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Major role of organic anion transporter 3 in the transport of indoxyl sulfate in the kidney

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Major role of organic anion transporter 3 in the transport of indoxyl sulfate in the kidney.

Background. Indoxyl sulfate is a uremic toxin that accumulates in the body because of the patient's inability to excrete it and it induces a number of uremic symptoms and leads to chronic renal failure. The functional failure of the excretion system for indoxyl sulfate causes its accumulation in blood. The purpose of the present study was to characterize the transport mechanism responsible for the renal excretion of indoxyl sulfate.

Methods. The [^{3}H]indoxyl sulfate transport mechanism was investigated using an in vivo tissue-sampling single-injection technique, the kidney uptake index (KUI) method. Rat organic anion transporter 3 (rOAT3)-expressing *Xenopus laevis* oocyte system was used for measuring [^{3}H]indoxyl sulfate uptake activity.

Results. Probenecid showed a concentration-dependent inhibitory effect on the uptake of [^{3}H]indoxyl sulfate using the KUI method, and uptake was inhibited by organic anions such as *para*-aminohippuric acid (PAH) and benzylpenicillin, by weak base such as cimetidine, and by uremic toxins, such as 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) and hippuric acid (HA). However, salicylic acid, indomethacin, 3,5,3′-triiodo-L-thyronine and indole acetic acid (IA) had no effect on the uptake. rOAT3-expressing oocytes exhibited uptake of [^{3}H]indoxyl sulfate by rOAT3 (K_m = 158 μmol/L). Moreover, a number of uremic toxins inhibited the uptake of [^{3}H]indoxyl sulfate by rOAT3.

Conclusions. These results suggest that rOAT3 is responsible for the renal uptake of indoxyl sulfate, and uremic toxins share the transport mechanism for indoxyl sulfate. Mutual inhibition of these uremic toxins via OAT3 may accelerate their accumulation in the body and, thereby, the progression of nephrotoxicity in uremia.

Serum indoxyl sulfate, a uremic toxin, is markedly increased in uremic patients [1, 2]. Its accumulation in the serum causes a number of pharmacological effects, such as inhibition of drug binding to serum albumin, erythropoiesis, and lymphocyte blast formation [3–6]. In particular, indoxyl sulfate is known to be the circulating substance that stimulates the progression of chronic renal failure [7, 8]. Oral administration of indoxyl sulfate to uremic rats stimulates the progression of glomerular sclerosis accompanied by a reduction in renal function [8]. Furthermore, oral administration of indoxyl sulfate to 5/6-nephrectomized rats promoted the progression of renal sclerosis associated with the increased gene expression of transforming growth factor (TGF)-β1, tissue inhibitor of metalloproteinase (TIMP)-1, and proα1(I) collagen mRNA in the renal cortex [9, 10]. The induction of these genes may contribute to renal sclerosis.

Indoxyl sulfate is mainly excreted via the kidney, but since uremic patients cannot efficiently excrete it in the urine, it accumulates in the body [2, 7]. Glomerular filtration of indoxyl sulfate is considered to be minimal because more than 96% is bound to albumin [11]. As far as transport in the kidney is concerned, indoxyl sulfate produces significant inhibition of *para*-aminohippuric acid (PAH) uptake by isolated renal tubules [12] and reduces the renal clearance of PAH [7, 8]. Thus, excretion of indoxyl sulfate into urine is thought to be mainly by tubular secretion, presumably by an organic anion transport system [12].

Rat renal organic anion transporter 1 (rOAT1) was isolated as the PAH transporter [13–15]. rOAT1 is expressed predominantly in the kidney and is localized on the basolateral membrane of the middle proximal tubules (S2) [16]. Subsequently two isoforms, named rOAT2 and rOAT3, have been identified [17, 18]. These isoforms in the kidney are believed to be located on the basolateral membrane [19]. As three OAT isoforms mediate the transport of many organic anions, including endogenous metabolites, drugs and xenobiotics, it is considered to be the most important physiological process involved in the basolateral uptake of organic anions in

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renal epithelial cells [20]. However, it is still not known which organic anion transporter is involved in indoxyl sulfate transport in the kidney.

To clarify the organic anion transporter involved in the transport of indoxyl sulfate is important to analyze the nephrotoxicity and uremic symptoms induced by indoxyl sulfate, since accumulation of indoxyl sulfate in serum occurs due to inhibition or failure of the transport activity of the organic anion transporter. Furthermore, inhibition of the transporter by indoxyl sulfate will lead to inhibition of the active tubular secretion of many other exogenous and endogenous organic acids that are transported by the same transporter as indoxyl sulfate. This inhibition may extend to other sites of organic acid transport, such as those in brain and liver, and induce many of the symptoms of uremia.

In the present study, we investigated the in vivo transport mechanism for indoxyl sulfate into the kidney using the KUI method. Moreover, to estimate the involvement of rOAT3 in the uptake transport of indoxyl sulfate into the kidney, we analyzed the uptake characteristics of indoxyl sulfate using *Xenopus* oocytes injected with cRNA derived from rOAT3.

METHODS

Animals

Adult male Wistar rats, weighing 230 to 280 g, were purchased from Charles River Laboratories (Yokohama, Japan). Mature female *Xenopus laevis* were purchased from Hamamatsu Kyozai (Hamamatsu, Japan) and maintained in a controlled environment as described by Goldin [21]. All experiments were approved by the Animal Care Committee, Graduate School of Pharmaceutical Sciences, Tohoku University.

Reagents

pGEM-HEN was a kind gift from Dr. T. Abe (Tohoku University, Japan). N-[1-^{14}C]butanol (1 mCi/mmol) was obtained from American Radiolabeled Chemicals, (St. Louis, MO, USA). [ring-^{3}H]Indoxyl sulfate ([^{3}H]Indoxyl sulfate, 6.5 Ci/mmol) was synthesized and purified by NEN Life Sciences (Boston, MA, USA). PAH, probenecid, salicylic acid, benzylpenicillin potassium salt, cimetidine, indomethacin, 2-hydroxycinnamic acid, 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, and quinolinic acid were obtained from Wako Pure Chemical (Osaka, Japan). Hippuric acid sodium salt was purchased from Nacalai Tesque Inc. (Kyoto, Japan). 3,5,3′-Triiodo-L-thyronine was purchased from Tokyo Kasei (Tokyo, Japan). Indoxyl sulfate potassium salt, estrone sulfate, indole acetic acid, and xylazine hydrochloride were purchased from Sigma Chemical (St. Louis, MO, USA). Ketamine hydrochloride was obtained from Sankyo Co. (Tokyo, Japan). Tissue-solubilizing reagent, Soluene-350, and scintillation cocktail, Hionic-fluor were purchased from Packard Co. (Meriden, CT, USA). 3-Carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) was synthesized as previously described [4]. All other chemicals were of analytical grade and used without further purification.

Kidney uptake index (KUI) study

An abdominal aorta injection technique to obtain a kidney uptake index (KUI) was performed as described in previous reports [22–24]. Briefly, rats were placed in the supine position and a laparotomy carried out after the animals had been anesthetized with an intramuscular injection of a mixture of ketamine (235 mg/kg) and xylazine (2.3 mg/kg). The upper part of the abdominal aorta was isolated, and the origin of the left renal artery was identified. The aorta was cannulated with a 27-gauge needle adjacent to the site of the origin of the left renal artery. About 300 μL buffered Ringer's solution (10 mmol/L HEPES, 141 mmol/L NaCl, 4 mmol/L KCl, and 2.8 mmol/L $CaCl_2$, pH 7.4) containing both 5 μCi/mL (0.78 μmol/L) [^{3}H]indoxyl sulfate and 0.5 μCi/mL [^{14}C]butanol (a freely diffusible reference) was rapidly injected (<1 sec). At 5 seconds after injection, the entire left kidney was removed. This kidney was minced and about 200 mg tissue was solubilized in 2.0 mL Soluene-350 at 55°C for three hours. After the addition of 300 μL hydrogen peroxide, 10 mL Hionic-flour was added and then the radioactivity was measured by double-isotope liquid scintillation counting in a liquid scintillation counter (LSC-5000; Aloka, Tokyo, Japan). The radioactivity of the injected solution was measured simultaneously.

Determination of KUI for the kidney

The KUI was defined by equation (1) and determined using equation (2) [22–24].

$$KUI = E_T/E_R \quad \text{(Eq. 1)}$$

$$KUI\ (\%) = \frac{(^{3}H/^{14}C\ dpm)\ \text{in kidney}}{(^{3}H/^{14}C\ dpm)\ \text{in injection solution}} \times 100 \quad \text{(Eq. 2)}$$

where E_T and E_R are the extraction of the test ([^{3}H]indoxyl sulfate) and reference ([^{14}C]butanol) compounds, respectively. E_T can be estimated when KUI and E_R are determined experimentally. Since the E_R value of [^{14}C]butanol has been reported as 60.4% for the kidney [22], the following equation is valid:

$$E_T = 0.604 \times (KUI) \quad \text{(Eq. 3)}$$

The apparent extraction of [^{3}H]indoxyl sulfate consists of intracellular uptake, distribution to the interstitial space, retention in the vascular space and glomerular

filtration. Therefore, intracellular extraction of [^{3}H]indoxyl sulfate is obtained by the following equation:

$$E = (E_T - E_{ns})/(100 - E_{ns}) \quad \text{(Eq. 4)}$$

where E represents the intracellular extraction (extraction only due to cellular uptake) and E_{ns} includes the extraction of the drug for distribution in this vascular, extracellular space, and for glomerular filtration. With regard to the kidney, an E_{ns} of 26.2% has been reported previously [22].

Expression of rOAT3 in *Xenopus laevis* oocytes

Polymerase chain reaction (PCR) primers were designed and synthesized based on the nucleotide sequence of rOAT3 [18], since full-length rOAT3 cDNA was not available at the beginning of this study. There was a sense strand with an EcoRI site, 5′-CGGAATTCCGGG TTCATCTTGCCTGGTGCCATG-3′ (position 104–126); antisense with an XbaI site, 5′-GCTCTAGAGCAAG AGGATTCTGTTGTTCTCA GCTA-3′ (position 1740–1764). Rat kidney total RNA was prepared from rat kidney using TRIzol Reagent (Gibco BRL, Grand Island, NY, USA). One microgram poly(A)$^+$RNA from rat kidney total RNA prepared using an mRNA purification kit (Amersham Pharmacia Biotech, Uppsala, Sweden) was reverse-transcribed with ReverTra Ace (Toyobo, Osaka, Japan) at 42°C for 50 minutes and then heated at 99°C for five minutes. The synthesized cDNA was used for subsequent PCR with a set of primers (1 μmol/L) according to the following profile: 98°C for 15 seconds, 65°C for 2 seconds, 74°C for 30 seconds, for 20 cycles. The PCR products were separated by electrophoresis on 1% agarose gels, and purified by a GENECLEAN II Kit (BIO 101, Vista, CA, USA). The purified PCR products were cut with EcoRI and XbaI (Takara, Shiga, Japan), ligated into pBluescript SK(+) (Strategene, La Jolla, CA, USA) for sequencing, and transformed into competent high *Escherichia coli* DH5α (Toyobo, Osaka, Japan). Both strands of the subcloned cDNA were sequenced with DNA sequencer (model 4200; Li-COR, Lincoln, NE, USA) and the cDNA clones with base sequences were 100% identical with rOAT3 [18]. Then, rOAT3 cDNA was subcloned into pGEM-HEN for in vitro transcription. The capped cRNA was synthesized in vitro using T7 RNA polymerase from plasmid DNA liberalized with Not I. Defolliculated oocytes were injected with 10 ng capped rOAT3 cRNA and incubated in Barth's solution (88 mmol/L NaCl, 1 mmol/L KCl, 0.33 mmol/L $Ca(NO_3)_2$, 0.4 mmol/L $CaCl_2$, 0.8 mmol/L $MgSO_4$, 2.4 mmol/L $NaHCO_3$, and 10 mmol/L HEPES) containing 50 μg/mL gentamicin and 2.5 mmol/L pyruvate, pH 7.4, at 18°C. After incubation for three days, uptake experiments were performed at 20°C in ND96 solution (96 mmol/L NaCl, 2 mmol/L KCl, 1.8 mmol/L $CaCl_2$, 1 mmol/L $MgCl_2$, and 5 mmol/L HEPES, pH 7.4).

Uptake by oocytes

The uptake experiment was initiated by replacing ND96 solution with that containing 2 μmol/L [^{3}H]indoxyl sulfate and terminated by the addition of ice-cold ND 96 buffer after the indicated time intervals. Oocytes were washed four times with ice-cold ND96, solubilized with 10% SDS, and the accumulated radioactivity was determined in a liquid scintillation counter (LS-6500; Beckman Instruments, Fullerton, CA, USA).

The kinetic parameters for the uptake of indoxyl sulfate via rOAT3 were estimated from equation (5):

$$v = V_{max} \times C/(K_m + C) \quad \text{(Eq. 5)}$$

where v is the uptake rate of the indoxyl sulfate [pmol/(h · oocyte)], C is the indoxyl sulfate concentration in the medium (mmol/L), K_m is Michaelis-Menten constant, and V_{max} is the maximum uptake rate [pmol/(h · oocyte)]. To obtain the kinetic parameters, the equation was fitted to the rOAT3-specific transport velocity, which was obtained by subtracting the transport velocity in water-injected oocytes from that in rOAT3-expressing oocytes, by an iterative nonlinear least squares method using a MULTI program [25]. The input data were weighted as the reciprocal of the observed values, and the Damping-Gauss Newton Method algorithm was used for fitting. The fitted line was converted to the v/C versus v form (Eadie-Scatchard plot).

For the inhibition study, the uptake of 2 μmol/L [^{3}H]indoxyl sulfate via rOAT3 was measured in the presence or absence of 1 mmol/L unlabeled compounds in ND96 solution. The specific uptake was obtained by subtracting the uptake into water-injected oocytes from the uptake into rOAT3-expressing oocytes.

Data analysis

Unless otherwise indicated, all data represent the mean ± SEM. An unpaired, two-tailed Student *t* test was used to determine the significance of differences between two group means. Statistical significance among means of more than two groups was determined by one-way ANOVA followed by the modified Fisher's least squares difference method. IC_{50} values were estimated by fitting the data to the nonlinear least-squares regression analysis program, MULTI.

RESULTS

Concentration-dependent inhibitory effect of probenecid on [^{3}H]indoxyl sulfate uptake by the kidney

In order to characterize the basolateral membrane transport systems proposed previously for indoxyl sulfate, it is necessary to examine the renal basolateral transport under physiological conditions. The tissue-

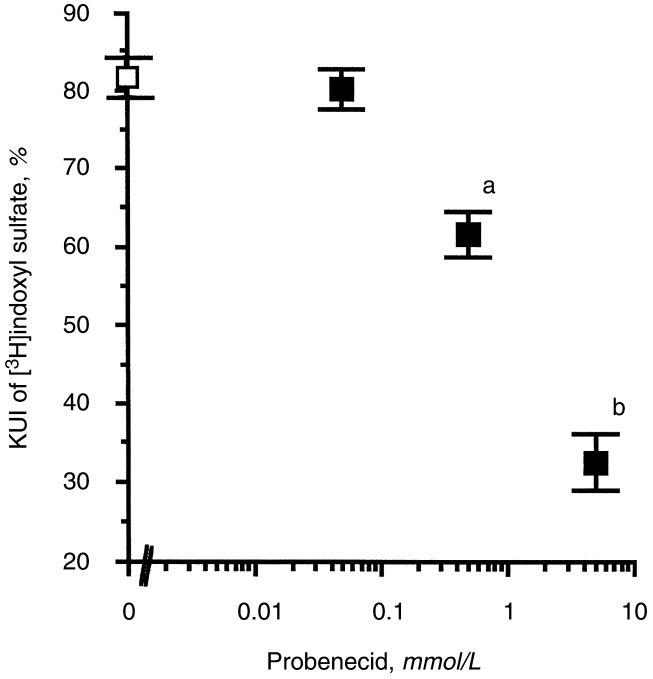

Fig. 1. Concentration-dependent inhibitory effect of probenecid on [^{3}H]indoxyl sulfate uptake by the kidney. [^{3}H]Indoxyl sulfate uptake was measured in the presence (■) and absence (□) of probenecid. Each point represents the mean ± SEM ($N = 4$ to 5). $^aP < 0.01$, $^bP < 0.001$, significantly different from the control uptake.

sampling single-injection technique, kidney uptake index (KUI) method, is known to be useful for measuring in vivo unidirectional influx rates across the basolateral membrane on the vascular side [22–24]. Figure 1 shows the effect of probenecid on [^{3}H]indoxyl sulfate uptake by the kidney measured by the KUI method. Probenecid, which is a potent inhibitor of organic anion transporters, including OAT and oatp families, had an inhibitory effect on [^{3}H]indoxyl sulfate uptake by the kidney that was concentration-dependent over the range 50 μmol/L to 5 mmol/L. The 5 mmol/L probenecid produced a significant reduction in [^{3}H]indoxyl sulfate uptake (40.6 ± 2.2% of the uptake in the control study). This result suggests that a carrier-mediated transport, an organic anion transporter, is involved in uptake of indoxyl sulfate in the kidney.

Inhibitory effect of various organic anions on [^{3}H]indoxyl sulfate uptake by the kidney

To characterize the uptake process for indoxyl sulfate by the kidney in vivo, the inhibitory effects of several compounds on [^{3}H]indoxyl sulfate uptake by the kidney were examined (Table 1). PAH, a substrate of rOAT1 (K_m = 14 μmol/L) [13] and rOAT3 (K_m = 65 μmol/L) [18], inhibited [^{3}H]indoxyl sulfate uptake by 72.1 ± 4.0%. Benzylpenicillin and cimetidine (K_m = 40 μmol/L for rOAT3), which are substrates of rOAT3 [18, 26], also reduced the uptake by 52.6 ± 0.9 and 51.3 ± 3.2%, respectively. These results suggest that rOAT3 mediates the transport of indoxyl sulfate in the kidney. Moreover, indomethacin is a potent inhibitor of rOAT1 [27], salicylic acid is a good substrate of rOAT2 [17] and 3,5,3′-triiodo-L-thyronine is a substrate of oatp3 [28]. These compounds had no effect on [^{3}H]indoxyl sulfate uptake. These results strongly suggest that rOAT3 at the basolateral membrane participates in indoxyl sulfate uptake by the kidney.

Uptake of indoxyl sulfate by rOAT3 in *Xenopus laevis* oocytes

To elucidate the involvement of rOAT3 in indoxyl sulfate transport in the kidney, the *Xenopus* oocytes expression system was studied. Figure 2 shows the indoxyl sulfate uptake activity by oocytes injected with cRNA derived from rOAT3 cDNA. This rOAT3-mediated indoxyl sulfate uptake was linear over at least 120 minutes, whereas the small diffusional component of indoxyl sulfate uptake remained constant (Fig. 2). Specific uptake of [^{3}H]indoxyl sulfate in rOAT3-expressing oocytes revealed saturable kinetics (Fig. 3), and the Eadie-Scatchard plot gave a single straight line (Fig. 3, inset), indicating that rOAT3 transports indoxyl sulfate. The estimated K_m and V_{max} values were 158 ± 0.1 μmol/L and 11.2 ± 0.1 pmol/(h · oocyte) (mean ± SD), respectively. As shown in Table 2, potent rOAT3 inhibitors, such as estrone sulfate (K_m = 2.3 μmol/L) [18] and probenecid, strongly inhibited the rOAT3-mediated uptake of [^{3}H]indoxyl sulfate. PAH, benzylpenicillin, and cimetidine also inhibited the uptake of [^{3}H]indoxyl sulfate, while salicylic acid, 3,5,3′-triiodo-L-thyronine, and indomethacin had minimal or no inhibitory effect. These differential inhibitory effects agreed with the in vivo results obtained by the KUI method as shown in Table 1, supporting the involvement of rOAT3 in indoxyl sulfate excretion transport in the kidney.

Inhibitory effect of uremic toxins on rOAT3-mediated indoxyl sulfate transport

Previous studies have reported that hippuric acid (HA) and CMPF appear to be transported by organic anion transporter since these uremic toxins inhibited PAH uptake in the kidney [12, 29, 30]. To examine the possibility that uremic toxins share the rOAT3-mediated indoxyl sulfate transport system, we analyzed the inhibitory effect of uremic toxins on [^{3}H]indoxyl sulfate uptake in the kidney using the KUI method (Table 3). CMPF and HA inhibited the uptake by 61.8 ± 2.1 and 56.7 ± 1.7%, respectively, while indole acetic acid (IA) did not.

Moreover, the effect of uremic toxins on the rOAT3-mediated uptake of [^{3}H]indoxyl sulfate was examined by the *Xenopus* oocytes expression system (Table 4). Uremic toxins, such as CMPF, HA, 2-hydroxycinnamic

Table 1. Inhibitory effect of several compounds on the intracellular uptake of [^{3}H]indoxyl sulfate by the kidney

Inhibitors	Conc. *mmol/L*	Number of investigations	Intracellular uptake of [^{3}H]indoxyl sulfate in the kidney[a]	
			%	*% of control*
Control		6	35.1 ± 2.8	100
para-Aminohippuric acid	5	6	9.81 ± 3.97[c]	27.9
3,5,3′-Triiodo-L-thyronine	1	4	32.6 ± 3.0	92.8
Salicylic acid	5	4	30.8 ± 2.3	87.8
Benzylpenicillin	5	4	16.6 ± 0.9[b]	47.4
Cimetidine	5	4	17.1 ± 3.2[b]	48.7
Indomethacin	1	4	34.5 ± 2.5	98.3

A solution containing both [^{3}H]indoxyl sulfate (0.78 μmol/L) and inhibitors was injected via the abdominal aorta.
[a]Each value represents the mean ± SEM
[b]$P < 0.01$, [c]$P < 0.001$, significantly different from control

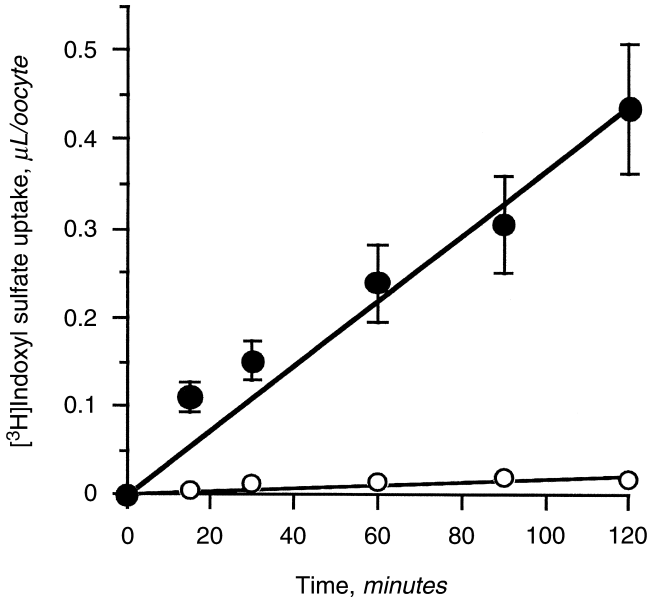

Fig. 2. Time-course of [^{3}H]indoxyl sulfate uptake by *X. laevis* oocytes injected with water or rOAT3 cRNA. The uptake of 2 μmol/L [^{3}H]indoxyl sulfate in water-injected oocytes (○) and rOAT3-expressing oocytes (●) were measured during a 120-minute incubation. Each bar represents the mean ± SEM (N = 10 to 12).

acid, 4-hydroxyphenylacetic acid, and 4-hydroxybenzoic acid inhibited rOAT3-mediated [^{3}H]indoxyl sulfate uptake, while quinolinic acid had no effect. To characterize the details of this inhibitory effect, a kinetic analysis of the inhibition profile was performed (Fig. 4). The IC_{50} values of probenecid, CMPF, and HA were 1.68 ± 0.03 μmol/L, 4.01 ± 1.07 μmol/L and 11.9 ± 2.6 μmol/L, respectively. The IC_{50} value of IA, 509 ± 96 μmol/L, is at least 50 times higher than the IC_{50} values of those compounds. These results suggest that at least CMPF and HA share rOAT3-mediated transport at the basolateral membrane in the kidney with indoxyl sulfate. In contrast, the transport system for IA and quinolinic acid is different from that for indoxyl sulfate.

DISCUSSION

Endogenous substances such as indoxyl sulfate, CMPF, and HA, which are referred to as uremic toxins, accumulate in the blood of patients with chronic renal failure [31]. In this condition, there are a number of physiological changes, such as glomerular hypertrophy and the development of glomerular sclerosis [32–35]. Circulating uremic toxins are thought to be one of the causes accelerating the progression of chronic renal failure [6]. It is well known that indoxyl sulfate stimulates progression of glomerular sclerosis and chronic renal failure [7–10]. Studies of the secretion of indoxyl sulfate in the renal proximal tubules have suggested that indoxyl sulfate is a substrate of the renal PAH transport system [7, 8, 12].

The present study provides in vivo evidence that indoxyl sulfate is transported by a carrier-mediated transport system across the renal basolateral epithelial cell membrane. Transport of [^{3}H]indoxyl sulfate into the kidney was significantly inhibited by probenecid using the KUI method (Fig. 1), and this supports the hypothesis that indoxyl sulfate is transported via a common carrier-mediated transport system across the renal basolateral membrane.

Recently, a rat renal organic anion transporter 1 (rOAT1) was isolated as the PAH transporter [13–15]. rOAT1 is expressed predominantly in the kidney and is localized at the basolateral membrane of the middle proximal tubules (S2) [16]. To date, two other isoforms, that is, rOAT2 and rOAT3, have been identified [17, 18], and these isoforms in kidney are believed to be located at the basolateral membrane [19]. [^{3}H]Indoxyl sulfate transport by the kidney is strongly inhibited by PAH, benzylpenicillin and cimetidine, which are substrates of rOAT3 [18, 26], but not by salicylic acid and indomethacin, which is a substrate of rOAT2 (K_m = 88.8 μmol/L) [17] and a potent inhibitor of rOAT1 (K_i = 10 μmol/L) [27], respectively (Table 1). Despite being a weak base, the transport by rOAT3 has been reported to be inhibited by cimetidine [18, 26]. Therefore, indoxyl sulfate

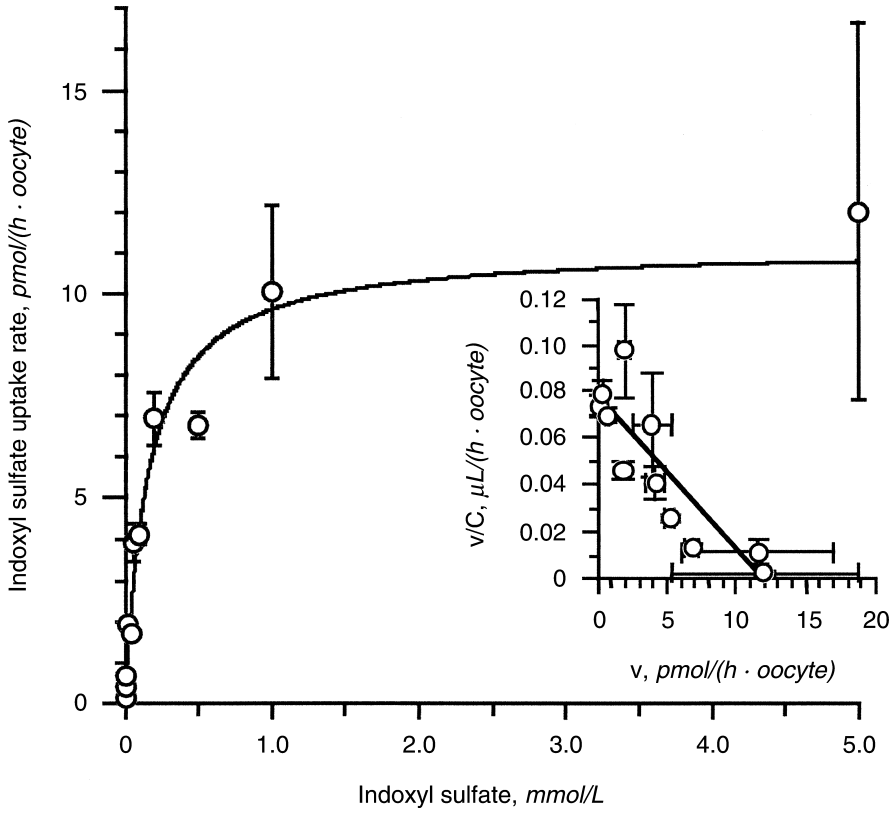

Fig. 3. Concentration-dependence of indoxyl sulfate uptake by *Xenopus* oocytes expressing rOAT3. Oocytes were injected with 50 nL containing 10 ng of rOAT3 cRNA or with an equal volume of water. They were cultured for three days at 20°C. The uptake rates of [^{3}H]indoxyl sulfate into rOAT3 cRNA-injected oocytes were measured at the concentration indicated after incubation for one hour at 20°C. Unspecific uptake by water-injected oocytes was subtracted from all uptake values. (*Inset*) the Eadie-Scatchard plot of the same data. The values indicated are the means of eight to ten oocyte determinations (means ± SEM).

Table 2. Inhibition study of [^{3}H]indoxyl sulfate in rOAT3-expressing oocytes

Inhibitors	% of control[a]	*N* determinations
Control	100 ± 7	25
Probenecid	2.58 ± 0.43[b]	19
Estrone sulfate	1.76 ± 0.88[b]	19
para-Aminohippuric acid	17.7 ± 3.9[b]	8
Benzylpenicillin	21.1 ± 5.4[b]	17
Cimetidine	26.1 ± 5.8[b]	17
Salicylic acid	68.1 ± 21.3	9
Indomethacin	103 ± 6	19
3,5,3′-Triiodo-L-thyronine	96.6 ± 17.3	9

The concentration of [^{3}H]indoxyl sulfate was 2 μmol/L and that of each inhibitor was 1 mmol/L. The uptake rates of [^{3}H]indoxyl sulfate into rOAT3 cRNA-injected oocytes were measured at one hour.

[a]Each value represents the mean ± SEM of *N* determinations.

[b]$P < 0.01$, significantly different from control

Table 3. Inhibitory effect of uremic toxins on the intracellular uptake of [^{3}H]indoxyl sulfate by the kidney

Inhibitors	Number of investigations	Intracellular uptake of [^{3}H]indoxyl sulfate in the kidney[a]	
		%	% *of control*
Control	7	30.9 ± 1.8	100
Hippuric acid	5	13.4 ± 1.7[b]	43.3
CMPF	5	11.8 ± 2.1[b]	38.2
Indole acetic acid	5	35.8 ± 1.6	116

A solution containing both [^{3}H]indoxyl sulfate and inhibitors at a concentration of 0.78 μmol/L and 5 mmol/L, respectively, was injected via the abdominal aorta.

[a]Each value represents the mean ± SEM.

[b]$P < 0.01$, significantly different from control

appears to undergo uptake by rOAT3 from the circulating blood to the kidney across the renal basolateral membrane. Recently, Cha et al isolated human organic anion transporter 3 (hOAT3) from the kidney and reported that estrone sulfate uptake by hOAT3 expressing oocytes was inhibited by PAH, probenecid, benzylpenicillin, cimetidine and indomethacin [36]. Although there appear to be species differences in the inhibitory effect of indomethacin, the substrate specificity of hOAT3 is similar to that of rOAT3 [19].

As kidney-specific organic anion transporters, OAT-K1 and OAT-K2, having a high degree homology with the organic anion transporting polypeptide (oatp) family, also were identified [37, 38]. Masuda et al reported that OAT-K1 and OAT-K2 were expressed at renal brush-border membranes and may participate in reabsorption to and/or secretion from tubular epithelial cells [20, 39]. Therefore, it might be that OAT-K1 and/or OAT-K2 are responsible for the transport of indoxyl sulfate at the renal brush border membrane. Moreover, Na^+-independent organic anion transporting polypeptide type 3 (oatp3) has been isolated from rat retina [28]. Oatp3 protein may be localized at the basolateral membrane in kidney because oatp3 mRNA is expressed in rat kidney.

Table 4. Inhibition of rOAT3-mediated [^{3}H]indoxyl sulfate uptake by uremic toxins

Inhibitors	% of control[a]	*N* determinations
Control	100 ± 1	10
CMPF	8.38 ± 1.72[b]	10
Hippuric acid	24.6 ± 1.5[b]	10
2-Hydroxycinnamic acid	39.5 ± 10.1[b]	8
4-Hydroxyphenylacetic acid	29.7 ± 2.5[b]	10
4-Hydroxybenzoic acid	22.1 ± 3.2[b]	10
Indole acetic acid	51.2 ± 7.1[b]	10
Quinolinic acid	136 ± 6	10

The concentration of [^{3}H]indoxyl sulfate was 2 μmol/L and that of each inhibitor was 1 mmol/L. The uptake rates of [^{3}H]indoxyl sulfate into rOAT3 cRNA-injected oocytes were measured at one hour.

[a]Each value represents the mean ± SEM of *N* determinations

[b]$P < 0.01$, significantly different from control

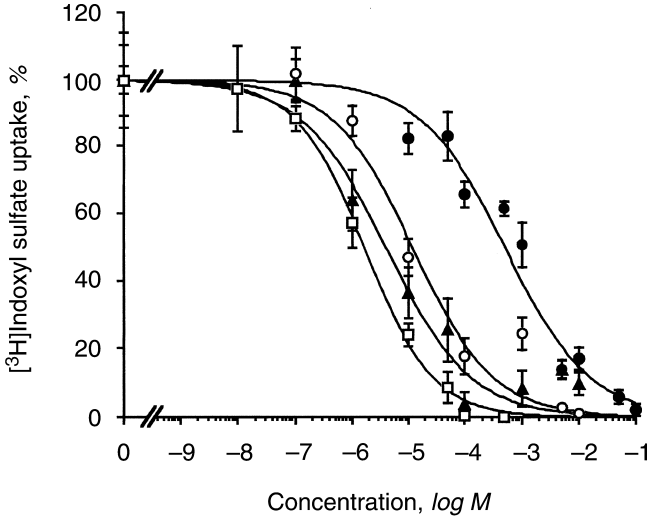

Fig. 4. Inhibition profile of rOAT3-mediated [^{3}H]indoxyl sulfate uptake by probenecid (□), CMPF (▲), hippuric acid (○), and indole acetic acid (●). The values are expressed as a percentage of [^{3}H]indoxyl sulfate uptake in rOAT3-expressing oocytes at one hour in the absence of inhibitor (mean ± SEM; $N = 8$ to 10).

Therefore, we carried out an investigation to see if oatp3 mediates indoxyl sulfate transport. However, we found that 3,5,3′-triiodo-L-thyronine, a substrate of oatp3 [28], had no inhibitory effect on [^{3}H]indoxyl sulfate uptake transport into the kidney (Table 1), suggesting that oatp3 is not involved in the uptake transport of [^{3}H]indoxyl sulfate.

To confirm that indoxyl sulfate is transported by rOAT3, we used the *Xenopus laevis* oocyte expression system. rOAT3-expressing oocytes exhibited significant uptake activity for [^{3}H]indoxyl sulfate (Fig. 2) and the [^{3}H]indoxyl sulfate uptake indicated a saturable process with a K_m of 158 μmol/L (Fig. 3). Under normal conditions, the concentration of unbound indoxyl sulfate in serum was reported to be 0.16 μmol/L [11]. These results demonstrate that indoxyl sulfate is a substrate of rOAT3 and suggests that rOAT3 is responsible for the uptake of indoxyl sulfate at the renal basolateral membrane. In addition, estrone sulfate, PAH, benzylpenicillin and cimetidine, which are substrates of rOAT3, inhibit the rOAT3-mediated uptake of [^{3}H]indoxyl sulfate (Table 2). In contrast, indomethacin, salicylic acid and 3,5,3′-triiodo-L-thyronine, do not exhibit any significant inhibitory effect on the uptake of [^{3}H]indoxyl sulfate. These results, along with a previous report [18, 26], agree with the results of the KUI study shown in Table 1. Considering the inhibitory effect of benzylpenicillin and cimetidine using the KUI method and the oocyte expression system, it appears that the contribution of rOAT3 is a major factor in the excretion of indoxyl sulfate in the proximal tubules. Miyazaki et al have recently reported that the administration of indoxyl sulfate increases TGF-β1 mRNA levels in the renal cortex of uremic rats [9, 10]. TGF-β1 stimulates the accumulation of extracellular matrix by increasing the production of extracellular matrix proteins, such as type I collagen, and inhibits its degradation by increasing the production of proteinase inhibitors, such as TIMP-1, in mesangial and tubulointestinal cells [40]. Thus, the indoxyl sulfate accumulating in the proximal tubular cells by the functional inhibition of rOAT3 causes the development of glomerular sclerosis and tubulointerstitial fibrosis, possibly also accelerating the progression of chronic renal failure.

It has been reported that HA and CMPF, uremic toxins, inhibit PAH transport in the kidney [12, 29, 30], and it has been suggested that these compounds also are transported by the organic anion transport system. In the present study, we demonstrated that HA and CMPF strongly inhibited the uptake of [^{3}H]indoxyl sulfate into the kidney using the KUI method (Table 3). Therefore, these uremic toxins may share the same uptake transport system as indoxyl sulfate in the kidney. Moreover, the uptake of [^{3}H]indoxyl sulfate by rOAT3-expressed oocytes is inhibited by HA and CMPF (Table 4). These results suggest that these compounds are candidate substrates of rOAT3. The IC_{50} values of HA and CMPF for the uptake of [^{3}H]indoxyl sulfate by rOAT3 were 11.9 ± 2.6 and 4.01 ± 1.07 μmol/L, respectively (Fig. 4), and these values were similar to that of probenecid (1.68 ± 0.03 μmol/L), which is a typical inhibitor of rOAT3.

These uremic toxins possess high protein-binding activity (indoxyl sulfate 96%; HA 55%; CMPF 99%) [11, 30] and only the unbound uremic toxins interact with rOAT3. The unbound concentration of uremic toxins in normal serum is low (indoxyl sulfate 0.16 μmol/L; HA 5.99 μmol/L; CMPF 0.15 μmol/L), suggesting that the transport of indoxyl sulfate by rOAT3 is not significantly reduced by those uremic toxins under normal conditions. Unbound serum concentrations of indoxyl sulfate, HA and CMPF in uremia have been reported to be 12.9, 121.9 and 3.7 μmol/L, respectively [11]. Estimated from

the results of the inhibition profile (Fig. 4), these uremic toxins inhibit the rOAT3-mediated transport of unbound indoxyl sulfate by more than 95% under uremic conditions, suggesting that the transport activity of OAT3 in the kidney is attenuated in patients with renal failure. The serum accumulation of uremic toxins could be accelerated because these substrates inhibit tubular secretion.

In contrast, IA, which is also a uremic toxin and a structural analog of indoxyl sulfate, failed to inhibit [^{3}H]indoxyl sulfate uptake into the kidney (Table 3), suggesting that the affinity of IA for rOAT3 is not as high as that of indoxyl sulfate. As show in Figure 4, the inhibitory activity of IA (IC_{50} = 509 ± 96 μmol/L) was at least 43-fold lower than that of HA and CMPF, whereas IA inhibited rOAT3-mediated transport of [^{3}H]indoxyl sulfate by 48.8 ± 7.1% in Table 4. The serum concentration of unbound IA in uremia was reported to be 3.92 μmol/L [11], suggesting that IA does not influence the transport of OAT3 in uremic patients.

Finally, other uremic toxins, such as 2-hydroxycinnamic acid, 4-hydroxyphenylacetic acid, 4-hydroxybenzoic acid, and quinolinic acid, were investigated. Previous studies have reported that these circulating uremic toxins are thought to be involved in a variety of uremic symptoms [31]. However, the transporter responsible for the uptake of these compounds has not been identified. Only the efflux transport of quinolinic acid from brain has been reported to be inhibited by probenecid, suggesting that quinolinic acid is transported by organic anion transporters [41]. The present study shows that these uremic toxins, but not quinolinic acid, could be substrates of rOAT3 and quinolinic acid might be transported by an organic anion transporter other than rOAT3, since all the compounds, except quinolinic acid, inhibited the rOAT3-mediated uptake of [^{3}H]indoxyl sulfate (Table 4). This evidence suggests that rOAT3 plays an important role in reducing the concentration of uremic toxins in the blood as part of a detoxication system.

Previously, Niwa et al proposed the protein metabolite hypothesis [42]. In this, the initial insult leads to a loss of functioning nephrons via a disease-specific pathophysiological process. A progressive decline in the glomerular filtration rate leads to increased circulating levels of endogenous protein metabolites, such as indoxyl sulfate, and the adverse effect of overload on the remaining nephrons. For example, indoxyl sulfate stimulates progressive chronic renal failure, leading to a further loss of nephrons. Thus, the vicious cycle of progressive renal injury is complete. Our present study supports this hypothesis. The accumulation of uremic toxins in proximal tubules, especially indoxyl sulfate, could be mediated by rOAT3. This finding will be very important not only in helping us better understand the development of nephrotoxicity, but also in improving the way we treat progressive renal failure caused by uremic toxins.

In conclusion, we report here that rOAT3 transports indoxyl sulfate and is responsible for the renal excretion of indoxyl sulfate. The present study suggests that uremic toxins share the rOAT3 transport system with indoxyl sulfate and this transport mechanism plays a key role in the development of nephrotoxicity in uremia.

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APPENDIX

Abbreviations used in this article are: CMPF, 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid; HA, hippuric acid; IA, indole acetic acid; KUI, kidney uptake index; OAT, organic anion transporter; PAH, *para*-aminohippuric acid.

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