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Interaction of human organic anion transporters with various cephalosporin antibiotics

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Abstract

Cephalosporin antibiotics are thought to be excreted into the urine via organic anion transporters (OATs). The purpose of this study was to elucidate the interaction of human-OATs with various cephalosporin antibiotics, using proximal tubule cells stably expressing human-OAT1, human-OAT3 and human-OAT4. Human-OAT1 and human-OAT3 are localized to the basolateral side of the proximal tubule, whereas human-OAT4 is localized to the apical side. The cephalosporin antibiotics tested were cephalothin, cefoperazone, cefazolin, ceftriaxone, cephaloridine, cefotaxime, cefadroxil and cefamandole. All of these cephalosporin antibiotics significantly inhibited organic anion uptake mediated by human-OAT1, human-OAT3 and human-OAT4. Kinetic analysis revealed that these inhibitions were competitive. The inhibition constant (K_i) values of cefoperazone, cefazolin, ceftriaxone and cephaloridine for human-OAT1 were much lower than those for human-OAT3 and human-OAT4, whereas the K_i values of cephalothin and cefotaxime for human-OAT3 were much lower than those for human-OAT1 and human-OAT4. Human-OAT4 mediated the bidirectional transport of estrone sulfate, an optimal substrate for human-OAT4. These results suggest that human-OAT1, human-OAT3 and human-OAT4 interact with various cephalosporin antibiotics, and that human-OAT1 and human-OAT3 play a distinct role in the basolateral uptake of cephalosporin antibiotics. Since the K_i value of cephaloridine for human-OAT4-mediated organic uptake was much higher than that for human-OAT1, the results indicate the possibility that human-OAT4 limits the efflux of cephaloridine, leading to the accumulation of cephaloridine and the induction of nephrotoxicity.

Keywords: Organic anion transporter; Proximal tubule; Cephalosporin antibiotic; Interaction

1. Introduction

The secretion of numerous organic anions, including endogenous metabolites, drugs and xenobiotics, is an important physiological function of the renal proximal tubules. The secretion of organic anions through the proximal tubular cells is achieved via unidirectional transcellular transport, involving the uptake of organic anions into the cells from the blood across the basolateral membrane, followed by extrusion across the brush-border membrane into the tubular fluid (Pritchard and Miller, 1993). Recently, cDNAs encoding members of the organic anion transporter (OAT) family have been successively cloned, including OAT1 (Sekine et al., 1997; Hosoyamada et al., 1999), OAT2 (Sekine et al., 1998), OAT3 (Kusuhara et al., 1999; Cha et al., 2001) and OAT4 (Cha et al., 2000). Among these clones, human-OAT1 and human-OAT3 were shown to be localized to the basolateral side of the proximal tubule (Hosoyamada et al., 1999; Cha et al., 2001), whereas human-OAT4 is localized to the apical side of the proximal tubule (Babu et al., in press).

Cephalosporin antibiotics are suggested to be not only filtered through the glomeruli but also actively secreted by the proximal tubules. Cephalosporins inhibited *para*-aminohippuric acid uptake in rat renal slices (Hori et al., 1982) and renal plasma membrane vesicles (Takano et al., 1989). Cephaloridine, a cephalosporin that possesses both anionic and cationic moieties, inhibited *para*-aminohippuric acid transport but not *N*-methylnicotinamide transport in basolateral membrane vesicles (Kasher et al., 1983). Thus, cephalosporin antibiotics are considered to be secreted by the proximal tubule via the *para*-aminohippuric acid transporter system (Moller and Sheikh, 1983; Ulrich et al., 1989). Consistent with these results, we have recently observed that rat-OAT1 as well as rat-OAT3 interacts with various cephalosporin antibiotics (Jariyawat et al., 1999; Takeda et al., 1999; Jung et al., in press).

The purpose of this study was to elucidate the interactions of human-OATs with various cephalosporin antibiotics, using cells derived from the second segment of the proximal tubule (S_2) that stably express human-OAT1,

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human-OAT3 and human-OAT4 (S_2 human-OAT1, S_2 human-OAT3 and S_2 human-OAT4, respectively).

2. Materials and methods

2.1. Materials

[^{14}C]*Para*-aminohippuric acid (53.1 mCi /mmol) and [^{3}H]estrone sulfate (53 Ci/mmol) were purchased from Perkin Elmer Life Sciences (Boston, MA, USA). Cephalosporin antibiotics were obtained from Sigma (St. Louis, MO, USA). Other materials used included fetal bovine serum, trypsin and geneticin from Invitrogen (Carlsbad, CA, USA) recombinant epidermal growth factor from Wakunaga (Hiroshima, Japan), insulin from Shimizu (Shizuoka, Japan), RITC 80-7 culture medium from Iwaki (Tokyo, Japan) and TfX-50 from Promega (Madison, WI, USA).

2.2. Cell culture and establishment of S_2 human-OAT1, S_2 human-OAT3 and S_2 human-OAT4

S_2 cells, derived from transgenic mice harboring the simian virus 40 large T-antigen gene, were established as described previously by us (Hosoyamada et al., 1996). S_2 is the segment of the proximal tubule where human-OAT1 and human-OAT3 have been shown to be localized (Hosoyamada et al., 1999; Cha et al., 2001). The establishment and characteristics of S_2 human-OAT1 and S_2 human-OAT3 were previously described by us (Takeda et al., 2000). S_2 human-OAT4 was established as follows. The full-length cDNA of human-OAT4 (Cha et al., 2000) was subcloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA, USA), a mammalian expression vector. S_2 human-OAT4 was obtained by transfecting S_2 cells with pcDNA3.1-human-OAT4 coupled with pSV2neo, a neomycin-resistant gene using TfX-50 according to the manufacturer's instructions. S_2 cells transfected with pcDNA3.1 lacking an insert and pSV2neo were designated S_2 pcDNA 3.1 and used as a control. These cells were grown in a humidified incubator at 33 °C and under 5% CO_2 in RITC 80-7 medium containing 5% fetal bovine serum, 10 μg/ml transferrin, 0.08 U/ml insulin, 10 ng/ml recombinant epidermal growth factor and 400 μg/ml geneticin. The cells were subcultured in a medium containing 0.05% trypsin-EDTA solution (containing in mM: 137 NaCl, 5.4 KCl, 5.5 glucose, 4 $NaHCO_3$, 0.5 EDTA and 5 HEPES; pH 7.2) and used for 25–35 passages. Clonal cells were isolated using a cloning cylinder and screened by using the optimal substrate for human-OAT4, i.e., [^{3}H]estrone sulfate (Cha et al., 2000). S_2 human-OAT4 exhibited a dose- and time-dependent increase in the uptake of estrone sulfate, and the uptake was saturable. In addition, estrone sulfate and probenecid, an organic anion transport inhibitor, inhibited [^{3}H]estrone sulfate uptake by S_2 human-OAT4. The S_2 monolayer was determined to be leaky based on the results of a study in which we estimated the rate of paracellular secretion from cells cultured on a permeable support, using D-[^{3}H]mannitol as an indicator. In addition, vertical sections of S_2 human-OAT4 stained with polyclonal antibody against human-OAT4 showed that the subcellular localization of human-OAT4 protein was mainly on the cell membrane (unpublished observation). Both the basolateral and apical portions of the membrane showed positive staining. For the generation of the antibody against human-OAT4, rabbits were immunized with keyhole limpet hemocyanin-conjugated synthesized peptide, GNRQEAVTVESTSL, corresponding to cysteine and the 14 amino acid residues of the COOH terminus of human-OAT4. Therefore, the cells were cultured on a solid support for these experiments.

2.3. Uptake experiments

Uptake experiments were performed as previously described (Takeda et al., 1999). The S_2 cells were seeded in 24-well tissue culture plates at a cell density of 1×10^5 cells/well. After 2 days of culture, the cells were washed three times with Dulbecco's modified phosphate-buffered saline (D-PBS) solution (containing in mM: 137 NaCl, 3 KCl, 8 Na_2HPO_4, 1 KH_2PO_4, 1 $CaCl_2$ and 0.5 $MgCl_2$; pH 7.4) and then preincubated in the same solution for 10 min in a water bath at 37 °C. S_2 human-OAT1-expressing cells were incubated in D-PBS containing 5 μM [^{14}C]*para*-aminohippuric acid, whereas S_2 human-OAT3 and S_2 human-OAT4-expressing cells were incubated in D-PBS containing 50 nM [^{3}H]estrone sulfate. The uptake was terminated by adding ice-cold D-PBS, and the cells were washed three times with the same solution. The cells in each well were lysed with 0.5 ml of 0.1 N sodium hydroxide and 2.5 ml of aquasol-2, and the radioactivity was determined using a β-scintillation counter (Aloka, LSC-3100).

2.4. Kinetic analysis

After the preincubation as described above, S_2 human-OAT1, S_2 human-OAT3 and S_2 human-OAT4-expressing cells were incubated in D-PBS containing either [^{14}C]*para*-aminohippuric acid or [^{3}H]estrone sulfate at various concentrations in the absence or presence of various cephalosporin antibiotics for 2 min. Based on the organic anion uptake under each condition, double reciprocal plot analyses were performed as previously described (Apiwattanakul et al., 1999). When the inhibition was competitive, the inhibition constant (K_i) values were calculated based on the following equation:

$$K_i = \text{concentration of cephalosporin antibiotic} / [(K_m \text{ of } \textit{para}\text{-aminohippuric acid or estrone sulfate with cephalosporin antibiotic} / K_m \text{ of } \textit{para}\text{-aminohippuric acid or estrone sulfate without cephalosporin antibiotic}) - 1].$$

2.5. Efflux study

The efflux study was performed as previously described (Takeda et al. 1999). The S_2 human-hOAT4-expresing cells and control cells were seeded in 24-well tissue culture plates at a cell density of 1×10^5 cells/well. After the cells were cultured for 2 days, the cells were washed three times with D-PBS and then preincubated in the same solution for 10 min in a water bath at 37 °C. Thereafter, the monolayers were incubated with 100 nM [^{3}H]estrone sulfate for 30 min at 37 °C, washed immediately with D-PBS, and incubated at 37 °C in 500 μl of D-PBS. After the incubation for the indicated periods, 50 μl of supernatant was collected. After the incubation, the medium was aspirated immediately and the cell monolayers were washed three times with the medium and solubilized in 0.5 ml of 0.1 N sodium hydroxide. The radioactivity in the supernatant and in the cell lysate was measured. The rate of efflux at each time point was calculated using the following formula: (effluxed [^{3}H]estrone sulfate by S_2human-OAT4 − effluxed [^{3}H]estrone sulfate by control)/([^{3}H]estrone sulfate accumulated by S_2 human-OAT4 at time 0 − [^{3}H]estrone sulfate accumulated by control at time 0).

2.6. Statistical analysis

Data are expressed as means ± S.E. Statistical differences were determined using Student's unpaired *t*-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of various cephalosporin antibiotics on organic anion uptake by S_2 human-OAT1, S_2 human-OAT3 and S_2 human-OAT4

We elucidated the effects of various cephalosporin antibiotics on organic anion uptake mediated by human-OAT1, human-OAT3 and human-OAT4. The cephalosporin antibiotics tested were cephalothin, cefoperazone, cefazolin, ceftriaxone, cephaloridine, cefotaxime, cefadroxil and cefamandole. As shown in Fig. 1, all of these cephalosporin antibiotics at 2 mM significantly inhibited the organic anion uptake mediated by human-OAT1 (A), human-OAT3 (B) and human-OAT4 (C) ($N = 4$, * $P < 0.001$, ** $P < 0.01$ and *** $P < 0.05$ vs. control, respectively). The organic anion uptake mediated by each transfectant was about 16 times (human-OAT1), 37 times (human-OAT3) and 31 times (human-OAT4) higher than that by control cells not expressing the transporters. Thus, even if cephalosporin antibiotics exerted an inhibitory effects on the organic anion uptake by the control cells, it would be negligible. On the basis of these results, we did not perform inhibition experiments with the control cells. In addition, by exchanging the substrates, we also examined the effects of cefazolin and cephaloridine at 2 mM on human-OAT1-mediated estrone sulfate uptake and human-OAT3-mediated *para*-aminohippuric acid uptake. The inhibition rates of cefazolin and cephaloridine on human-OAT1-mediated estrone sulfate uptake were 66.5 ± 4.5% and 79.5 ± 8.6% of that of the control, respectively ($N = 4$), whereas those of cefazolin and cephaloridine on human-OAT3-mediated *para*-aminohippuric uptake were 28.0 ± 0.52% and 56.0 ± 3.3% of that of the control, respectively ($N = 4$).

3.2. Kinetic analysis of the inhibitory effects of various cephalosporin antibiotics on organic anion uptake by S_2 human-OAT1, S_2 human-OAT3 and S_2 human-OAT4

To further elucidate the inhibitory effects of various cephalosporin antibiotics on human-OAT1-, human-OAT3- and human-OAT4-mediated organic anion uptake, the kinetics of inhibition of *para*-aminohippuric acid uptake by S_2 human-OAT1, estrone sulfate uptake by S_2 human-OAT3 and estrone sulfate uptake by S_2 human-OAT4 for these cephalosporin antibiotics was analyzed. The organic anion uptake mediated by human-OAT1, human-OAT3 and

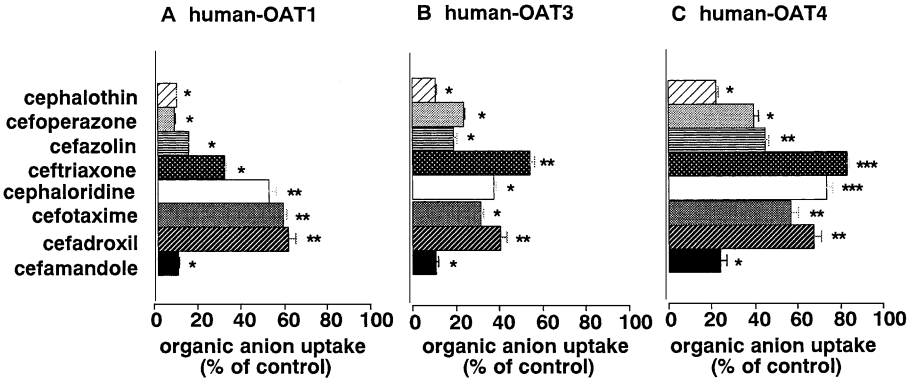

Fig. 1. Effects of various cephalosporin antibiotics on organic anion uptake by S_2 human-OAT1 (A), S_2 human-OAT3 (B) and S_2 human-OAT4 (C)-expressing cells. These cells were incubated with medium containing 5 μM [^{14}C] *para*-aminohippuric acid or 50 nM [^{3}H]estrone sulfate for 2 min in the absence or presence of various cephalosporin antibiotics at 2 mM. Each value represents the mean ± S.E. of four determinations. * $P < 0.001$, ** $P < 0.01$ and *** $P < 0.05$ vs. control, respectively.

Table 1
The K_i values of various cephalosphorin antibiotics for human-OAT1-, human-OAT3- and human-OAT4-mediated organic anion uptake

Drug	Human-OAT1		Human-OAT3		Human-OAT4	
	K_i value (μM)	inhibitor (mM)	K_i value (μM)	inhibitor (mM)	K_i value (μM)	inhibitor (mM)
cephalothin	0.22	0.5	0.04	0.1	0.20	0.5
cefoperazone	0.21	0.5	1.89	2	2.80	2
cefazolin	0.18	0.5	0.55	0.5	1.74	2
ceftriaxone	0.23	0.5	4.39	5	2.38	2
cephaloridine	0.74	2	2.46	2	3.63	2
cefotaxime	3.13	5	0.29	1	6.15	5
cefadroxil	6.14	5	8.62	5	N.D.[a]	5
cefamandole	0.03	0.1	0.046	0.1	1.14	2

S_2 human-OAT1, S_2 human-OAT3 and S_2 human-OAT4-expressing cells were incubated in a solution containing various concentrations of [^{14}C]*para*-aminohippuric acid or [^{3}H]estrone sulfate, in the absence or presence of various cephalosporin antibiotics. The K_i values were estimated from the Lineweaver–Burke plot.

[a] The effects of cefadroxil on human-OAT4-mediated organic anion uptake were not determined, because the inhibitory effects were weak.

human-OAT4 at various concentrations was determined in the absence or presence of various cephalosporin antibiotics. For the accurate estimation of K_i values shown in Table 1, when the inhibition rates for 2 mM cephalosporin antibiotics on organic anion uptake were less than 50% of that of the control as shown in Fig. 1, the effects of various concentrations of cephalosporin antibiotics on organic anion uptake were examined and the dose-dependent inhibitory effects were observed (data not shown). On the basis of these results, we used the concentration of various cephalosporin antibiotics at which the inhibition rate was 30–40% of that of the control as listed in Table 1, and performed an additional kinetic analysis. Fig. 2 shows the analysis of the Lineweaver–Burke plot of the effects of cephalothin on human-OAT1-(A), human-OAT3-(B) and human-OAT4-(C)-mediated organic anion uptake. The results revealed that the maximum velocity of the organic anion uptake (*y*-intercept) was not altered by cephalothin, whereas the K_m values (the slope) were increased by cephalothin. These results indicate that cephalothin competitively inhibited the organic anion uptake mediated by human-OAT1, human-OAT3 and human-OAT4. The mode of inhibition for the other drugs was also competitive (data not shown). The table shows the K_i values of cephalosporin antibiotics tested with human-OAT1, human-OAT3 and human-OAT4-expressing cells.

3.3. Efflux of estrone sulfate in S_2 human-OAT4

In order to determine whether human-OAT4 mediates the efflux of organic anion, we measured the efflux of estrone sulfate from S_2 human-OAT4-expressing cells and preincubated in a solution containing [^{3}H]estrone sulfate. As shown in Fig. 3, when the efflux from the control cells was subtracted, S_2 human-OAT4 mediated the efflux of estrone sulfate ($N=3$).

4. Discussion

Human-OAT1 and human-OAT3 were recently cloned and characterized as multispecific OATs and were found to mediate the active transport of organic anions from the interstitium to the cells in the basolateral membrane of the proximal tubule (Hosoyamada et al., 1999; Cha et al., 2001). Both transporters mediate the basolateral uptake of various drugs and endogenous substances such as nonsteroidal anti-inflammatory drugs, antitumor drugs, H_2-receptor antago-

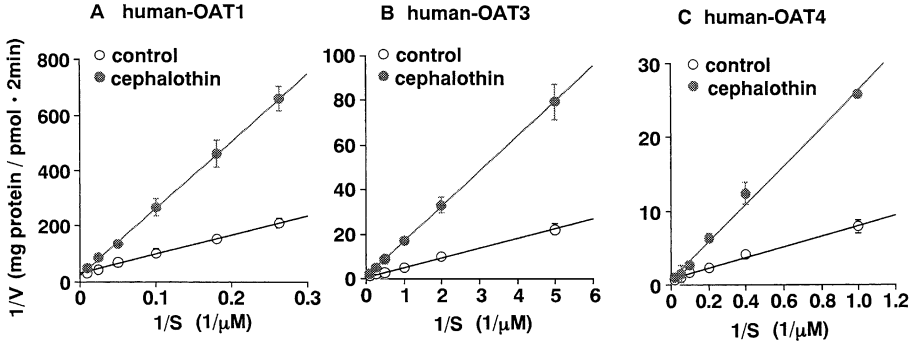

Fig. 2. Kinetic analysis of inhibition by cephalothin of organic anion uptake by S_2 human-OAT1, S_2 human-OAT3 and S_2 human-OAT4. Organic anion uptake was measured at various concentrations of [^{14}C]*para*-aminohippuric acid in S_2 human-OAT1 (A) and [^{3}H]estrone sulfate in S_2 human-OAT3 (B) and S_2 human-OAT4 (C) in the presence or absence of cephalothin at 0.5 mM for human-OAT1 and human-OAT4 and at 0.1 mM for human-OAT3 for 2 min at 37 °C. Lineweaver–Burke plot analyses were performed. Each value represents the mean ± S.E. of four determinations.

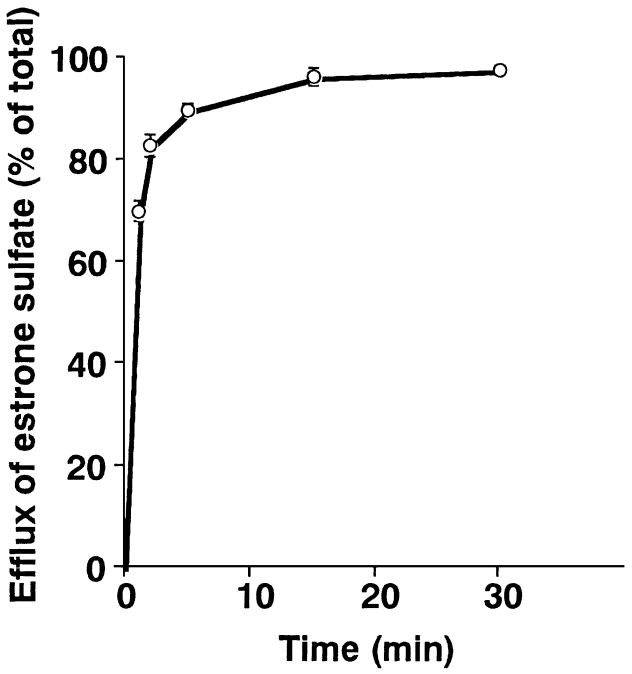

Fig. 3. Efflux of estrone sulfate by human-OAT4. After a 30-min incubation in solution containing 100 nM [^{3}H]estrone sulfate at 37 °C, S_2 human-OAT4-expressing and S_2 pcDNA 3.1 (control) cells were incubated at 37 °C for 30 min. The amount of [^{3}H]estrone sulfate in the supernatant and in the cell lysate was determined. The rate of efflux at each time point was calculated with the following formula: (effluxed [^{3}H]estrone sulfate by S_2 human-OAT4 − effluxed [^{3}H]estrone sulfate by control)/([^{3}H]estrone sulfate accumulated by S_2 human-OAT4 at time 0 − [^{3}H]estrone sulfate accumulated by control at time 0). Each value represents the mean ± S.E. of four determinations.

nists, prostaglandins, diuretics, angiotensin-converting enzyme inhibitors and beta-lactam antibiotics (Hosoyamada et al., 1999; Cha et al., 2001). Some differences in characteristics exist between human-OAT1 and human-OAT3, such as substrate specificity and localization: human-OAT1 is located on the basolateral side of the S_2 segment of the proximal tubule (Hosoyamada et al., 1999), whereas human-OAT3 is located on the first, second and third segments (S_1, S_2 and S_3, respectively) of the proximal tubule (Cha et al., 2001). In addition, human-OAT1, but not human-OAT3, exhibits transport properties as an exchanger (Hosoyamada et al., 1999; Cha et al., 2001). The human-OAT4 mediates the apical transport of various anionic drugs, but exhibits a relatively narrow substrate recognition spectrum compared with human-OAT1 and human-OAT3 (Cha et al., 2000; Babu et al., in press).

All of the cephalosporin antibiotics tested significantly inhibited organic anion uptake by S_2 human-OAT1, S_2 human-OAT3 and S_2 human-OAT4. These results suggest that human-OAT1, human-OAT3 and human-OAT4 interact with these drugs. These results are similar to those for these drugs on the uptake of *para*-aminohippuric acid in oocytes or cells expressing rat-OAT1 (Jariyawat et al., 1999; Takeda et al., 1999) and the uptake of estrone sulfate in oocytes or cells expressing rat-OAT3 (Kusuhara et al., 1999; Jung et al., in press).

The K_i values of cefoperazone, cefazolin, ceftriaxone and cephaloridine for human-OAT1-mediated organic anion uptake were much lower than those for human-OAT3-mediated organic anion uptake (more than threefold; Zhang et al., 1999), whereas those of cephalothin and cefotaxime for human-OAT3-mediated organic anion uptake were much lower than those for human-OAT1-mediated organic anion uptake. It is possible that human-OAT1 and human-OAT3 play a distinct role in the transport of various cephalosporin antibiotics in the basolateral membrane of the proximal tubule, although the level of expression of these transporters must also be considered. In addition, for rat-OATs, the K_i value of cephalothin for OAT3 was much lower than that for OAT1 (more than threefold), but those of cefoperazone, cephaloridine and cefazolin were comparable between OAT1 and OAT3 (within threefold; Jung et al., in press). Furthermore, the K_i value of cefazolin for human-OAT1 was much lower than that for rat-OAT1 (more than threefold, Jung et al., in press). These results suggest that there are some interspecies differences in the interactions of OATs with cephalosporin antibiotics.

As described above, the inhibitory effects of cefazolin and cephaloridine were attenuated when human-OAT1-mediated organic anion uptake was estimated using estrone sulfate and compared with that using *para*-aminohippuric acid as well as when human-OAT3-mediated organic anion uptake was estimated using *para*-aminohippuric acid and compared with that using estrone sulfate. The reason for this may be associated with the changes in the affinity of these transporters for substrates. *Para*-aminohippuric acid and estrone sulfate were identified as optimal substrates for human-OAT1 and human-OAT3 (Hosoyamada et al., 1999; Cha et al., 2001), the K_m values of which in transfectants were 15.8 and 7.5 μM, respectively (Takeda et al., 2000). In contrast, the K_m value of human-OAT1-mediated estrone sulfate uptake was 315.5 μM, whereas that of human-OAT3-mediated *para*-aminohippuric acid uptake was 45.0 μM (unpublished observation).

Cephaloridine is actively taken up via OATs in the basolateral membrane of the proximal tubule. However, cephaloridine is not readily transported across the apical membrane, which results in the accumulation of cephaloridine at high concentrations, leading to selective damage of the proximal tubular cells (Tune and Fernholt, 1973; Wold et al., 1979; Tune, 1997). Our present results show that the K_i value of cephaloridine for human-OAT4 was much lower than that for human-OAT1 (more than threefold; Zhang et al., 1999). In addition, we found that the human-OAT4 mediates the efflux of organic anions. Taking these results into consideration, it is possible that the human-OAT4 limits the cephaloridine efflux in the apical membrane of the proximal tubule. In contrast, cephalothin is thought to be also taken up via OATs, but is rapidly secreted from the

apical membrane (Tune et al., 1974; Tune, 1997). The resulting low and transient intracellular concentrations of cephalothin may explain its minimal toxicity to the kidney. Consistent with this, our present results show that the K_i values of cephalothin for human-OAT1 and human-OAT4 were low and comparable. Thus, it is suggested that the human-OAT4 plays an important role in determining the toxicity of cephalosporin antibiotics.

In conclusion, human-OAT1, human-OAT3 and human-OAT4 interacted with various cephalosporin antibiotics. These interactions may be associated with the transport of these cephalosporin antibiotics in the proximal tubule.

Acknowledgements

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