear to be mediated by the activation of nuclear receptors that lead to increased expression of mRNA and subsequent protein synthesis. Disorders of the thyroid gland are among the most common endocrine disorders, and are known as hyperthyroidism and hypothyroidism. It was reported that pharmacokinetic properties of various drugs were altered in patients with thyroid disorders (Shenfield, 1981; O'Connor and Feely, 1987). As for Pgp substrates, plasma concentrations of digoxin were decreased in patients with hyperthyroidism as compared with euthyroid patients. Such an altered pharmacokinetics of digoxin in hyperthyroid patients has been explained by an increase in renal clearance (Lawrence et al., 1977) and volume of distribution (Shenfield et al., 1977; Shenfield, 1981). The reason for the increased renal clearance of digoxin is considered to be a facilitation of tubular secretion (Bonelli et al., 1978); however, the mechanisms underlying the altered pharmacokinetics of digoxin in thyroid disease have not been fully elucidated.

Previous studies have shown that thyroid hormone regulates the expression levels of various membrane transporters such as the fructose transporter GLUT5 (Matosin-Matekalo et al., 1999), the peptide transporter PEPT1 (Ashida et al., 2002), Na^+/K^+-ATPase (Giannella et al., 1993), and the Na^+/H^+ exchanger NHE1 (Li et al., 2002). Therefore, we hypothesized that the alteration in the plasma concentration of digoxin in patients with thyroid disorders might be due to changes in Pgp expression by thyroid hormone. In the present study, to elucidate the influence of a hyperthyroid state on the expression of Pgp in various tissues, we investigated Pgp and mdr1a/1b mRNA levels in liver, kidney, jejunum, and ileum from euthyroid and hyperthyroid rats.

Materials and Methods

Materials. L-Thyroxine (T_4) was purchased from Sigma-Aldrich (St. Louis, MO). Monoclonal antibody C219 and anti-villin polyclonal antibody were obtained from CIS Bio International (Gif-sur-Yvette, France) and Santa Cruz

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ABBREVIATIONS: Pgp, P-glycoprotein; T_4, L-thyroxine; bsep, bile salt export pump; PCR, polymerase chain reaction.

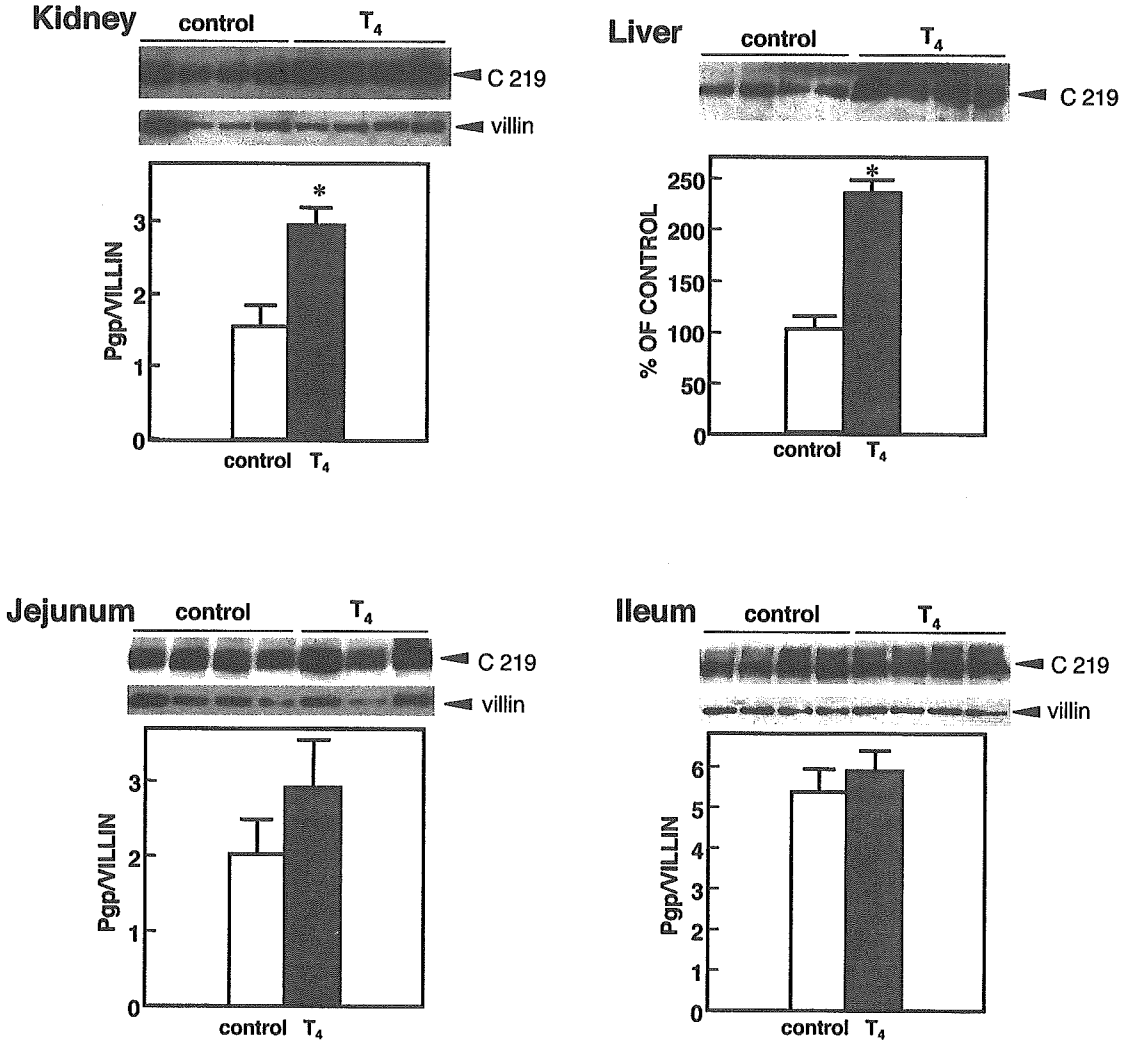

FIG. 1. Western blot analysis of the crude membranes from rat tissues for Pgp. Upper panel, immunoblotting of crude membranes from kidney, liver, jejunum, and ileum of hyperthyroid rats (T_4) or euthyroid rats (control). Lower panel, densitometric quantification of Pgp. Each column represents the mean ± S.E. of 3~7 rats. *, $p < 0.05$, significantly different from control.

Biotechnology, Inc. (Santa Cruz, CA), respectively. All other chemicals used were of the highest purity available.

Animals. The animal experiments were performed in accordance with the Guideline for Animal Experiments of Kyoto University. Eight-week-old male Wistar rats were housed in a temperature- and humidity-controlled room and fed rat chow ad libitum. Hyperthyroidism was induced by adding T_4 (12 mg/l) to the drinking water for 21 days as previously described (Ashida et al., 2004). After treatment, the kidney, liver, jejunum, and ileum were excised. Blood was also collected for measurement of plasma level of T_3. Plasma T_3 levels in euthyroid and hyperthyroid rats were measured by an Enzyme Immuno Assay method (IMx; Dainabot, Tokyo, Japan). Plasma T_3 levels in euthyroid and hyperthyroid rats were 0.32 ± 0.02 and 2.40 ± 0.18 ng/ml, respectively (mean ± S.E., $n = 6$). In addition, hyperthyroid rats lost an average of 0.055 kg in response to T_4 treatment (data not shown).

Western Blot Analysis. Isolation of crude plasma membrane fractions from each tissue and Western blot analysis were performed as described previously (Ogihara et al., 1996). Monoclonal antibody C219 and anti-villin polyclonal antibody were used as primary antibodies.

RNA Isolation, Semiquantitative Reverse Transcription-Polymerase Chain Reaction (PCR), and Competitive PCR. RNA isolation, reverse transcription, and competitive PCR procedures were performed as described previously (Masuda et al., 2000) with some modifications. The specific primer sets (5 μM) used were as follows: for rat mdr1a primers, 5′-GATGGAATTGATAATGTGGACA-3′ and 5′-AAGGATCAGGAACAATAAA-3′; for rat mdr1b primers, 5′-GAAATAATGCTTATGAATCCCAAA G-3′ and 5′-GGTTTCATGGTCGTCGTCTCTTGA-3′ (Zhang et al., 1996); for rat bile salt export pump (bsep) primers, 5′-GAGGTTACTTAATAGCCTACG-3′ and 5′-CATCTATCATCACAGTTCCC-3′; and for rat mdr2 primers, 5′-AAGAATTTGAAGTTGAGCTAAGTGA-3′ and 5′-TGGTTTCCACATCCAGCCTAT-3′. For the detection of mdr1a and mdr1b, semilogarithmic serial dilutions of mimic competitor DNA from 50 to 0.01 amol were added.

Statistical Analysis. Data were analyzed statistically using the nonpaired *t* test. Probability values of less than 5% were considered significant.

Results and Discussion

To investigate the effect of thyroid hormone on the expression of Pgp, we examined the Pgp expression by Western blotting. As shown in Fig. 1, the expression of Pgp was remarkably increased in crude membranes of hyperthyroid rat kidney and liver (1.9-fold and 2.3-fold, respectively) as compared with those of euthyroid rats. In contrast, hyperthyroidism caused a slight increase in the expression of Pgp in rat jejunum and ileum (1.1-fold and 1.4-fold, respectively). These results suggest that the differences in the behavior of thyroid hormone in each tissue are involved in tissue selectivity of Pgp induction. Similarly, it was reported that MDR1 mRNA was elevated in a dexamethasone-treated human hepatoma cell line but not in a nonhepatoma cell line, suggesting that the hormonal regulation of *mdr* gene expression is gene- and cell type-specific (Zhao et al., 1993).

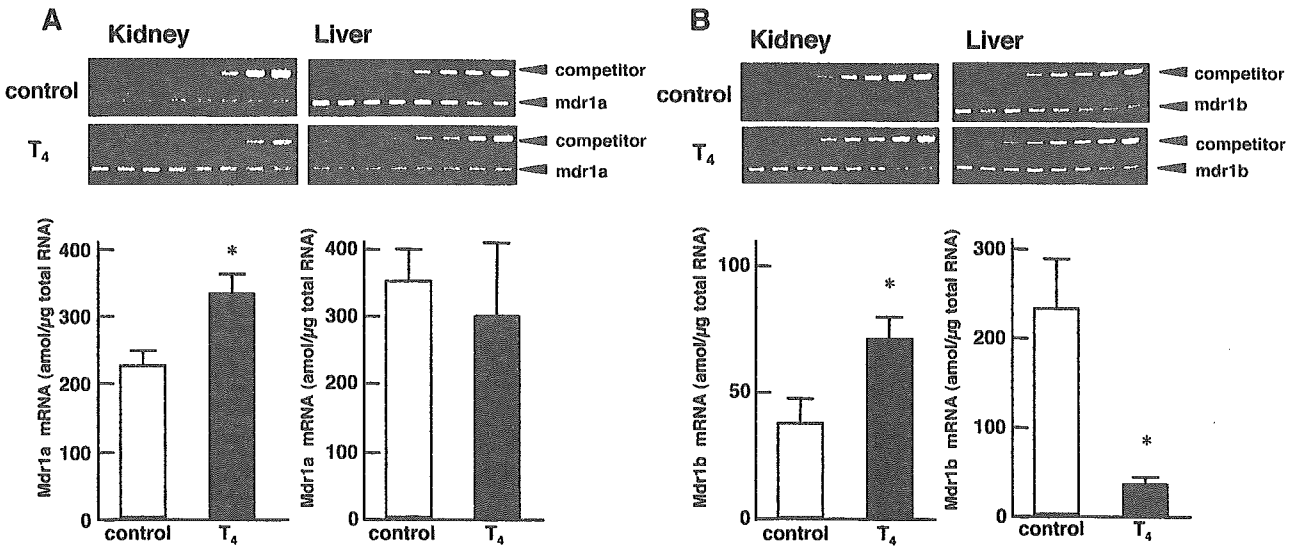

FIG. 2. Effect of hyperthyroidism on the expression of mdr1a (A) and mdr1b (B) mRNA in rat kidney and liver. Upper panel, typical results of agarose gel electrophoresis of the PCR products from each tissue of hyperthyroid rats (T_4) or euthyroid rats (control). Eight points were selected with serial dilutions of mdr1a/1b mimic competitor cDNA from 0.01 to 25 amol. Lower panel, densitometric quantification of mdr1a and mdr1b mRNA. Each column represents the mean ± S.E. of four rats. *, $p < 0.05$, significantly different from control.

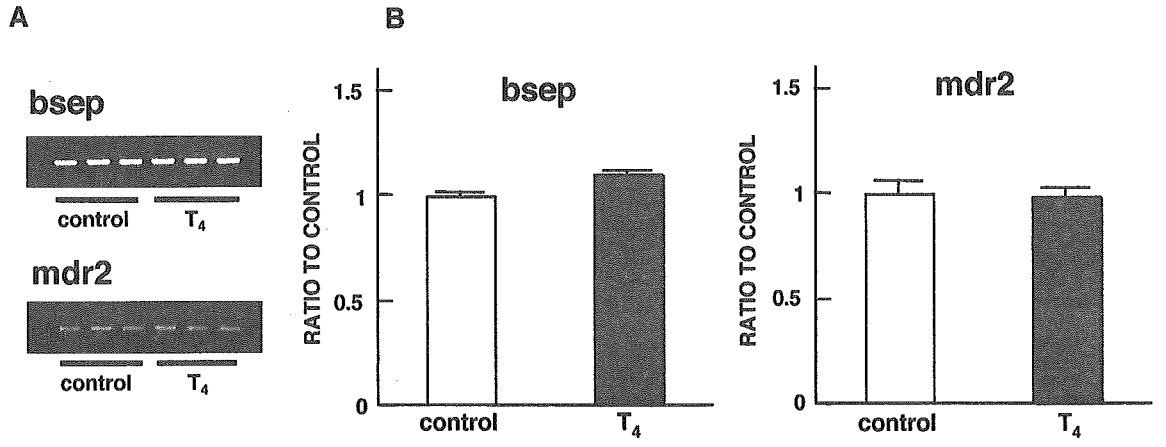

FIG. 3. Effect of hyperthyroidism on the expression of bsep and mdr2 mRNA in rat liver. A, typical results of agarose gel electrophoresis of the PCR products from liver of hyperthyroid rats (T_4) or euthyroid rats (control). B, densitometric quantification of bsep and mdr2 mRNA. Each column represents the mean ± S.E. of three rats.

Their report supports our results suggesting that the regulation of Pgp expression by thyroid hormone was possibly tissue-specific.

We then examined the expression of mdr1a and mdr1b mRNA using competitive PCR in each tissue. As shown in Fig. 2, the expression of both mdr1a and mdr1b mRNA was significantly increased in hyperthyroid rat kidney as compared with the control. Since the action of thyroid hormone appears to be mediated by the activation of nuclear receptors, which leads to increased levels of mRNA and subsequent protein synthesis (Ribeiro et al., 1995), it is likely that the induction of Pgp expression in the hyperthyroid kidney is mediated by the increased transcription of mdr1a/1b mRNA. On the other hand, hyperthyroidism caused a significant decrease in the expression of mdr1b mRNA in the liver but did not affect the expression of mdr1a mRNA. Since it was reported that C219 antibody reacted with not only Pgp but bsep and mdr2 (Childs et al., 1995) and bsep and mdr2 are expressed in the liver, we examined whether the immunoreactive protein bands obtained using C219 antibody reflected the induction of these transporters in hyperthyroid liver. As shown in Fig. 3, however, the expression of bsep and mdr2 mRNA did not change in hyperthyroid rat liver. Although the precise mechanism is not clear at this stage, thyroid hormone may regulate Pgp expression via nontranscriptional control in the liver. Further studies are needed to elucidate the regulation of Pgp and MDR1 by thyroid hormone in the liver.

Siegmund et al. (2002) examined the effect of levothyroxine administration on human intestinal Pgp expression. They demonstrated that duodenal MDR1 mRNA expression and immunoreactive Pgp were increased by levothyroxine administration, although the increase in MDR1 mRNA was not significant. However, changes in the expression of Pgp were not associated with major alterations in the pharmacokinetics of talinolol, a substrate of Pgp. In addition, the effect of thyroid hormone on the expression of Pgp in other tissues was not elucidated. In the present study, we observed a significant increase in Pgp expression in the kidney and liver (Fig. 2) and a slight increase in the intestine (Fig. 4) in hyperthyroid rats. Our results suggest that the decrease in the serum concentration of digoxin in hyperthyroidism is attributable to the increased expression of Pgp in the kidney and/or liver. Further studies are needed to assess the pharmacokinetics of Pgp substrates in hyperthyroid rats.

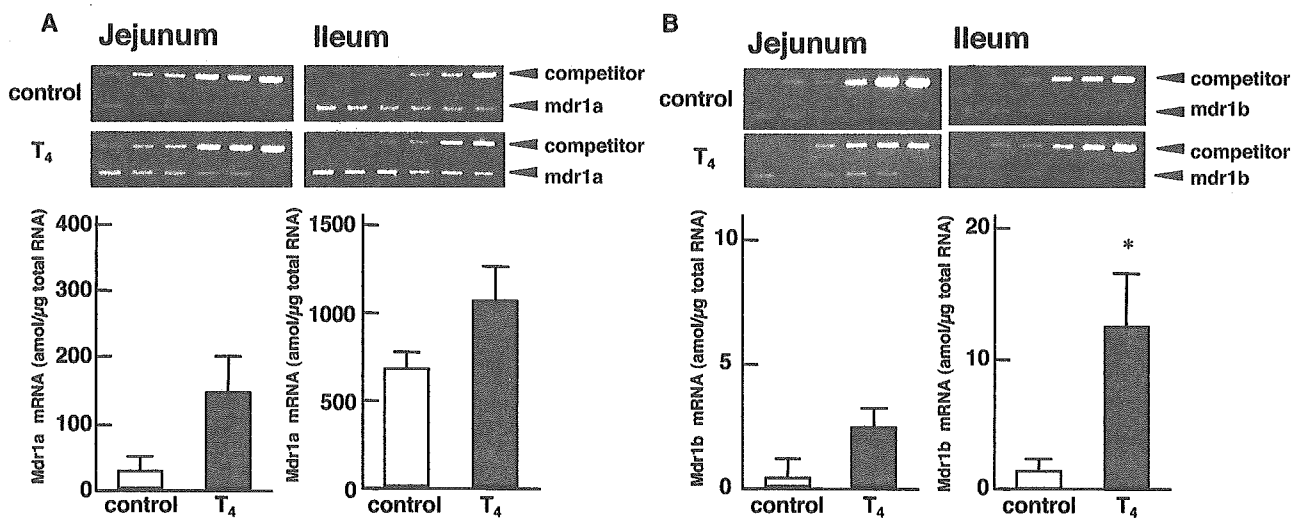

FIG. 4. Effect of hyperthyroidism on the expression of mdr1a (A) and mdr1b (B) mRNA in rat jejunum and ileum. Upper panel, typical results of agarose gel electrophoresis of the PCR products from each tissue of hyperthyroid rats (T_4) or euthyroid rats (control). Six points were selected with serial dilutions of mdr1a/1b mimic competitor cDNA from 0.25 to 50 amol. Lower panel, densitometric quantification of mdr1a and mdr1b mRNA. Each column represents the mean ± S.E. of four rats. *, $p < 0.05$, significantly different from control.

For obvious ethical reasons, Siegmund et al. (2002) administered levothyroxine in doses that do not cause thyrotoxicosis. In the present study, the expression of Pgp was only slightly increased in rat intestinal tissues, although we used hyperthyroid rats. It is known that the gene encoding Pgp differs between humans and rats. Pgp is encoded by *MDR1* in humans and by *mdr1a* and *mdr1b* in rats. Therefore, it is supposed that the mechanisms by which thyroid hormone induces Pgp expression differ between species, and Pgp expression in human intestinal tissues may increase dramatically under thyrotoxicosis.

In conclusion, thyroid hormone induces Pgp expression in a tissue-selective manner. In addition, the modulation of mdr1a/1b mRNA expression in the hyperthyroid state varied among tissues. These results provide useful information for elucidating the drug interaction and pharmacokinetic variability in thyrotoxicosis.

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Regulation of human peptide transporter 1 (PEPT1) in gastric cancer cells by anticancer drugs

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Abstract

Human peptide transporter 1 (PEPT1) mediates the cellular uptake of di- and tripeptides and peptide-like drugs in the small intestine. In the present study, we examined the regulation of PEPT1 by anticancer drugs in the gastric cancer cell line MKN45. PEPT1 was expressed and functioned in MKN45 cells. The transport activity and mRNA expression of the facilitative glucose transporter 1 (GLUT1) were significantly decreased by 5-fluorouracil treatment, but those of PEPT1 were slightly increased. Cisplatin treatment affected neither PEPT1 nor GLUT1 activity. In conclusion, PEPT1 expressed in MKN45 cells are resistant against the cellular injury induced by 5-fluorouracil and cisplatin.

Keywords: Peptide transporter; Gastric cancer; 5-Fluorouracil; Cisplatin

1. Introduction

The consumption of nutrients such as glucose and amino acids is augmented in cancer cells to meet the rapid metabolic turnover. Accordingly, following malignant transformation, the expression and function of several nutrient transporters are expected to be activated in order to increase the accumulation of nutrients.

Human peptide transporter 1 (PEPT1) is normally expressed in the small intestine and kidney and mediates the transport of di- and tripeptides using the H^+-gradient as a driving force. PEPT1 also plays important roles as a drug transporter to mediate the transport of peptide-like drugs such as β-lactam antibiotics and the anticancer drug bestatin [1]. Previously, it was reported that PEPT1 is expressed in some human cancer cell lines such as AsPC-1 and Capan-2 (pancreatic cancer) [2] and SK-ChA-1 (cholangiocarcinoma) [3]. Using the broad substrate specificity of PEPT1, the development of selective drug delivery systems to cancer cells has been attempted [4]. For example, Neumann et al. [5] demonstrated that the endogenous photosensitizer δ-aminolevulinic acid is accumulated specifically in bile duct tumor cells before photodynamic therapy. These findings suggest that PEPT1 may be

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