Role of Organic Anion Transporters in the Tubular Transport of Indoxyl Sulfate and the Induction of its Nephrotoxicity

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Abstract. In uremic patients, various uremic toxins are accumulated and exert various biologic effects on uremia. Indoxyl sulfate (IS) is one of uremic toxins that is derived from dietary protein, and serum levels of IS are markedly increased in both uremic rats and patients. It has been previously reported that the accumulation of IS promotes the progression of chronic renal failure (CRF). This study demonstrates the role of rat organic anion transporters (rOATs) in the transport of IS and the induction of its nephrotoxicity. The administration of IS to 5/6-nephrectomized rats caused a faster progression of CRF, the induction of its nephrotoxicity. The administration of IS to organic anion transport inhibitors. The addition of IS induced a decrease in the viability of S2 rOAT1 and S2 rOAT3 as compared with the mock, which was rescued by probenecid. These results suggest that rOAT1 and rOAT3 play an important role in the transcellular transport of IS and the induction of its nephrotoxicity.

There have been many reports that protein restriction delays the progression of chronic renal failure (CRF) in both experimental uremic rats (1,2) and undialyzed uremic patients (3,4). To date, various possible mediators of the effect of dietary protein on the progression of renal insufficiency have been indicated, including angiotensin II (5,6), transforming growth factor-β (7), eicosanoids (8), and glucocorticoids (9,10).

In addition to these compounds, biologically active circulating uremic toxins derived from dietary protein have been proposed to play an important role in the progression of renal damage (11–13). We previously identified indoxyl sulfate (IS) as a uremic toxin derived from dietary protein. IS is metabolized by the liver from indole, which is produced from tryptophan by intestinal flora including Escherichia coli. We previously found that there is a marked elevation in the serum levels of IS in 5/6-nephrectomized (5/6-NPX) uremic rats and in uremic patients (11,12). In addition, a low-protein diet was shown to reduce the serum levels of IS in uremic rats (12,14). Furthermore, administration of AST-120, an oral absorbent, decreased the intensity of IS staining in the proximal tubules as well as the serum and urinary concentrations of IS; it also improved the slope of the time-dependent 1/serum creatinine plot (11,15). We have thus proposed a protein metabolite theory such that the increased serum concentration of uremic toxins, including IS and subsequent accumulation of those within the renal tubules in CRF possess the potential to exacerbate the deterioration of renal function (11,12). However, the mechanism by which IS is accumulated in the renal tubular cells and that of its nephrotoxicity remain unknown.

In the kidney, carrier-mediated secretory pathways for organic anions exist in the tubular cells (16,17). Various anionic drugs and substances are taken up into the tubular cells by basolateral multispecific organic anion transporters (OATs) and subsequently effluxed into the tubular lumen by luminal OATs. During transepithelial transport, organic anions are transiently accumulated in the proximal tubular cells. Over the past few years, we and others have identified various OATs, including OAT1 (18,19), OAT2 (20), OAT3 (21,22), and OAT4 (23). In addition, OAT homologues might also contribute to apical efflux of organic anions like PAH in vivo, i.e., organic anion-transporting polypeptide ( oatp1) (24), OAT-K1 (25), OAT-K2 (26), multidrug resistance-associated protein 2 (MRP2) (27,28), and human-type I sodium-dependent inorganic phosphate transporter (NPT1) (29). Among them, rat
OAT1 (rOAT1) and rat OAT3 (rOAT3) are the major OATs that are localized in the basolateral membrane of the proximal and distal tubules (30–32).

On the basis of its physicochemical properties, i.e., IS possesses an anionic moiety (Figure 1), we hypothesized that IS is a substrate for OATs and that the specific OAT involved may be related to the progression of CRF. We investigated the localization of accumulated IS and sites of rOAT1 and rOAT3 expression in 5/6-NPX rats after administration of IS using immunohistochemistry. We also elucidated the role of rOAT1 and rOAT3 in the transport of IS and the induction of its nephotoxicity by using cells stably expressing rOAT1 and rOAT3.

Materials and Methods

In Vivo Experimental Design

Male Sprague-Dawley rats (Clea, Tokyo, Japan) aged 7 wk were anesthetized, and five sixths of the normal kidney mass was infarcted, which induced CRF in most of the rats (33,34). Two days after the operation, the treated rats showed increased serum concentrations of creatinine (1.0 approximately 1.4 mg/dl), blood urea nitrogen (BUN) (67 approximately 92 mg/dl), and reduced levels of creatinine clearance (0.67 approximately 1.12 ml/min). Sham-operated rats as well as operated ones were given an intraperitoneal infusion of antibiotics (50 to 100 μl of 5000 U/ml penicillin-streptomycin; Life Technologies BRL, New York, NY).

Fourteen rats were paired and assigned to two separate groups: (1) 5/6-NPX rats (n = 7) and (2) 5/6-NPX rats orally administered IS in water daily at a dose of 50 mg/kg body wt and additionally receiving 1 ml of 5000 U/ml penicillin-streptomycin; Life Technologies BRL, New York, NY). The characterization of the monoclonal anti-IS antibody was described previously (15). There was no positive staining in the renal cortex when the primary antibody was preincubated with IS (data not shown) or when the primary antibody was omitted from the protocol. A rabbit polyclonal antibody was raised against a synthesized polypeptide of the carboxyl terminal of rOAT1 and rOAT3. The specificity of the antibody was described elsewhere (30,32).

Immunohistochemical Studies in 5/6-NPX Rat Kidney

Immunostaining of IS in renal tissue sections (1-μm-thick) was performed by using cells stably expressing rOAT1 and rOAT3 (S2 rOAT1 and S2 rOAT3). The sections were incubated with polyclonal antibodies against rOAT1 (1:2000 dilution) and rOAT3 (1:250 dilution), respectively. The sections were incubated with horseradish peroxidase (HRP)–conjugated secondary antibody against rabbit Ig (Dako, Glostrup, Denmark), and the HRP was detected using a diaminobenzidine (DAB)-H2O2 solution.

Cell Culture and Establishment of S2 Cells Stably Expressing rOAT1 and rOAT3 (S2 rOAT1 and S2 rOAT3)

An immortalized cell line derived from the second segment of the proximal tubule (S2) cells was established from the kidney of transgenic mice harboring the simian virus 40 large T-antigen gene as described previously (35,36). S2 cells do not express mouse OATs by RT-PCR (data not shown) and show a faint uptake of para-amino hippuric acid (PAH). Moreover, although protein kinase C activation downregulates estrone sulfate (ES) uptake by S2 rOAT3, other cells derived from the same transgenic mice did not show such regulation (37). Therefore, we assumed this cell line to be appropriate for the transfection. The establishment of S2 rOAT1 and S2 rOAT3 was carried out as described previously (36,37). Briefly, the full-length cDNAs of rOAT1 (18) and rOAT3 (21) were subcloned into pcDNA3.1(+) (Invitrogen, San Diego, CA). S2 cells were transfected with pcDNA3.1-rOAT1 and pcDNA3.1-rOAT3 coupled with pSV2neo, a neomycin resistance gene, using TFX-50 according to the manufacturer’s instructions. S2 cells transfected with pcDNA3.1 lacking an insert and pSV2neo were designated S2 pcDNA3.1 and used as a control. The cells were grown in a humidified incubator at 33°C under 5% CO2 using RITC 80 -7 medium (Iwaki Co., Tokyo, Japan) 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 genetin. The cells were subcultured in a medium containing 0.05% trypsin-EDTA solution (containing 137 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 4 mM NaHCO3, 0.5 mM EDTA, and 5 mM HEPES; pH 7.2). Clonal cells were isolated by using a cloning cylinder and screened by determining the optimal substrate for each transporter, i.e., [14C]PAH (53.1 mCi/mmol; Perkin Elmer Life Sciences, Boston, MA) (18) and [3H]ES (53 Ci/mmol; Perkin Elmer Life Sciences) for rOAT3 (21). Staining of vertical sections of S2 rOAT1 and S2 rOAT3 with polyclonal antibodies against rOAT1 and rOAT3, respectively, showed that the subcellular localization of rOAT1 and rOAT3 proteins was mainly on the cell membrane (36,38). Both the basolateral and apical portions of the membrane showed positive staining. Therefore, the cells were cultured on a solid support for subsequent experiments.

Uptake Experiments in S2 rOAT1 and S2 rOAT3

Uptake experiments were performed as described previously (36–38). The cells were seeded in 24-well tissue culture plates at a cell density of 1 x 10³ cells/well. After culturing for 2 d, they were washed three times with Dulbecco modified phosphate-buffered saline (D-PBS) solution (containing 137 mM NaCl, 3 mM KCl, 8 mM Na2HPO4, 1 mM KH2PO4, 1 mM CaCl2, and 0.5 mM MgCl2; pH 7.4). They were then preincubated in the same solution at 37°C for 10 min prior to the addition of IS (30 μM) at 37°C for 10 min prior to the addition of IS (30 μM).
Kinetic Analyses
After preincubation as described above, S2 rOAT1 and S2 rOAT3 were incubated in a solution containing [14C]PAH or [3H]ES at different concentrations in the absence or presence of IS (Sigma, St. Louis, MO) at 37°C for 2 min. On the basis of the level of organic anion uptake under each condition, analyses of Lineweaver-Burk plot as well as Eadie-Hofstee plot were performed as described previously (39). When the inhibition was competitive, Ki values were calculated based on the following equation,

\[
Ki = \frac{[\text{IS}]}{[\text{Km}] - [\text{IS}]} - 1
\]

Measurement of Intracellular IS Content
S2 cells were seeded in 6-well tissue culture plates at a cell density of 3 x 10^5 cells/well, cultured, and preincubated as in the uptake experiments. The cells were thereafter incubated in a solution containing 100 μM IS in the absence or presence of 1 mM probenecid or 1 mM clastatin at 37°C for 5, 15, and 30 min. After washing the cells with IS-free PBS, the intracellular concentrations of IS were determined using HPLC (40).

Cytotoxicity Assays
Cell viability was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay as described previously (38). S2 cells were incubated in a solution in the absence or presence of IS at 33°C for 24 or 48 h. In addition, to elucidate the protective effect of probenecid on IS-induced cytotoxicity, the cells were incubated in a solution containing IS in the absence or presence of 1 mM probenecid. After the incubation, 1 ml of 0.5% MTT was added to the media. The cells were then further incubated at 33°C for 4 h. After the cells were lysed with isopropanol/HCl solution, the optical density was measured at 570 nm using that at 630 nm as a reference (Beckman, Du-640).

Statistical Analyses
Results are expressed as the means ± SEM. To compare values among the three groups, ANOVA and Fisher’s least significance difference (LSD) test were performed. Results were considered statistically significant when the P value was less than 0.05.

Results
Biochemical Parameters in Vivo
As shown in Table 1, significant increases in systolic BP, serum creatinine, BUN, serum IS, and urinary protein excretion and significant decrease in creatinine clearance level were observed in 5/6-NPX rats compared with sham rats. In addition, 5/6-NPX rats administered with IS showed further in-
creases in serum creatinine, BUN, serum IS, urinary IS excretion, and urinary protein excretion, with a decrease in creatinine clearance being much more predominant than in 5/6-NPX rats. Renal function deteriorated time-dependently until the 16th wk; remnant kidneys from 5/6-NPX rats with or without administration of IS were therefore used for immunohistologic analyses at week 16.

Localization of OAT1, OAT3, and IS in Renal Cortex by Immunohistochemistry

Immunohistochemical analyses were performed to determine the localization of rOAT1, rOAT3, and IS in various sections of the renal cortex. In the control, rOAT1 is localized in the basolateral membrane of the S2 segment of the proximal tubule but not in the S1 segment (Figure 2A), whereas rOAT3 is localized in the basolateral membrane of both S1 and S2 segments, the thick ascending limb of Henle (TAL), and the distal convoluted tubule (DCT) (Figure 2D) as well as the S3 segment (data not shown). The 5/6-NPX rats administered with IS showed increased intensity of staining for rOAT3 in the renal cortex as compared with control, and rOAT1 was found to be slightly induced in the S1 segment. IS was weakly stained in the proximal tubule but not in the glomeruli of control rats. In 5/6-NPX rats, IS was strongly localized in the S2 segments of proximal tubule and glomerular epithelial cells and weakly localized in the S1 segment, TAL, and DCT. The 5/6-NPX rats administered with IS showed a marked increase in IS immunoreactivity not only in the S1 and S2 segments of the proximal tubule but also in the glomerular epithelial cells, DCT, and TAL with macula densa. These data confirm the localization of IS and demonstrate that IS administration markedly enhances

![Figure 2: Immunostaining of rOAT1, rOAT3, and IS in the renal cortex of control rats (Sham; A, D, and G), uremic rats (5/6-NPX; B, E, and H), and uremic rats to which IS was administered (5/6-NPX+IS; C, F, and I). Serial sections from each rat kidney were stained using a polyclonal anti-rOAT1 antibody (A, B, C), a polyclonal anti-rOAT3 antibody (D, E, F), and a monoclonal anti-IS antibody (G, H, I). These figures are representatives of typical samples from seven rats. The S1 segment of the proximal tubule (stars) was connected to the glomerulus, followed by the S2 segment. The thick ascending limb of the loop of Henle (TAL) is represented by triangles, the distal convoluted tubule (DCT) by circles, and the macula densa by arrows. Magnification x 150.](image-url)
the tubular uptake of IS in the remnant kidney. In DCT and TAL with macula densa, IS staining was found to be colocalized with the staining of rOAT3 but not of rOAT1.

Effects of Various Concentrations of IS on rOAT1- and rOAT3-Mediated Organic Anion Uptake

As shown in Figure 3, S2 rOAT1 (A) and S2 rOAT3 (B)
exhibited a time-dependent uptake of PAH and ES, respectively. In addition, S2 rOAT1 and S2 rOAT3 exhibited a dose-dependent increase in PAH and ES uptake, respectively (data not shown). Eadie-Hofstee analyses showed that the Michaelis constant (Km) for PAH uptake was 17.6 ± 5.6 μM in S2 rOAT1; for ES uptake, it was 4.30 ± 1.20 μM in S2 rOAT3, values which are similar to those in the Xenopus laevis oocyte expression system (18,21). As shown in Figure 3, IS inhibited the rOAT1-mediated (C) and rOAT3-mediated (D) organic anion uptake in a dose-dependent manner (n = 4; *P < 0.01 versus control). In contrast, IS did not significantly inhibit PAH uptake and/or ES uptake in S2 pcDNA 3.1 (n = 4; NS). As shown in Figure 4, kinetic analyses of the inhibitory effects were performed using Lineweaver-Burk plot (A and C) as well as Eadie-Hofstee plot (B and D). The results of these kinetic analyses revealed that the mode of inhibition was competitive. The inhibition constant (Ki) values for rOAT1 and rOAT3 were determined to be 34.2 μM (n = 4) and 74.4 μM (n = 4), respectively.

**HPLC Determination of IS Content in S2 rOAT1 and S2 rOAT3**

To determine whether rOAT1 and rOAT3 mediate the uptake of IS, we measured the intracellular content of IS using HPLC. As shown in Figure 5, S2 rOAT1 (A) and S2 rOAT3 (B) showed a significantly higher intracellular IS content compared with that in S2 pcDNA 3.1 (n = 4; *P < 0.01 versus S2 pcDNA 3.1). In addition, we have examined the effects of organic anion transport inhibitors on IS uptake mediated by rOAT1 and rOAT3. Organic anion transport inhibitors used were probenecid and cilastatin (41). Probenecid and cilastatin dose-dependently inhibited the organic anion uptake by S2 rOAT1 and S2 rOAT3 at concentrations up to 2 mM (data not shown). As shown in Figures 5A and 5B, probenecid and cilastatin at 1 mM significantly inhibited IS uptake mediated by rOAT1 and rOAT3 (n = 4; **P < 0.01 versus S2 rOAT1 or S2 rOAT3; ###P < 0.01 versus S2 rOAT1 or S2 rOAT3). Both probenecid and cilastatin exhibited no significant inhibitory effects on IS uptake by S2 pcDNA 3.1 (data not shown).

**Effects of IS on the Viability of S2 rOAT1 and S2 rOAT3 in the Absence or Presence of Probenecid**

We determined whether accumulated IS within the cells induced toxicity in S2 rOAT1 and S2 rOAT3. For this purpose, we examined the effects of various concentrations of IS on the viability of S2 rOAT1, S2 rOAT3, and S2 pcDNA 3.1 using the MTT colorimetric assay. As shown in Figure 6, IS after 24-h culture significantly decreased the viability of S2 rOAT1 (A), but not that of S2 rOAT3 (B), compared with that of S2 pcDNA 3.1, respectively. In contrast, the viability after 48 hr culture was significantly decreased in both S2 rOAT1 and S2 rOAT3 compared with that of S2 pcDNA3.1 (Figure 6, A and B; n = 4; **P < 0.01 versus S2 pcDNA3.1). To confirm the role of rOAT1 and rOAT3 in the IS-induced nephrotoxicity, we evaluated the effects of probenecid, an organic anion transport inhibitor (41), on the IS-induced decrease in the viability.

**Figure 4. Kinetic analysis of the inhibitory effects of IS on rOAT1- and rOAT3-mediated organic anion uptake.** S2 rOAT1 (A and B) and S2 rOAT3 (C and D) were incubated at 37°C for 2 min in a solution containing various concentrations of [14C]PAH and [3H]ES in the absence (○) or presence (■, 250 μM; □, 1000 μM) of IS. Lineweaver-Burke plot analysis (A and C) and Eadie-Hofstee plot analysis (B and D) were performed. Each value represents the mean ± SEM of four determinations from one typical experiment.

**Figure 5. Intracellular IS content in S2 cells determined by HPLC.** Confluent S2 rOAT1 (A), S2 rOAT3 (B), and S2 pcDNA 3.1 in six-well plates were incubated in a solution containing 100 μM IS in the absence or presence of 1 mM probenecid or 1 mM cilastatin at 37°C for 2, 5, 15, and 30 min. After washing the cells with a solution, the intracellular IS contents were determined using HPLC. Each value represents the mean ± SEM of three determinations from one typical experiment. *P < 0.01 versus S2 pcDNA3.1; #P < 0.05 versus S2 rOAT1 or S2 rOAT3; ##P < 0.01 versus S2 rOAT1 or S2 rOAT3.
cell viability (data not shown). Probenecid alone did not show any significant effects on the function. Although immunologic disorders may play a major role in the development of tubulointerstitial damage, we have previously proposed that the accumulation of uremic toxins in the tubular cells also plays an important role, especially in progressive CRF (15).

Discussion

In the progression of CRF, tubulointerstitial damage develops even if the glomerulus is the primary site of injury, and the level of tubulointerstitial damage has been suggested to be a good indicator to predict prognosis (42, 43). Progressive damage to the renal interstitium destroys extensive amounts of kidney tissue and results in considerable reduction of renal function. Although immunologic disorders may play a major role in the development of tubulointerstitial damage (44), we have previously proposed that the accumulation of uremic toxins in the tubular cells also plays an important role, especially in progressive CRF (15).

Both rOAT1 and rOAT3 mediate the basolateral uptake of various drugs and endogenous organic anions such as nonsteroidal antiinflammatory drugs, antitumor drugs, H2-receptor antagonists, prostaglandins, diuretics, angiotensin-converting enzyme inhibitors, and β-lactam antibiotics (18, 21). Some characteristic differences exist between rOAT1 and rOAT3, such as substrate specificity, affinity, and localization. rOAT1 is localized at the basolateral side of the S2 segment of the proximal tubules (30); rOAT3 is found in the first, second, and third segments (S1, S2, and S3) of the proximal tubules (32). In addition, rOAT1, but not rOAT3, exhibits transport properties that are typical of an exchanger (18, 21).

IS is a small and relatively hydrophobic organic anion (Figure 1) that possesses the typical chemical structure accepted by the OATs. IS administration to 5/6-NPX rats accelerated the progression of CRF as indicated by the increased serum concentrations of creatinine and BUN and the decreased creatinine clearance levels compared to those of 5/6-NPX rats (Table 1). The serum concentration of IS in IS-administered 5/6-NPX rats (mean, 156 approximately 226 μM) was comparable to those of hemodialysis patients (mean, 249 μM) (13). Immunohistochemical analyses revealed that rOAT1 and rOAT3 in control rats were localized to the basolateral membrane of renal tubules, whereas IS was weakly detected. In contrast, IS staining in 5/6-NPX rats was more marked in the renal tubules, where rOAT1 and rOAT3 were also detected. IS staining was more remarkable in 5/6-NPX rats treated with IS. On the basis of these results, we suggest that the increased serum IS concentration leads to the accumulation of IS within the renal tubules by the uptake of IS via rOAT1 and rOAT3 by the mechanisms confirmed by the in vitro experiment carried out in this study. The accumulated IS may subsequently accelerate the progression of tubulointerstitial damage. The rOAT3 protein was more broadly distributed than that of rOAT1, i.e., not only in the proximal tubules but also in the distal tubules. In addition, the Ki value of IS for rOAT3-mediated organic anion uptake was about twice of that for rOAT1. It is thus possible that rOAT3 plays a dominant role for the uptake of IS in CRF to cause nephrotoxicity. IS staining was colocalized with rOAT3 but not with rOAT1 (Figure 2). Considering the Ki values of rOAT1 and rOAT3 for IS, it is possible that the colocalization of rOAT3 with IS is a phenomenon that is seen only at high IS levels after rOAT1 becomes saturated and rOAT3 becomes the major contributor.

We also observed the changes in the levels of rOAT1 and rOAT3 proteins by immunohistochemistry and found that the expression of rOAT3 seems to be increased in 5/6-NPX rats compared to normal rats, whereas that of rOAT1 was increased slightly (Figure 2). This finding appears to reflect the previous observation that the secretory capacity for organic anion was increased per nephron basis when the nephron mass decreased (45). The changes of expressions of both rOAT1 and rOAT3 in CRF rats should also be quantified in the future investigation using Western and Northern blot analyses. Although the present study mainly focused on IS as an endogenous organic anion, a similar mechanism may operate for other anionic uremic toxins. In uraemia, various anionic uremic toxins

Figure 6. As shown in Figure 6, 1 mM probenecid significantly reversed the decrease in the viability of S2 rOAT1 (A) and S2 rOAT3 (B) treated with IS (n = 4; #P < 0.05). Addition of 1 mM probenecid alone did not show any significant effects on the cell viability (data not shown).

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may accumulate in the proximal tubular cells, some of which may be as potentially nephrotoxic as IS.

Although the progressive nature of chronic renal disease has been extensively investigated in 5/6-NPX rats, the mechanisms responsible for the compensatory hypertrophy are still controversial (46–48). It has been suggested that the adaptations associated with the compensatory changes are maladaptive and result in eventual glomerulosclerosis of the initially normal remnant nephrons. In this study, both toxic effects of IS and progression of adaptive changes in the remnant nephrons may coexist in the nephrectomized model; it therefore seems difficult to distinguish the contributions of these two pathogenic mechanisms. We assume that the increased expression level of OAT3 is an adaptive change that takes place during the accumulation of serum organic acids such as IS, although its upregulation mechanism requires further in vitro investigation.

Regarding extrarenal distribution of OAT1 and OAT3, it has been demonstrated using Northern blot analyses or reverse transcription-PCR that OAT1 (18,49) and OAT3 (21) are expressed not only in the kidney but also in the brain. It is therefore important to investigate the OAT-mediated transport of such uremic toxins as IS across the blood-brain barrier and to clarify the mechanism of neurologic symptoms of uremic syndrome in CRF patients.

IS inhibited rOAT1- and rOAT3-mediated organic anion uptake, and those inhibitions were competitive. The Ki values were comparable with the serum concentration of IS in uremic rats, i.e., 55 approximately 226 µM (Table 1). These results suggest that IS interacts with rOAT1 and rOAT3 not only in vitro but also in vivo. It is important to note that approximately 90% of IS is bound to albumin in vivo; the interaction between IS and these transporters is therefore not perfectly consistent with the in vitro model. However, the observed high level of IS secretion into the urine in 5/6-NPX rats could not be explained by glomerular filtration only (Table 1). A further in vitro study is necessary to investigate the interaction between these transporters and IS in the presence of albumin.

The addition of IS at concentrations relevant to in vivo conditions resulted in a significant decrease in the viability of S2 rOAT1 and S2 rOAT3, whereas no significant change was observed in that of S2 pcDNA 3.1. In addition, this decrease in viability was inhibited by the simultaneous administration of probenecid. The concentration of probenecid used in the current study (1 mM) was shown in previous studies to inhibit cephaloridine-induced and ochratoxin A–induced nephrotoxicity in S3 cells stably expressing rOAT1 (38,50). Furthermore, as described above, HPLC analyses revealed that rOAT1 and rOAT3 mediate the accumulation of IS, which was inhibited by probenecid and cilastatin (Figure 5). On the basis of these results, we suggest that IS is taken up via rOAT1 and rOAT3, accumulates within the cells, and induces cytotoxicity. The extent of viability in S2 rOAT1 and S2 rOAT3 treated with IS and probenecid for 48 h was approximately 40% more than that treated with IS alone. On the other hand, the amount of IS in S2 rOAT1 and S2 rOAT3 treated with IS and probenecid for 30 min was approximately 80% less than that treated with IS alone. This apparent discrepancy may be due to the difference in experimental setting, i.e., 30-min incubation in D-PBS for uptake experiments and 48-h culture in RITC containing FBS and growth factors for viability experiments. In addition, although viability was significantly decreased in S2 rOAT1 but not in S2 rOAT3 for the 24-h culture, the extents of viability for the corresponding 48-h cultures between S2 rOAT1 and S2 rOAT3 were similar. This phenomenon was also observed in S2 rOAT3 treated with cephaloridine (38) and in S2 hOAT1 and S2 hOAT3 treated with ochratoxin A (unpublished observation), although the reason for this remains unclear. The intracellular mechanisms of nephrotoxicity induced by IS accumulation should be further elucidated.

In conclusion, we clarify that IS is one of the important uremic toxins that are transported by OAT1 and OAT3. Thus, accumulated IS may cause cellular functions to decrease, although its intracellular mechanisms await further investigation.

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References
11. Niwa T, Nomura T, Sugiyama S, Miyazaki T, Tsukushi S, Tsutsui S: The protein metabolite hypothesis, a model for the


43. Risdon RA, Sloper JC, deWardener HE: Relationship between renal function and histological changes found in renal-biopsy
specimens from patients with persistent glomerular nephritis. 


