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European Journal of Pharmacology 466 (2003) 13–20
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Interactions of human organic anion as well as cation transporters with indoxyl sulfate

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Received 14 February 2003; accepted 21 February 2003

Abstract

Various uremic toxicants including indoxyl sulfate exert a number of biological effects on uremic patients. In order to elucidate the molecular mechanisms for the pharmacokinetics of indoxyl sulfate in human, we examined the interactions of human organic anion transporters (human-OATs) and human organic cation transporters (human-OCTs) with indoxyl sulfate using stable transfectants. Indoxyl sulfate inhibited human-OAT1, human-OAT3 and human-OAT4, but not human-OAT2, human-OCT1 and human-OCT2. Kinetic analysis revealed that the K_i values for human-OAT1, human-OAT3 and human-OAT4 were 22.7, 168.7 and 181.3 μM, respectively. Human-OAT1 and human-OAT3 mediated the uptake of indoxyl sulfate and human-OAT4 mediated not only the uptake but also the efflux of indoxyl sulfate. In conclusion, by comparing the K_i values with the plasma concentration of unbound indoxyl sulfate, it was predicted that human-OAT1 and human-OAT3 mediate the transport of indoxyl sulfate in vivo. In addition, it was suggested that human-OAT1 and human-OAT3 are involved in the urinary excretion of indoxyl sulfate, the exacerbation of renal dysfunction and the induction of uremic encephalopathy by indoxyl sulfate.

Keywords: Organic anion transporter; Organic cation transporter; Proximal tubule; Indoxyl sulfate; Uremia

1. Introduction

Uremia is characterized by a variety of symptoms including azotemia, overhydration, acidosis, anemia, bleeding tendency, hypertension, cardiac failure and neuropathy (Niwa, 1996). These symptoms are thought to be caused by the accumulation of uremic toxicants in the blood. We have previously identified indoxyl sulfate as an uremic toxicant derived from dietary protein. Indoxyl sulfate is metabolized by the liver from indole, which is produced from tryptophan by intestinal flora including *Escherichia coli*. Indoxyl sulfate is normally excreted into the urine primarily by proximal tubular secretion and partly by glomerular filtration (Niwa, 2001). Consistent with this, we have found that there is a marked elevation in the serum levels of indoxyl sulfate in 5/6-nephrectomized uremic rats and in uremic patients (Niwa and Ise, 1994; Niwa et al., 1997a). Administration of AST-120, an oral absorbent, decreased the intensity of indoxyl sulfate staining in the proximal tubules, as well as the serum and urinary concentrations of indoxyl sulfate, and prevented the progression of renal dysfunction in rats (Niwa et al., 1997a; Miyazaki et al., 2000). Thus, we have proposed that the increased serum concentration of uremic toxicants including indoxyl sulfate and the subsequent accumulation of those within the renal tubules in chronic renal failure may exacerbate the deterioration of renal function (Niwa and Ise, 1994; Niwa et al., 1997b). In addition, indoxyl sulfate has been reported to cause a number of biological effects on uremic patients including the inhibition of drug binding to serum albumin, erythropoiesis and lymphocyte blast formation (Sakai et al., 1995, 2001; Tsutsumi et al., 2000).

The secretion of numerous organic anions and cations, including endogenous metabolites, drugs and xenobiotics, is an important physiological function of the renal proximal

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doi:10.1016/S0014-2999(03)01530-9

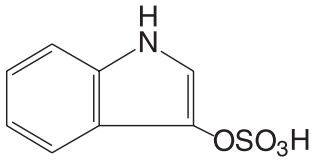

Fig. 1. Chemical structure of indoxyl sulfate.

tubule. The process of secreting organic anions and cations through the proximal tubule cells is achieved via unidirectional transcellular transport involving the uptake of organic anions and cations into the cells from the blood across the basolateral membrane, followed by extrusion across the brush-border membrane into the proximal tubule fluid (Pritchard and Miller, 1993). Recently, cDNAs encoding the human organic anion transporter (human-OAT) family have been successively cloned, including human-OAT1 (Reid et al., 1998; Hosoyamada et al., 1999), human-OAT2 (Enomoto et al., 2002a), human-OAT3 (Cha et al., 2001) and human-OAT4 (Cha et al., 2000). The human organic cation transporters (human-OCTs) isolated thus far are human-OCT1 (Gorboulev et al., 1997), human-OCT2 (Gorboulev et al., 1997) and human-OCT3 (Wu et al., 2000).

On the basis of its physicochemical properties, indoxyl sulfate possesses an anionic moiety (Fig. 1). Consistent with this, we have previously demonstrated that rat-OAT1 and rat-OAT3 mediate the uptake of indoxyl sulfate and the induction of nephrotoxicity using in vitro and in vivo models (Enomoto et al., 2002b). However, the molecular mechanisms for the pharmacokinetic handling of indoxyl sulfate in human, including renal excretion and tissue distribution, remain unclear. Thus, we examined the interactions of human-OATs and human-OCTs with indoxyl sulfate using proximal tubule cells stably expressing human-OAT1, human-OAT2, human-OAT3, human-OAT4, human-OCT1 and human-OCT2. We also investigated the interaction of indoxyl sulfate with rat-OAT2, a rodent counterpart of human-OAT2, which has been described to differ from human-OAT2 in its localization (Kojima et al., 2002).

2. Materials and methods

2.1. Materials

[^{14}C]*para*-aminohippuric acid (1.86 GBq/mmol), [^{3}H]prostaglandin $F_{2\alpha}$ (6808 GBq/mmol), [^{3}H]estrone sulfate (1961 GBq/mmol) and [^{14}C]tetraethylammonium (2.035 GBq/mmol) were purchased from Perkin Elmer (Boston, MA). Indoxyl sulfate and probenecid were obtained from Sigma (St. Louis, MO). Other materials used included fetal bovine serum, trypsin and geneticin from Invitrogen (Carlsbad, CA), 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). Pravastatin was a kind gift from Sankyo Pharmaceutical (Tokyo, Japan).

2.2. Cell culture

The second segment of the proximal tubule (S_2) cells was established by culturing the microdissected S_2 segment derived from transgenic mice harboring the temperature-sensitive simian virus 40 large T-antigen gene. The establishment and characterization of S_2 human-OAT1, S_2 human-OAT2, S_2 human-OAT3, S_2 human-OAT4, S_2 rat-OAT2, S_2 human-OCT1 and S_2 human-OCT2 were reported previously (Takeda et al., 2001, 2002; Kimura et al., 2002; Babu et al., 2002a,b; Enomoto et al., 2002a,b). Briefly, the full-length cDNAs of human-OAT1, human-OAT2, human-OAT3, human-OAT4, rat-OAT2, human-OCT1 and human-OCT2 were subcloned into pcDNA 3.1 (Invitrogen, Carlsbad, CA), a mammalian expression vector. S_2 human-OAT1, S_2 human-OAT2, S_2 human-OAT3, S_2 human-OAT4, S_2 rat-OAT2, S_2 human-OCT1 and S_2 human-OCT2 were obtained by transfecting S_2 cells with pcDNA3.1-human-OAT1, pcDNA3.1-human-OAT2, pcDNA3.1-human-OAT3, pcDNA3.1-human-OAT4, pcDNA3.1-rat-OAT2, pcDNA3.1-human-OCT1 and pcDNA3.1-human-OCT2, using TfX-50 according to the manufacturer's instructions. S_2 cells transfected with pcDNA3.1 lacking an insert were designated as S_2 pcDNA 3.1 (mock), and used as control. These cells were grown in a humidified incubator at 33 °C and under 5% CO_2 using RITC 80-7 medium containing 5% fetal bovine serum, 10 mg/ml transferrin, 0.08 U/ml insulin, 10 ng/ml recombinant epidermal growth factor and 400 mg/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 determining the optimal substrate for each transporter, i.e., [^{14}C]*para*-aminohippuric acid for human-OAT1 (Hosoyamada et al., 1999), [^{3}H]prostaglandin $F_{2\alpha}$ for human-OAT2 and rat-OAT2 (Enomoto et al., 2002a), [^{3}H]estrone sulfate for human-OAT3 and human-OAT4 (Cha et al., 2000, 2001) and [^{3}H]tetraethylammonium for human-OCT1 and human-OCT2 (Gorboulev et al., 1997; Kimura et al., 2002).

2.3. Uptake experiments

Uptake experiments were performed as previously described (Takeda et al., 2001, 2002; Kimura et al., 2002; Babu et al., 2002a,b; Enomoto et al., 2002a,b). The S_2 cells were seeded in 24-well tissue culture plates at a density of 1×10^5 cells/well. After the cells were cultured for 2 days, the cells were washed three times with Dulbecco's modified

phosphate-buffered saline (D-PBS) solution (containing in mM: 137 NaCl, 3 KCl, 8 $NaHPO_4$, 1 KH_2PO_4, 1 $CaCl_2$ and 0.5 $MgCl_2$; pH 7.4) and then preincubated in the same solution in a water bath at 37 °C for 10 min. The cells were then incubated in a solution at 37 °C for the indicated period of time. The uptake was stopped by the addition of 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 radioactivity was determined using a β-scintillation counter (LSC-3100, Aloka, Tokyo, Japan).

2.4. Inhibition study

After preincubation as described above, S_2 human-OAT1, S_2 human-OAT2, S_2 human-OAT3, S_2 human-OAT4, S_2 rat-OAT2, S_2 human-OCT1 and S_2 human-OCT2 were incubated in a solution containing either [^{14}C]*para*-aminohippuric acid for 2 min (human-OAT1), [^{3}H]prostaglandin $F_{2\alpha}$ for 20 s (human-OAT2) and for 2 min (rat-OAT2), [^{3}H]estrone sulfate for 2 min (human-OAT3 and human-OAT4) or [^{3}H]tetraethylammonium for 5 min (human-OCT1 and human-OCT2) in the absence or presence of various concentrations of indoxyl sulfate at 37 °C.

2.5. Kinetic analysis

After preincubation as described above, S_2 human-OAT1, S_2 human-OAT3 and S_2 human-OAT4 were incubated in a solution containing 5 or 10 μM [^{14}C]*para*-aminohippuric acid (human-OAT1) or 50 or 100 nM [^{3}H]estrone sulfate (human-OAT3 and human-OAT4) in the absence or presence of 100 and 200 μM indoxyl sulfate at 37 °C for 2 min. Analyses of Dixon plots were performed.

2.6. Measurement of intracellular indoxyl sulfate content

Measurement of intracellular indoxyl sulfate content was performed as previously described by us (Enomoto et al., 2002b). S_2 cells were seeded in 24-well tissue culture plates at a cell density of 1×10^5 cells/well, cultured and preincubated as described above. Then, the cells were incubated in a solution containing 100 μM indoxyl sulfate in the absence or presence of 1mM probenecid or 1mM pravastatin at 37 °C for 0.5, 1, 2.5, 5, 10 or 15 min. After washing the cells with indoxyl-sulfate-free D-PBS, the intracellular concentrations of indoxyl sulfate were determined by high-performance liquid chromatography (HPLC) (Niwa et al., 1988).

2.7. Efflux study

The efflux study was performed as previously described (Takeda et al., 2002; Babu et al., 2002a,b). The S_2 human-hOAT4 and mock 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 500 μM indoxyl sulfate for 30 min at 37 °C, washed immediately with D-PBS and incubated at 37 °C in 500 μl of D-PBS. After incubation for the indicated periods, 50 μl of supernatant was collected. After the

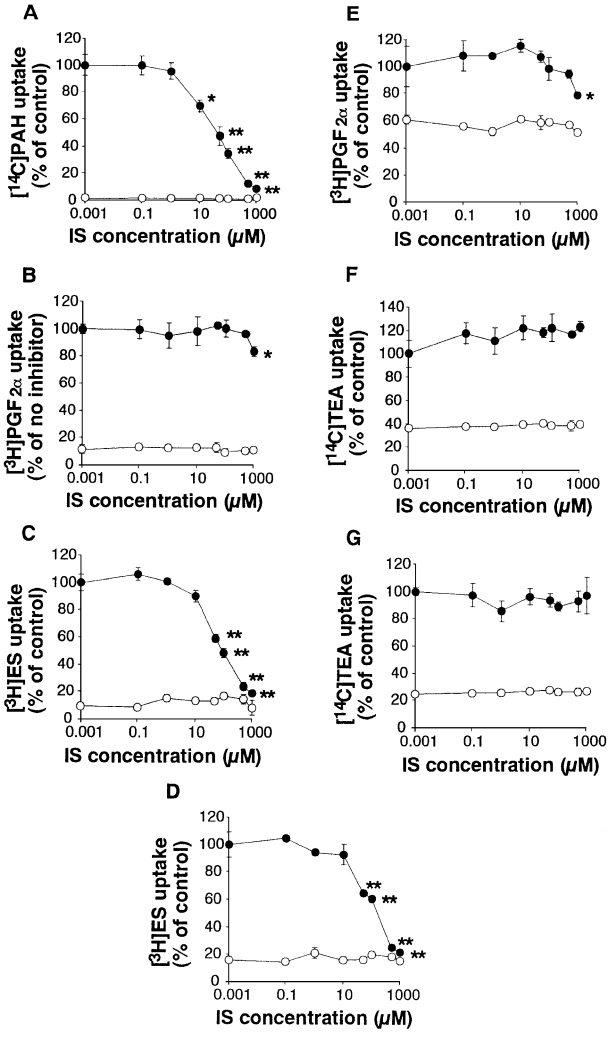

Fig. 2. Effects of various concentrations of indoxyl sulfate (IS) on the organic anion uptake mediated by human-OATs and rat-OAT2, and the organic cation uptake mediated by human-OCTs. S_2 human-OAT1 (A), S_2 human-OAT2 (B), S_2 human-OAT3 (C), S_2 human-OAT4 (D), S_2 rat-OAT2 (E), S_2 human-OCT1 (F), S_2 human-OCT2 (G) and mock were incubated in solution containing 5 μM [^{14}C]*para*-aminohippuric acid (PAH; human-OAT1), 50 nM [^{3}H]prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (human-OAT2 and rat-OAT2), 50 nM [^{3}H]estrone sulfate (ES; human-OAT3 and human-OAT4) or 5 μM [^{14}C]tetraethylammonium (TEA; human-OCT1 and human-OCT2) in the presence of various concentrations of IS at 37 °C for 2 min (human-OAT1, human-OAT3, human-OAT4 and rat-OAT2), 20 s (human-OAT2) or 5 min (human-OCT1 and human-OCT2). Closed circles: OATs or OCTs; open circle: mock. Each value represents the mean ± S.E. of six monolayers from two separate experiments. *$P<0.05$ and **$P<0.01$ vs. control.

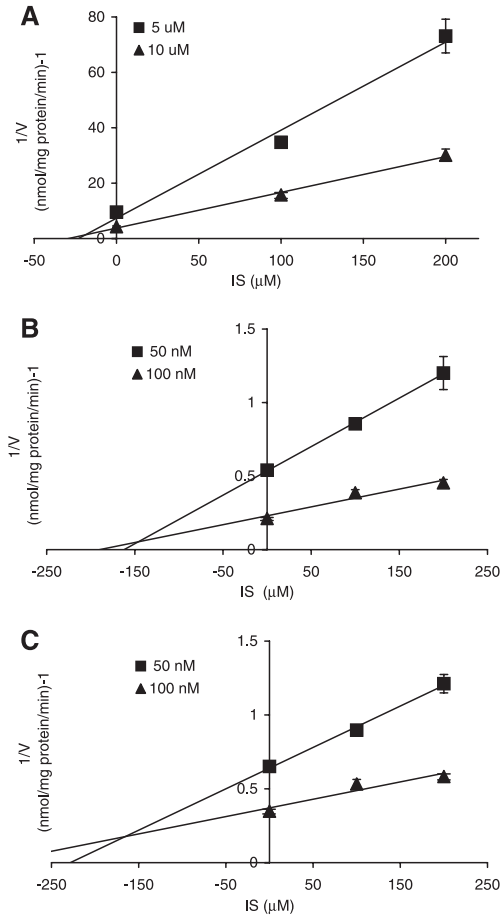

Fig. 3. Kinetic analyses of the effects of indoxyl sulfate (IS) on organic anion uptake mediated by human-OATs. S_2 human-OAT1, S_2 human-OAT3 and S_2 human-OAT4 were incubated in solution containing 5 or 10 μM [^{14}C]*para*-aminohippuric acid (human-OAT1) or 50 or 100 nM of [^{3}H]estrone sulfate (human-OAT3 and human-OAT4) in the absence or presence of 100 and 200 μM IS at 37 °C for 2 min. Analyses of Dixon plots were performed. (A) human-OAT1, (B) human-OAT3 and (C) human-OAT4. Each value represents the mean ± S.E. of six monolayers from two separate experiments.

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 amount of indoxyl sulfate 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 indoxyl sulfate by S_2 human-OAT4 − effluxed indoxyl sulfate by mock)/(indoxyl sulfate accumulated by S_2 human-OAT4 at time 0 − indoxyl sulfate accumulated by mock at time 0).

2.8. Statistical analysis

Data are expressed as means ± S.E. Statistical differences were determined using one-way analysis of variance with Dunnett's post-hoc test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Effects of indoxyl sulfate on organic anion uptake mediated by human-OATs and rat-OAT2, and organic cation uptake mediated by human-OCTs

We examined the inhibitory effects of various concentrations of indoxyl sulfate on the organic anion uptake mediated by human-OAT1, human-OAT2, human-OAT3, human-OAT4 and rat-OAT2, and the organic cation uptake mediated by human-OCT1 and human-OCT2. As shown in Fig. 2, indoxyl sulfate dose-dependently inhibited the organic anion uptake mediated by human-OAT1 (A), human-OAT3 (C) and human-OAT4 (D), whereas it inhibited the organic anion uptake by human-OAT2 (B) and rat-OAT2 (E) at 1 mM only (*$P < 0.05$ and **$P < 0.01$ vs. control). In contrast, indoxyl sulfate did not inhibit the organic cation uptake mediated by human-OCT1 (F) and human-OCT2 (G) (NS).

3.2. Kinetic analysis of the effects of indoxyl sulfate on organic anion uptake mediated by human-OATs

In order to further elucidate the inhibitory effects of indoxyl sulfate on the organic anion uptake mediated by human-OAT1, human-OAT3 and human-OAT4, we performed the analyses of Dixon plots. As shown in Fig. 3, indoxyl sulfate competitively inhibited the organic anion transport mediated by human-OAT1 (A), human-OAT3 (B) and human-OAT4 (C). The K_i values of indoxyl sulfate for human-OAT1, human-OAT3 and human-OAT4 are listed in Table 1.

Table 1
Kinetic parameters of indoxyl sulfate for OATs

	K_i	IC_{50}	K_m
μM			
Human-OAT1		83.0 ± 5.00[a]	
	22.7 ± 8.67	47.3 ± 2.19	
Rat-OAT1	34.2[b]	50.2 ± 1.56[b]	
Human-OAT3	168.7 ± 32.6	80.4 ± 12.4	
Rat-OAT3	74.4[b]	61.6 ± 2.83[b]	136.7 ± 32.7
			158 ± 0.1[c]
Human-OAT4	181.3 ± 37.6	188 ± 27.7	

For K_i experiments, S_2 human-OAT1, S_2 human-OAT3 and S_2 human-OAT4 were incubated in a solution containing various concentrations of [^{14}C]*para*-aminohippuric acid (human-OAT1) or [^{3}H]estrone sulfate (human-OAT3 and human-OAT4) in the absence or presence of 500 μM indoxyl sulfate at 37 °C for 2 min. For IC_{50} experiments, S_2 human-OAT1, S_2 human-OAT3 and S_2 human-OAT4 were incubated in a solution containing 5 μM [^{14}C]*para*-aminohippuric acid (human-OAT1) or 50 nM [^{3}H]estrone sulfate (human-OAT3 and human-OAT4) in the absence or presence of various concentrations of indoxyl sulfate at 37 °C for 2 min. Each value represents the mean ± S.E. from six monolayers from two separate experiments.

[a] Motojima et al. (2002).
[b] Enomoto et al. (2002b).
[c] Deguchi et al. (2002).

3.3. Indoxyl sulfate uptake mediated by human-OATs

In order to determine whether hOATs mediate the uptake of indoxyl sulfate, we evaluated the uptake activities of indoxyl sulfate by hOATs by HPLC. The uptake rates of indoxyl sulfate by human-OAT1 (A), human-OAT3 (B) and human-OAT4 (C) were significantly higher than those by mock (Fig. 4, $*P<0.01$ vs. mock). In addition, we examined the effects of organic anion transport inhibitors, probenecid and pravastatin (Takeda et al., 2001; Hasegawa et al., 2002; Enomoto et al., 2002a) on indoxyl sulfate uptake by human-OATs. As shown in Fig. 4, probenecid and pravastatin significantly inhibited indoxyl sulfate uptake mediated by human-OAT1 (A), human-OAT3 (B) and human-OAT4 (C) ($**P<0.01$ vs. human-OATs).

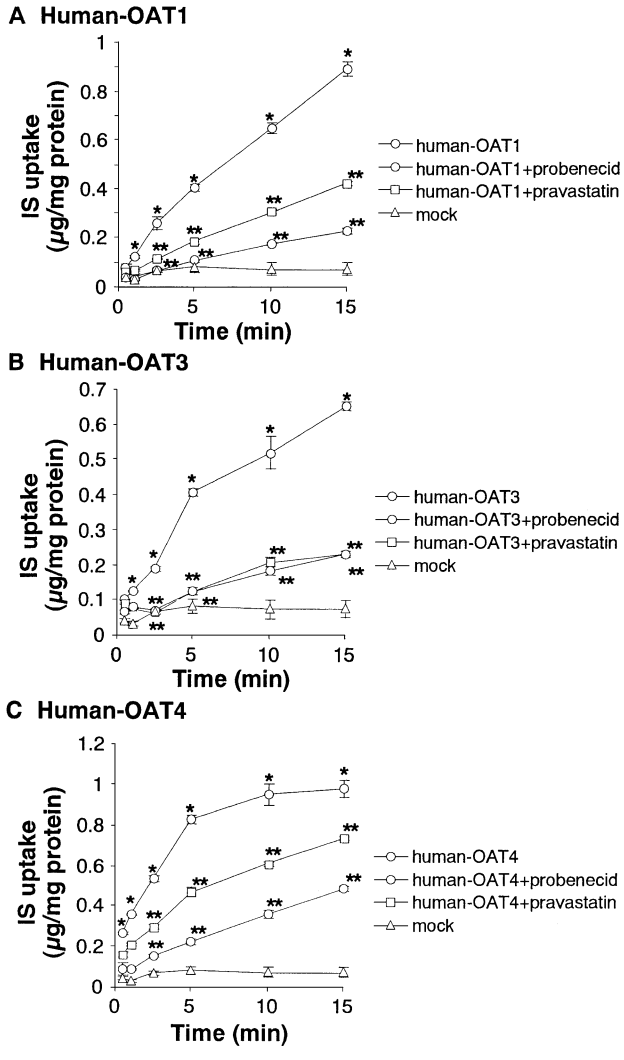

Fig. 4. Indoxyl sulfate (IS) uptake by human-OATs. S_2 human-OAT1, S_2 human-OAT3, S_2 human-OAT4 and mock were incubated in solution containing at 37 °C for 0.5, 1, 2.5, 5, 10 or 15 min. Each value represents the mean ± S.E. of six monolayers from two separate experiments. $*P<0.01$ vs. mock and $**P<0.01$ between human-OATs and human-OATs in the presence of probenecid or pravastatin.

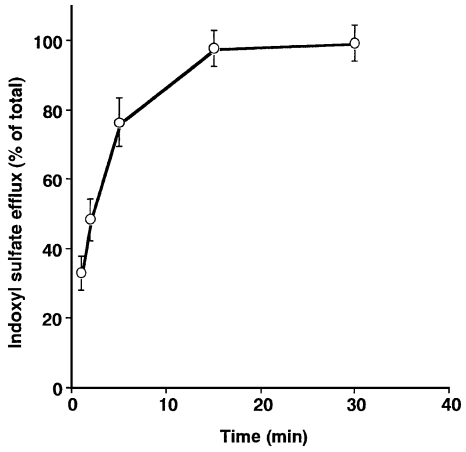

Fig. 5. Efflux of indoxyl sulfate by human-OAT4. After a 30-min incubation in solution containing 500 μM indoxyl sulfate at 37 °C, S_2 human-OAT4 and mock were incubated at 37 °C for 30 min. The amount of indoxyl sulfate in the supernatant and in the cell lysate was determined. The rate of efflux at each time point was calculated with the following formula (effluxed indoxyl sulfate by S_2 human-OAT4 − effluxed indoxyl sulfate by mock)/(indoxyl sulfate accumulated by S_2 human-OAT4 at time 0 − indoxyl sulfate accumulated by mock at time 0). Each value represents the mean ± S.E. of four determinations from one typical experiment of two separate experiments.

3.4. Efflux of indoxyl sulfate in S_2 human-OAT4

In order to determine whether human-OAT4 mediates the efflux of indoxyl sulfate, we measured the efflux of indoxyl sulfate from S_2 human-OAT4 and mock preincubated in a solution containing indoxyl sulfate. As shown in Fig. 5, when the efflux from mock was subtracted, S_2 human-OAT4 mediated the efflux of indoxyl sulfate ($N=4$).

4. Discussion

Human-OAT1 and human-OAT3 have been shown to mediate the transport of nonsteroidal antiinflammatory drugs, antitumor drugs, histamine H_2-receptor antagonist, prostaglandins, diuretics, angiotensin-converting enzyme inhibitors and β-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 within the proximal tubule (Hosoyamada et al., 1999; 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). Human-OAT2, also shown to be localized at the basolateral side of the proximal tubule, mediates the transport of organic anions including salicylate and prostaglandin $F_{2\alpha}$ (Enomoto et al., 2002a). In contrast to human-OAT2, rat-OAT2 is localized at the apical side of the medullary thick ascending limb of Henle's loop (Kojima et al., 2002) and mediates the transport of prostglandin $F_{2\alpha}$ with similar affinity to human-OAT2 (unpublished observa-

tion). In addition, rat-OAT2 also mediates the transport of salicylate, α-ketoglutarate, prostaglandin E_2, methotrexate, acetylsalicylate and *para*-aminohippuric acid (Sekine et al., 1998). Human-OAT4 also mediates the apical transport of various anionic drugs (Babu et al., 2002a,b); however, this transporter exhibits relatively narrow substrate recognition compared to human-OAT1 and human-OAT3 (Cha et al., 2000). Human-OCT1 is localized at the liver but not the kidney and mediates the polyspecific pH-independent transport of organic cations (Gorboulev et al., 1997; Motohashi et al., 2002). In contrast, human-OCT2 is mainly localized at the basolateral side of the proximal tubule and mediates pH-independent, electrogenic and polyspecific transport of organic cations (Gorboulev et al., 1997; Motohashi et al., 2002). Using stable cell lines, we elucidated the interactions of human-OATs, rat-OAT2 and human-OCTs with indoxyl sulfate.

Human-OAT1, human-OAT3 and human-OAT4 interacted with indoxyl sulfate, consistent with the previous report that indoxyl sulfate inhibited *para*-aminohippuric acid transport by human-OAT1 (Motojima et al., 2002). On the other hand, although we have previously demonstrated that anionic compounds such as prostaglandin E_2 and prostaglandin $F_{2\alpha}$ are transported by human-OCT1 and human-OCT2 (Kimura et al., 2002), human-OCT1 and human-OCT2 did not interact with indoxyl sulfate.

The serum concentration of indoxyl sulfate in hemodialysis patients was reported to be approximately 249 μM (Niwa et al., 1994) and approximately 90% of indoxyl sulfate is bound to albumin (Niwa et al., 1991). Therefore, the serum concentration of unbound indoxyl sulfate in uremic patients is thought to be approximately 25 μM. As shown in Table 1, human-OAT1 and human-OAT3 interacted with indoxyl sulfate with K_i values of 22.7 and 168.7 μM, respectively. In addition, human-OAT1 and human-OAT3 mediated the transport of indoxyl sulfate, which was inhibited by organic anion transport inhibitors. Thus, it is predicted that human-OAT1 and human-OAT3 mediate the transport of indoxyl sulfate in vivo, leading to the urinary excretion of indoxyl sulfate or the accumulation of indoxyl sulfate associated with the exacerbation of renal dysfunction.

Human-OAT4 mediated the uptake and the efflux of indoxyl sulfate. The results are consistent with the previous report that human-OAT4 mediates not only the uptake, but also the efflux of estrone sulfate, ochratoxin A and tetracycline (Takeda et al., 2002; Babu et al., 2002a,b). It is suggested that human-OAT4 mediates the efflux and the reabsorption of indoxyl sulfate in the apical side of the proximal tubule.

In uremia, various forms of neurological disorders, such as uremic encephalopathy occur (Moe and Sprague, 1994). It was reported that increased concentrations of indoxyl sulfate in cerebrospinal fluid and serum were observed in uremic patients (Muting, 1965). In addition, indoxyl sulfate concentration in the cerebrospinal fluid of uremic patients with coma was about twofold higher than that of uremic patients without coma. In this regard, we found that mRNA for human-OAT1 and human-OAT3 are expressed in the brain (Hosoyamada et al., 1999; Cha et al., 2001), and recently found that human-OAT1 and human-OAT3 are localized at choroid plexus epithelial cells immunohistochemically (unpublished observation). In the current study, we found that human-OAT1 and human-OAT3 mediate the transport of indoxyl sulfate. Based on these lines of evidence, it is possible that human-OAT1 and human-OAT3 mediate the efflux of indoxyl sulfate from the cerebrospinal fluid across the blood-cerebrospinal fluid barrier, which may be associated with the induction of uremic encephalopathy.

Although perinatal outcome in patients undergoing chronic hemodialysis has exhibited improvement, it was reported that when the period of dialysis before pregnancy is shorter, the infant's chances of survival are higher (Nakabayashi et al., 1999). In this regard, human-OAT4 was shown to be localized at the placenta using northern blot analysis (Cha et al., 2000). In the current study, we found that human-OAT4 mediated the transport of indoxyl sulfate. Thus, although precise immunohistochemical analysis of human-OAT4 in the human placenta should be performed, it is possible that human-OAT4 mediates the delivery of indoxyl sulfate into the fetus, thereby impairing the infant's chances of survival.

In uremic patients, "uremic myopathy" including muscle weakness and muscle atrophy occurs (Quintanilla and Sahgal, 1984). In this regard, human-OAT3 was shown to be expressed in the skeletal muscle using northern blot analysis (Cha et al., 2001). In the current study, we found that human-OAT3 mediated the transport of indoxyl sulfate. Although precise immunohistochemical analysis should be performed, it is possible that human-OAT3 is involved in the accumulation of indoxyl sulfate within the skeletal muscle, which may be associated with the induction of uremic myopathy.

Indoxyl sulfate uptake by rat-OAT1 and rat-OAT3 has already been reported by us (Enomoto et al., 2002b) and Deguchi et al. (2002). We also determined the K_m value for rat-OAT3-mediated indoxyl sulfate uptake to be 136.7 ± 32.7 μM. The value was consistent with that of Deguchi et al. (2002), however, it was twice as much as K_i and IC_{50}. One possible explanation that accounts for this discrepancy is that there may be several substrate recognition sites in the rat-OAT3. By comparing the kinetic parameters of indoxyl sulfate for OAT1 and OAT3 between human and rat as shown in Table 1, we found that there was no significant difference in the interactions of OAT1 and OAT3 with indoxyl sulfate between human and rat (within threefold difference; Zhang et al., 1998). In addition, as shown in Fig. 2, the profile of the inhibition effects of indoxyl sulfate on the organic anion uptake mediated by human-OAT2 as well as rat-OAT2 are similar. Based on these, there appears to be no interspecies difference in the interaction of OATs with indoxyl sulfate between human and rat. Studies have been and will be performed to clarify the role of indoxyl sulfate in the

exacerbation of renal dysfunction and uremic symptoms. In this regard, the current experimental results provide useful information for extrapolating the results of experiments using rats into humans.

So far, as toxic anion metabolites, it was reported that 3-carboxy-4-methyl-5-propyl-2-furanpropionic acid also inhibits *para*-aminohippuric acid uptake in rat kidney slices (Henderson and Lindup, 1992). In addition, hippuric acid, *p*-hydroxyhippuric acid, *o*-hydroxyhippuric acid, indoleacetic acid, *p*-hydroxyphenylacetic acid and uric acid were shown to inhibit organic anion transport mediated by human-OAT1 (Motojima et al., 2002). Among them, the IC_{50} values for hippuric acid, *p*-hydroxyhippuric acid and *o*-hydroxyhippuric acid were reported to be 20, 25 and 27 μM, respectively. Since the affinities of these uremic toxicants for human-OAT1 are similar to that of indoxyl sulfate, it is possible that they compete for indoxyl sulfate transport mediated by human-OAT1 in uremia.

In conclusion, by comparing the kinetic parameters with the plasma concentration of unbound indoxyl sulfate in uremic patients, it was predicted that human-OAT1 and human-OAT3 mediate the transport of indoxyl sulfate in vivo. In addition, the current results suggest that human-OAT1 and human-OAT3 are involved in the urinary excretion of indoxyl sulfate, the exacerbation of renal dysfunction and the induction of uremic encephalopathy by indoxyl sulfate. Furthermore, there seems to be no interspecies difference in the interactions of OATs with indoxyl sulfate between human and rat.

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