reduced by FCCP, although the values at 30 min were similar in the absence or presence of FCCP.

To determine whether [^{14}C]tetraethylammonium uptake depends on membrane potential, the effect of a K^+ diffusion potential generated by valinomycin on [^{14}C]tetraethylammonium uptake was examined. As shown in Fig. 6*B*, the H^+ gradient-stimulated [^{14}C]tetraethylammonium uptake was not altered by the presence of valinomycin. Furthermore, we also examined the effect of a K^+ diffusion potential generated by valinomycin on rMATE1-mediated [^{14}C]tetraethylammonium uptake in the absence of H^+ gradient ($[H^+]_{in} = [H^+]_{out}$, pH 7.5). [^{14}C]tetraethylammonium uptake was not significantly changed with or without valinomycin at 30 s (with valinomycin, 1.77 ± 0.21; without valinomycin, 1.84 ± 0.14 pmol·mg protein^{-1}·30 s^{-1}; n = 3) and at 1 min (with valinomycin, 3.10 ± 0.18; without valinomycin, 3.48 ± 0.68 pmol·mg protein^{-1}·min^{-1}; n = 3). These results indicate that the inside-negative membrane potential does not affect [^{14}C]tetraethylammonium uptake by rMATE1, suggesting the electroneutral antiport of H^+ and [^{14}C]tetraethylammonium.

DISCUSSION

Transport studies in brush-border and basolateral membrane vesicles from renal epithelial cells have been successfully utilized to characterize a number of transport systems under well-defined in vitro conditions. The membrane vesicle studies are particularly useful for identifying the driving forces of secondary active transport systems, compared with other analyses. This is because the ionic composition inside or outside membrane vesicles is easily manipulated, and ion gradients and membrane potential can be provided artificially. In fact, it was clearly demonstrated that organic cation transport systems at the renal brush-border membranes are driven by an outwardly directed H^+ gradient (4, 19, 23). Recent cloning and functional studies of MATE1 from various species have suggested that an oppositely directed H^+ gradient was a driving force of tetraethylammonium transport by MATE1 (3, 13, 15, 20), but there had been no evidence of a direct coupling of organic cation transport to H^+.

In the present study, by using membrane vesicles from HEK-rMATE1 cells, we provide the first direct evidence that MATE1 mediates the H^+-coupled uphill transport of [^{14}C]tetraethylammonium. Furthermore, this stimulation disappeared in the presence of a protonophore, FCCP, indicating that MATE1 functions as the H^+/organic cation antiporter. The K^+ diffusion potential generated by valinomycin had no effect on [^{14}C]tetraethylammonium uptake by membrane vesicles from HEK-rMATE1 cells with or without an H^+ gradient. This is consistent with a report that the tetraethylammonium uptake by brush-border membrane vesicles was not enhanced by inside-negative membrane potential (19). Taken together, it is suggested that the antiport of H^+ and tetraethylammonium via rMATE1 is electroneutral and that the stoichiometry might be 1:1.

In our previous study (20), using rMATE1-transiently expressing cells without NH_4Cl pretreatment, we assessed the time course of [^{14}C]tetraethylammonium uptake (pH 8.4), pH profile of [^{14}C]tetraethylammonium uptake, and substrate specificity at the pH 8.4. In the present study, using HEK-rMATE1 cells with NH_4Cl pretreatment, the transport characteristics for rMATE1 were analyzed by [^{14}C]tetraethylammonium (pH 7.4) or various compounds (pH 7.4). It was reported that the intracellular pH of HEK293 cells is ~7.2 and transiently acidified to 6.0–6.5 by NH_4Cl pretreatment (11). These distinct experimental conditions may have affected the different transport characteristics of rMATE1. For example, we previously reported that the intracellular accumulation of [^{14}C]tetraethylammonium via rMATE1 showed a time-dependent increase. In the present study, [^{14}C]tetraethylammonium intracellular accumulation by rMATE1 peaked at 30–60 s and then gradually decreased. This may be due to the consumption of the outward H^+ gradient within 30–60 s and subsequent back flux of [^{14}C]tetraethylammonium via MATE1. In addition, [^{3}H]1-methyl-4-phenylpyridinium acetate and [^{14}C]procainamide were transported by rMATE1 in the present study, but not in the previous study. This may be due to the lack of a strong enough driving force to transport [^{3}H]1-methyl-4-phenylpyridinium acetate and [^{14}C]procainamide in the previous conditions.

In conclusion, we generated HEK293 cells stably expressing rMATE1, and clearly demonstrated that the driving force of tetraethylammonium transport by rMATE1 is an oppositely directed H^+ gradient using membrane vesicles from this stable transfectant. These findings can provide important information about the renal tubular secretion of organic cations, and these experimental strategies may be useful for elucidating the mechanisms of action used by single transporters in heterologous expression systems.

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Short Communication

Renal Transport of Adefovir, Cidofovir, and Tenofovir by SLC22A Family Members (hOAT1, hOAT3, and hOCT2)

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Purpose. The nephrotoxicity of the nucleotide antivirals adefovir, cidofovir and tenofovir is considered to depend on the renal tubular transport of them. Although it is known that the antivirals are substrates of the human renal organic anion transporter hOAT1 (SLC22A6), there is no information available on other organic ion transporters. The aim of the present study was to investigate whether the other renal organic anion transporter hOAT3 (SLC22A8) and organic cation transporter hOCT2 (SLC22A2) transport the antivirals.
Materials and Methods. Uptake experiments were performed using HEK293 cells transfected with cDNA of the organic ion transporters.
Results. The uptake of adefovir, cidofovir and tenofovir in monolayers stably expressing hOAT3 increased time-dependently, compared with control. Probenecid, a typical inhibitor of organic anion transporters, completely inhibited their transport. The amounts of the antivirals taken up by hOAT3 were much lower than those by hOAT1. The transient expression of hOCT2 did not increase uptake of the antivirals.
Conclusion. These results indicate that adefovir, cidofovir and tenofovir are substrates of hOAT3 as well as hOAT1, but that quantitatively hOAT1 is the major renal transporter for these drugs.

KEY WORDS: antivirals; nephrotoxicity; organic anion transporter; renal transport.

INTRODUCTION

The SLC22A superfamily comprises organic anion transporters and organic cation transporters, and is responsible for the tissue distribution and disposition of various organic compounds including endogenous metabolites, toxins, xenobiotics and drugs (1,2). So far, cDNAs of the human organic anion transporters 1–4 (hOAT1-4), the urate transporter (URAT) and the human organic cation transporters 1–3 (hOCT1-3), hOCTN1 and hOCTN2 have been isolated and characterized in terms of function and expression. The SLC22A members are known to transport clinically important drugs such as cephalosporins, diuretics, non-steroidal anti-inflammatory drugs, H_2 receptor antagonists, antivirals and antitumor agents, and the clinical relevance of each transporter has been assessed (3,4). In the members, hOAT1 (SLC22A6), hOAT3 (SLC22A8) and hOCT2 (SLC22A2) are highly expressed at the basolateral membrane in the renal proximal tubules, indicating that they play important roles in the tubular uptake of organic compounds from circulation (5).

hOAT1, hOAT3 and hOCT2 are determinants of the renal toxicity of cephaloridine, ochratoxin A, cisplatin and so on (6,7). In other words, the basolateral uptake of these compounds via the organic ion transporters in the renal proximal tubules is the first step in their nephrotoxicity. Similarly, the acyclic nucleotide analogues adefovir, cidofovir and tenofovir, which are currently used to treat infections of hepatitis B virus, cytomegalovirus and human immunodeficiency virus, respectively, induce renal impairment triggered by their renal tubular transport (8). It is shown that they are substrates of hOAT1 (9) and that the expression of hOAT1 is required for the cytotoxicity of adefovir and cidofovir (10), although there is no report on the transport of the antivirals by other SLC22A members.

Recently, we showed that mRNA expression levels of hOAT3 in the kidney cortex were higher than those of hOAT1 (5), and that mRNA levels of hOAT3 but not of hOAT1 correlated with the elimination rates of cefazolin and phenolsulfonphthalein in patients with renal diseases (11,12). Accordingly, to understand the renal basolateral transport mechanisms for the antivirals in detail, information on hOAT3 is required. The purpose of this study is to investigate the transport of adefovir, cidofovir and tenofovir by hOAT3. In addition, the contribution of hOCT2 was also examined.

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ABBREVIATIONS: hOAT, human organic anion transporter; hOCT, human organic cation transporter; MRP, multidrug resistance protein.

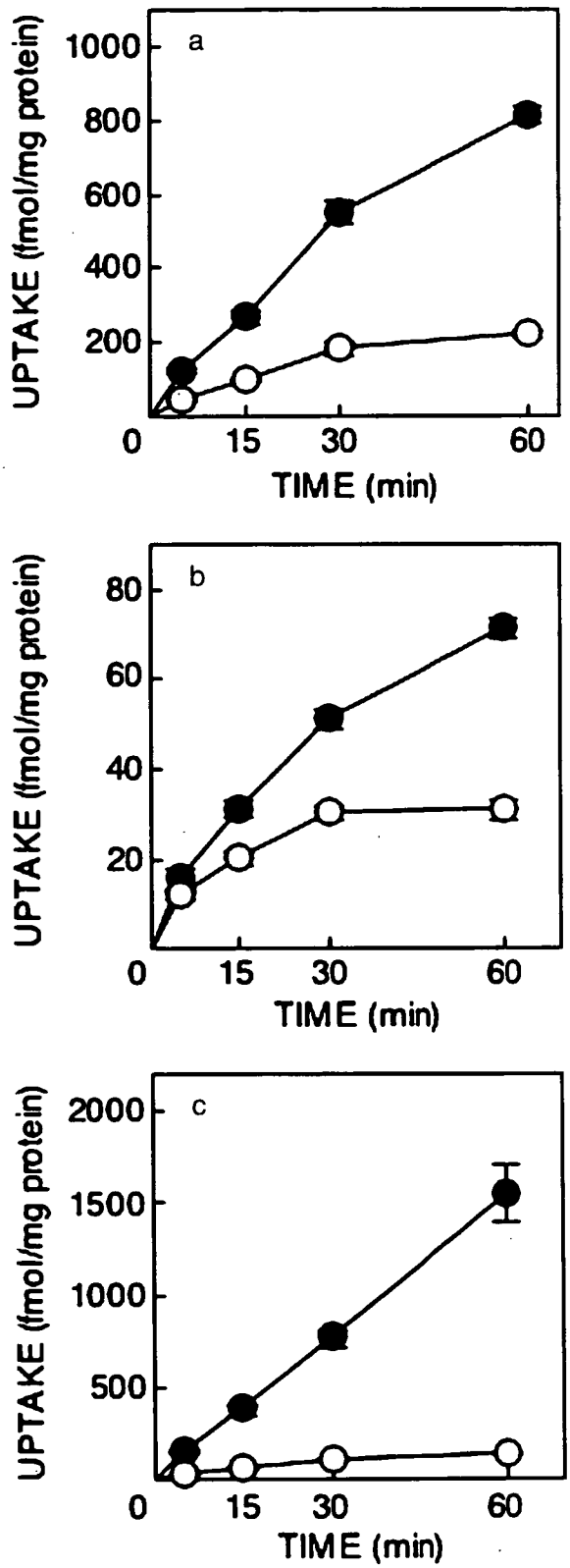

Fig. 1. Time-dependent uptake of adefovir (**a**), cidofovir (**b**) and tenofovir (**c**) by HEK-pBK and HEK-hOAT3. HEK-pBK (*open circle*) and HEK-hOAT3 (*closed circle*) were incubated with 111 nM [^{3}H]adefovir, 66.7 nM [^{3}H]cidofovir or 100 nM [^{3}H]tenofovir for the periods indicated. Each point represents the mean±S.E. of the uptake amounts of each antiviral in three monolayers. When the error bar is not shown, it is smaller than the symbol.

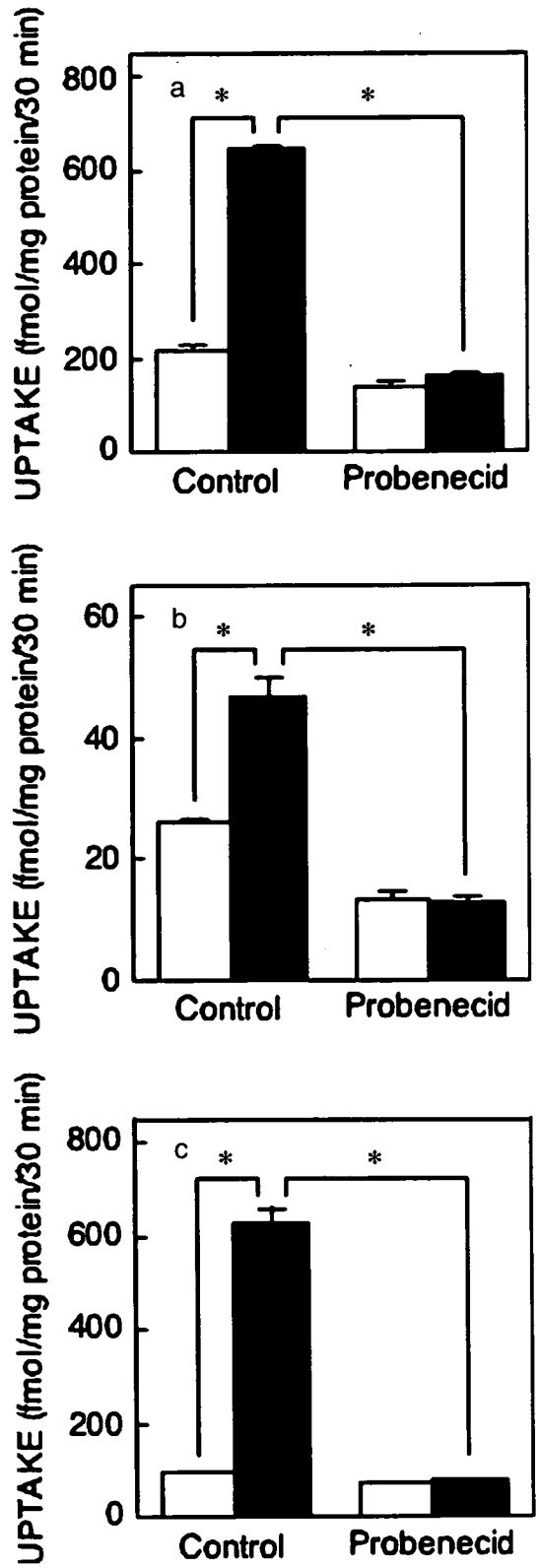

Fig. 2. Effect of probenecid on hOAT3-mediated uptake of adefovir (**a**), cidofovir (**b**) and tenofovir (**c**). HEK-pBK (*open column*) and HEK-hOAT3 (*closed column*) were incubated with 111 nM [^{3}H]adefovir, 66.7 nM [^{3}H]cidofovir or 100 nM [^{3}H]tenofovir in the absence (control) or presence of 1 mM probenecid for 30 min. Each column represents the mean±S. E. of the uptake amounts of each antiviral in three monolayers. *, $P<0.001$, significantly different.

Table I. Uptake of Adefovir, Cidofovir and Tenofovir by HEK293 Cells Stably Expressing hOAT1 or hOAT3

Compounds	Control	hOAT1 μl/mg protein/5 min	hOAT3
p-Aminohippurate	2.19±0.08	24.9±0.6**	3.38±0.17
Estrone sulfate	1.41±0.03	1.98±0.05*	14.4±0.1**
Adefovir	0.369±0.007	46.1±0.5**	1.38±0.08
Cidofovir	0.135±0.010	22.5±0.4**	0.295±0.024
Tenofovir	0.260±0.018	26.0±0.7**	1.22±0.07

HEK-pBK (control), HEK-hOAT1 and HEK-hOAT3 were incubated with 5 μM *p*-[^{14}C]aminohippurate, 17.5 nM [^{3}H]estrone sulfate, 111 nM [^{3}H]adefovir, 66.7 nM [^{3}H]cidofovir or 100 nM [^{3}H]tenofovir for 5 min. Each value represents the mean ± S. E. of the uptake amounts of each compound in three monolayers.
**, $P<0.001$, significantly different from control.
*, $P<0.01$, significantly different from control.

MATERIALS AND METHODS

Materials

p-[^{14}C]Aminohippurate (1.9 GBq/mmol) was obtained from NEN™ Life Science Products Inc. (Boston, MA, USA). [^{3}H]Estrone sulfate, ammonium salt (2.1 TBq/mmol) and [^{14}C]tetraethylammonium bromide (88.8 MBq/mmol) were from Perkin-Elmer Life Sciences Inc. (Boston, MA, USA). [^{3}H]Adefovir (9 Ci/mmol), [^{3}H]cidofovir (15 Ci/mmol) and [^{3}H]tenofovir (10 Ci/mmol) were purchased from Moravek Biochemicals Inc. (Brea, CA, USA). Probenecid was obtained from Sigma (St. Louis, MO, USA). All other chemicals used were of the highest purity available.

Functional Analyses of hOAT1 and hOAT3

The functions of hOAT1 and hOAT3 were evaluated according to our former report (13), using HEK293 cells stably transfected with pBK-CMV vector containing hOAT1 cDNA, hOAT3 cDNA or no cDNA, named HEK-hOAT1, HEK-hOAT3 and HEK-pBK, respectively. In brief, 48 h after the cells were seeded on poly-D-lysine-coated 24-well plates at a density of 2×10^5 cells/well, the uptake of organic compounds by the cells was examined. The composition of the incubation medium was as follows: 145 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM D-glucose and 5 mM HEPES (pH 7.4). After the preincubation of the cells with 0.2 ml of the incubation medium at 37°C for 10 min, the medium was replaced with 0.2 ml of the incubation medium containing test compounds. At the end of the incubation, the medium was aspirated, and then the cells were washed twice with 1 ml of ice-cold incubation medium. The cells were lysed in 250 μl of 0.5 N NaOH solution, and the radioactivity in aliquots was determined in 3 ml of ACSII (Amersham International, Buckingham shire, UK). The protein contents of the solubilized cells were determined by the method of Bradford using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine γ-globulin as a standard.

Table II. Uptake of Adefovir, Cidofovir and Tenofovir by HEK293 Cells Transiently Expressing hOCT2

Compounds	Control	hOCT2 μl/mg protein/30 min
Tetraethylammonium	12.8±0.8	97.7±9.0*
Adefovir	0.964±0.062	0.857±0.026
Cidofovir	0.189±0.023	0.161±0.007
Tenofovir	0.674±0.049	0.653±0.017

HEK293 cells transfected with empty pCMV6-XL4 (control) or hOCT2 cDNA were incubated with 5 μM [^{14}C]tetraethylammonium, 111 nM [^{3}H]adefovir, 66.7 nM [^{3}H]cidofovir or 100 nM [^{3}H]tenofovir for 30 min. Each value represents the mean±S. E. of the uptake amounts of each compound in three monolayers.
*, $P<0.001$, significantly different from control.

Functional Analysis of hOCT2

As previously reported (14), the transient expression system with HEK293 cells was used to examine whether hOCT2 transports the antivirals. Briefly, 1 day after HEK293 cells were seeded on poly-D-lysine-coated 24-well plates at a density of 2×10^5 cells/well, the cells were transfected with plasmid cDNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Forty-eight hours after the transfection, uptake experiments were performed as above described.

Statistical Analysis

Data were statistically analyzed with a one-way analysis of variance followed by Scheffe's test.

RESULTS AND DISCUSSION

First, we investigated the time-dependent uptake of adefovir, cidofovir and tenofovir by HEK-pBK and HEK-hOAT3. As shown in Fig. 1, the expression of hOAT3 enhanced the amounts of these antivirals taken up into the cells, and their uptake by hOAT3 was increased time-dependently. Fig. 2 depicts the effect of probenecid, a typical inhibitor of renal organic anion transporters, on the transport of the antivirals by hOAT3. Probenecid significantly inhibited the hOAT3-mediated uptake of the antivirals. These results indicate that hOAT3 recognizes adefovir, cidofovir and tenofovir as substrates.

Table I represents the amounts of adefovir, cidofovir and tenofovir taken up by HEK-pBK, HEK-hOAT1 and

HEK-hOAT3, including those of *p*-aminohippurate and estrone sulfate, the representative substrates of hOAT1 and hOAT3, respectively. As previously reported (9), hOAT1-mediated transport of adefovir, cidofovir and tenofovir was observed. The uptake amounts of the antivirals via hOAT1 were much greater than those via hOAT3. It is important to compare the kinetic parameters of the hOAT1-mediated versus hOAT3-mediated transport of adefovir, cidofovir and tenofovir. However, we could not perform the experiments on the concentration-dependent uptake, because their unlabeled compounds were not commercially available. Information on the expression levels of hOAT1 and hOAT3 in HEK293 cells would make the data in Table I more significant. We previously quantified mRNA levels of each transporter in HEK-hOAT1 and HEK-hOAT3 to be 64.9 and 225.6 amol/μg total RNA, respectively (13). Because the transport activities of the typical substrates by each transfectant in the present study tended to be similar to those in our previous report (13), it was considered that the mRNA levels of hOAT3 in HEK-hOAT3 used in this study could be higher than those of hOAT1 in HEK-hOAT1. Taking these findings into account, it is suggested that hOAT1 is a potent transporter of adefovir, cidofovir and tenofovir, compared with hOAT3, under the conditions tested in this study. Furthermore, the facts that the blood levels of adefovir and tenofovir in patients are comparable to the concentrations examined in the present study could indicate that hOAT1 is a key transporter mediating the entry of adefovir and tenofovir into the renal tubules from blood.

As described in the Introduction, we recently quantified mRNA levels of drug transporters in normal parts of the kidney cortex from nephrectomized patients and in the renal biopsy specimens of patients with renal diseases. Two important findings were made. First, mRNA levels of hOAT3 were about 3-time higher than those of hOAT1 in the normal kidney cortex (5). Second, mRNA levels of only hOAT1 among hOAT1-4 were significantly lower in the biopsy specimens, compared in the normal segments of the kidney cortex, suggesting that hOAT1 expression is readily influenced by renal diseases (11). Therefore, there is a possibility that the contribution of hOAT3 to tubular uptake of the antivirals could be greater in patients with renal failure.

Now, multidrug resistance protein 4 (MRP4) is thought to be a candidate which transports these antivirals from the proximal epithelial cells into the lumen, because MRP4-mediated efflux of adefovir from cells was observed (15,16) and the functional expression of Mrp4 in the brush-border membrane of the renal proximal tubule was recognized using Mrp4-knockout mice (17). Our previous study using serial sections showed that the localization of hOAT1 and hOAT3 was not completely identical in the proximal tubules (5). An investigation of the exact distribution of MRP4 in the renal proximal tubules and a comparison with hOAT1 and hOAT3 would facilitate elucidation of the mechanisms behind the nephrotoxicity of adefovir, cidofovir and tenofovir.

The transport of adefovir, cidofovir and tenofovir by hOCT2 was also examined in this study. As represented in Table II, no uptake of these antivirals via hOCT2 was observed. Previously, we reported that cimetidine was transported by hOAT1 and hOAT3 as well as hOCT2, and that hOAT3 but not hOAT1 or hOCT2 transported famotidine (18). Because famotidine and cimetidine exist partly in the cationic forms at the experimental pH, the results of the study were surprising, and the substrate recognition of the SLC22A family might be complicated. Although our previous report indicated that preconceived ideas on the substrate recognition of SLC22A members should be reconsidered, the findings of the present study were consistent with the preconception that hOCT2 would not transport the anionic antivirals. hOCT2 is not likely to contribute to the elimination of adefovir, cidifovir and tenofovir.

In conclusion, this is the first report representing that adefovir, cidofovir and tenofovir are substrates of hOAT3 as well as hOAT1. Furthermore, it is suggested that hOAT1 rather than hOAT3 plays a crucial role in the basolateral uptake of the antivirals into the renal proximal tubules. These findings provide useful information for the elucidation of the molecular mechanisms of disposition and nephrotoxicity of adefovir, cidofovir and tenofovir.

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Pharmacokinetic significance of luminal multidrug and toxin extrusion 1 in chronic renal failure rats

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ABSTRACT

Functional and expressional depression of the rat organic cation transporter rOCT2 after 5/6 nephrectomy (Nx) is accompanied by the decreased plasma level of testosterone in the male rats. Though vectorial transport across the tubular epithelial cells is important in the secretion of cationic compounds, there has been no imformation about the luminal organic cation transporter in disease state. In the present study, the role of luminal multidrug and toxin extrusion 1 (rMATE1) was examined using female rats with or without Nx, avoiding the influence of testosterone. The tubular secretion of cimetidine was markedly decreased in female Nx rats as well as male rats. Unlike in the male rats, the plasma level of testosterone and the expression of basolateral rOCT2 were unchanged in the female rats after Nx. On the other hand, the expression of rMATE1 was markedly decreased in both male and female Nx rats, and the level of rMATE1, but not of rOCT2, correlated well with the tubular secretion of cimetidine in the female rats ($r = 0.74$). Immunohistochemical analysis revealed that rMATE1 and Na^+/H^+ exchanger (NHE) 3 were localized at the brush-border membrane of proximal tubules. The level of NHE3 was also markedly depressed in both male and female Nx rats, suggesting that the expression level on the luminal rMATE1 in combination with NHE3 was indicated to be a crucial factor for the tubular secretion of cimetidine.

1. Introduction

The tubular transport of organic substances plays an essential role in the removal of *xenobiotics* including drugs and numerous chemicals contained in our environment and some metabolites. Basolateral types of organic anion and cation transporters, OAT1 (SLC22A6), OAT3 (SLC22A8), OCT1 (SLC22A1) and OCT2 (SLC22A2), have been cloned and characterized [1–4]. These transporters are indicated to mediate the basolateral entry of various anionic or cationic drugs into the proximal tubular epithelial cells [5]. Recently, a renal luminal type of H^+/organic cation antiporter, multidrug and toxin extrusion 1 (MATE1), has been cloned, and considered to be responsible for the final step in the excretion of organic cations [6–8]. However, the pharmacokinetic and pathophysiological role of the luminal MATE1 in combination with the basolateral OCT2 is not clear. Furthermore, there has been no information regarding the change in luminal MATE1 during chronic renal failure.

More gene product of rat (r) OCT2 is found in the male kidney than female kidney, and the expression of rOCT2 in the kidney is regulated by the plasma level of testosterone [9,10]. We previously found that the renal tubular secretion of *para*-aminohippuric acid and cimetidine were markedly decreased in male 5/6 nephrectomized (Nx) rats, the protein expression of rOCT2 but not of rOCT1, rOAT1, or rOAT3 was depressed in male Nx rats [11]. Furthermore, a lowered plasma level of testosterone was likely to be responsible for the depressed rOCT2 expression in chronic renal failure.

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Based on these findings, the female rats were hypothesized to be a good animal model with which to evaluate the pharmacokinetic significance of the luminal organic cation transport system, avoiding the influence of hormonal regulation of the basolateral rOCT2. In the present study, we examined the renal handling of cimetidine, a substrate for the renal organic cation transport system, in rats after Nx. Furthermore, the expression levels of renal organic ion transporers were examined to clarify the responsible factor in the tubular secretion of cationic drugs in the kidney.

2. Materials and methods

2.1. Experimental animals

For ablation of the renal mass, male and female Wistar albino rats (180–200 g) were anesthetized with sodium pentobarbital (50 mg/kg) and the kidneys were exposed under aseptic conditions via a ventral abdominal incision. The right kidney was removed, the posterior and anterior apical segmental branches of the left renal artery were individually ligated, and the abdominal incision was closed with 4-0 silk sutures. In the sham-operated animals, the peritoneal cavity was exposed, and both kidneys were gently manipulated. To examine the effect of the administration of testosterone on drug pharmacokinetics and the renal expression of transporters, the sham-operated and Nx rats were administered a subcutaneous injection of testosterone (0.5 mg testosterone enanthate (T) dissolved in 200 μL corn oil/rat, T(+)) or vehicle (200 μL corn oil/rat, T(−)) at 1, 4, 7, 10 and 13 days after surgery. Except during the subcutaneous administration of testosterone every 3 days, animals were allowed access to water and standard rat chow for 2 weeks.

Rats were maintained in metabolic cages for 24 h before the in vivo experiment, to determine urine output and urinary levels of creatinine. The blood urea nitrogen (BUN) concentration was determined by the urease/indophenol method. The levels of creatinine in plasma and urine were determined with the Jaffé reaction. For measurements, we used assay kits from Wako Pure Chemical Industries (Osaka, Japan). The plasma testosterone and 17β-esrtadiol level was measured with an enzyme immunoassay kit (Cayman Chemical Co., MI, USA). The experiments with animals were performed in accordance with the *Guidelines for Animal Experiments of Kyoto University*.

2.2. Infusion experiment

Rats were anesthetized with an intraperitoneal administration of 50 mg/kg sodium pentobarbital. Catheters were inserted into the right femoral artery and the left femoral vein with polyethylene tubing (Intramedic PE-50, Becton Dickinson and Co., Parsippany, NJ, USA) filled with a heparin solution (100 U/mL) for blood sampling and drug administration, respectively. Urine was collected from the urinary bladder catheterized with PE-50 tubing. Thereafter, cimetidine was administered as a bolus via the femoral vein and incorporated into the infusion solution as described [12]. The loading and maintenance doses of cimetidine including 4% mannitol were 317 μmol/kg and 21.8 μmol/mL, respectively. The infusion rate was 2.2 mL/h using an automatic infusion pump (Natsume Saisakusho, Tokyo, Japan). Mannitol was used to maintain a sufficient and constant urine flow rate. After a 30-min equilibration period, urine samples were collected three times at 10 min intervals, and blood samples were obtained at the midpoint of urine collection. The plasma was immediately separated from erythrocytes by centrifugation. At the end of the experiment, an adequate volume of blood was collected from the abdominal aorta to examine the plasma protein binding rate, and the kidneys were removed to determine the tissue concentrations of cimetidine and the expression of renal drug transporters. The concentrations of cimetidine in plasma, urine, and the renal homogenate were determined by high performance liquid chromatography [13]. The plasma unbound fraction (fu) of cimetidine was determined by ultrafiltration using a micropartition system (MRS-1, Amicon Inc., Beverly, MA, USA), as described [12]. The free fraction of cimetidine was expressed as the ratio of the concentration in the ultrafiltrate to that in plasma.

2.3. Analytical methods

Pharmacokinetic parameters were calculated using standard procedures for each experimental period. The total plasma clearance (Ctot) was calculated by dividing the infusion rate by the steady-state plasma concentration (Cpss) at the midpoint of urine collection. Renal clearance (Cren) was obtained by dividing the urinary excretion rate by Cpss. The renal clearance of unbound cimetidine (Cr.f) was determined by dividing Cren by the fu of cimetidine. The glomerular filtration rate (GFR) was assumed to be equal to the Cren of creatinine. The renal secretory clearance of unbound cimetidine was calculated by substracting GFR from Cr.f.

2.4. Polyclonal antibody against rMATE1 and Western blot analysis

Polyclonal antibody was raised against the synthetic peptide that corresponded to the C-terminus of MATE1, which is fully conserved in human and rat [8]. The crude plasma membrane fractions were prepared from rat kidneys, as described previously [14]. The crude plasma membrane fractions were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA, USA) by semi-dry electroblotting. The blots were blocked with 5% non-fat dry milk and 5% bovine serum albumin in phosphate-buffered saline (PBS, 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1 mM KH_2PO_4, and 12 mM K_2HPO_4, pH 7.5) containing 0.5% Tween 20 (PBS-T) for OAT1, OAT3, OCT1, OCT2 and Na^+/K^+-ATPase or 5% non-fat dry milk in Tris-buffered saline (TBS, 20 mM Tris and 137 mM NaCl) containing 0.5% Tween 20 (TBS-T) for MATE1. The blots were then incubated overnight at 4 °C with primary antibody specific for rOAT1[11], rOAT3 [11], rOCT1 [15], rOCT2 [9], rMATE, NHE3 (CHEMICON International Inc., Temecula, CA, USA), Na^+/K^+-ATPase (Upstate Biotechnology Inc., Lake Placid, NY, USA), or with an antibody preabsorbed with the synthetic antigen peptide (20 μg/mL) for rMATE1. The blots were washed three times with PBS-T or TBS-T, and the bound antibody was detected on X-ray film by enhanced chemiluminescence (ECL) with

horseradish peroxidase-conjugated secondary antibodies and cyclic diacylhydrazides (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA). Na^+/K^+-ATPase was examined as a positive control. The relative amounts of the bands in each lane were determined densitometrically using NIH Image 1.61 (National Institutes of Health, Bethesda, MD), and the densitometric ratios relative to each control (Sham or Sham T(−)) were used as the reference and accorded an arbitrary value of 1.0, respectively.

2.5. *Immunohistochemical analysis*

The animals were anesthetized and the kidneys were perfused via the abdominal aorta, first with saline containing 50 U/mL of heparin and then with 4% paraformaldehyde in PBS. Fixed tissues were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen rapidly in liquid nitrogen. Sections (5 μm thick) were cut and covered with a blocking agent Blocking One® (Nacalai Tesque, Kyoto, Japan) containing 1 mg/mL RNase A (Nacalai Tesque) at 37 °C for 30 min. The covered sections were incubated at 37 °C for 60 min with antiserum specific for MATE1 (1:100 dilution) or anti-NHE3 antibody (1:200). Following two washings each with 3 × PBS and regular PBS, sections were incubated with Cy3-labeled donkey anti-rabbit IgG (CALTAG Laboratory, San Francisco, CA, USA), Alexa 488-Phalloidin (Molecular Probe, Eugene, OR, USA), and 4′,6-diamidino-2-phenylindole (DAPI; Wako, Osaka, Japan) at 37 °C for 60 min. These sections were examined and captured with a BZ-8000 (KEYENCE, Osaka, Japan) at 150× magnification.

2.6. *Statistical analysis*

All data are expressed as means ± S.E. Data from the Western blot analysis are expressed in arbitrary units of densitometry/25 μg protein. Comparisons were made using the unpaired t-test. $P < 0.05$ was considered significant.

3. Results

3.1. *Renal functional data after administration of testosterone in female rats with chronic renal failure*

As shown in Table 1, body weight tended to decline and 24 h urine volume was markedly increased in female Nx rats. The levels of BUN and creatinine in plasma (Pcr) were significantly increased, and the creatinine clearance (Ccr) was markedly decreased in female Nx rats in comparison with sham-operated controls. These parameters were consistent with our previous report using male Nx rats [11]. Unlike in the male rats, plasma level of testosterone was comparable between female sham-operated rats and Nx rats. Although an elevation in the plasma level of testosterone on the administration of testosterone was confirmed to occur both in sham-operated and in Nx rats, the plasma concentration of 17β-estradiol was not significantly changed. In addition, none of the renal functional data changed significantly after the administration of testosterone in female rats.

3.2. *Pharmacokinetics of cimetidine after administration of testosterone in female rats with chronic renal failure*

Table 2 shows the pharmacokinetic parameters of cimetidine in female rats with or without treatment with testosterone. The steady-state plasma concentration (Cpss) of cimetidine was markedly elevated in the Nx rats in comparison with sham-operated controls. The total plasma clearance (Ctot) of cimetidine was significantly decreased in female Nx rats compared with the controls. The renal clearance (Cren) of cimetidine in female sham-operated rats was about 60% of that in male sham-operated rats according to our previous report [11]. In female rats, the Cren of cimetidine was markedly decreased by Nx to 43% of that in the sham-operated controls. The ratio between the renal concentration and Cpss of cimetidine (Kp) was shown to be significantly decreased in Nx females compared to the sham-operated controls. Similar to the renal functional data in Table 1, there was no significant influence on the pharmacokinetic parameters of cimetidine in female rats with or without the administration of testosterone.

As shown in Fig. 1, the tubular secretory clearance (Csec) of cimetidine in female sham-operated rats was about 50% of that in the sham-operated males. In male and female Nx rats, it was markedly decreased. After the administration of testosterone, the Csec of cimetidine in male Nx rats recovered significantly to be 80% of that in male controls, but that in female Nx rats did not change at all.

3.3. *Gender difference in protein expression of rOCT2, rMATE1 and NHE3*

At first, a primary band with a size of 66 kDa was detected using the antibody raised against rMATE1 (Fig. 2(A)). The

Table 1 – Renal functional data and plasma testosterone and 17β-estradiol levels after the administration of testosterone in female rats

	Body weight (g)	Urine volume (mL/24 h)	Pcr (mg/dL)	Ccr (mL/min kg)	BUN (mg/dL)	Testosterone (ng/mL)	17β-Estradiol (pg/mL)
Sham T(−)	212 ± 10	18.2 ± 2.2	0.52 ± 0.08	6.0 ± 1.0	16.7 ± 0.7	0.1 ± 0.0	51.3 ± 10.1
Sham T(+)	228 ± 9	17.8 ± 2.0	0.55 ± 0.07	5.2 ± 0.8	18.1 ± 0.9	5.5 ± 0.7**	30.1 ± 14.4
Nx T(−)	199 ± 7	30.3 ± 5.4**	1.20 ± 0.12**	2.3 ± 0.3**	59.4 ± 5.6**	0.2 ± 0.0	58.6 ± 14.4
Nx T(+)	207 ± 6	31.7 ± 3.3**	1.12 ± 0.11**	2.4 ± 0.3**	58.7 ± 5.3**	5.0 ± 0.6**	38.7 ± 8.3

Values are means ± S.E. for 6–10 rats; Pcr, plasma creatinine; Ccr, creatinine clearance; BUN, blood urea nitrogen; Sham T(−), sham-operated rats administered vehicle; Sham T(+), sham-operated rats administered testosterone, Nx T(−), 5/6 nephrectomized rats administered vehicle; Nx T(+), 5/6 nephrectomized rats administered testosterone. **$P < 0.01$, significantly different from Sham T(−) rats.

Table 2 – Pharmacokinetic parameters of cimetidine in infusion experiments after the administration of testosterone in female rats

	Cpss (μmol/L)	fu	Ctot (mL/min kg)	Cren (mL/min g kid)	Kp
Sham T(−)	254 ± 12	0.74 ± 0.04	15.5 ± 0.9	1.4 ± 0.1	1.6 ± 0.0
Sham T(+)	260 ± 20	0.76 ± 0.01	13.8 ± 0.6	1.4 ± 0.2	1.6 ± 0.1
Nx T(−)	409 ± 17**	0.70 ± 0.05	9.5 ± 0.3**	0.6 ± 0.1**	0.9 ± 0.1**
Nx T(+)	405 ± 30**	0.78 ± 0.04	9.4 ± 0.7**	0.6 ± 0.2**	1.0 ± 0.1**

Each value represents the mean ± S.E. of six rats; Cpss, steady-state plasma concentration; Ctot, total clearance; Cren, renal clearance; fu, plasma unbound fraction; Kp, tissue to plasma concentration ratio. Sham T(−), sham-operated rats administered vehicle; Sham T(+), sham-operated rats administered testosterone, Nx T(−), 5/6 nephrectomized rats administered vehicle; Nx T(+), 5/6 nephrectomized rats administered testosterone. **$P < 0.01$, significantly different from Sham T(−) rats.

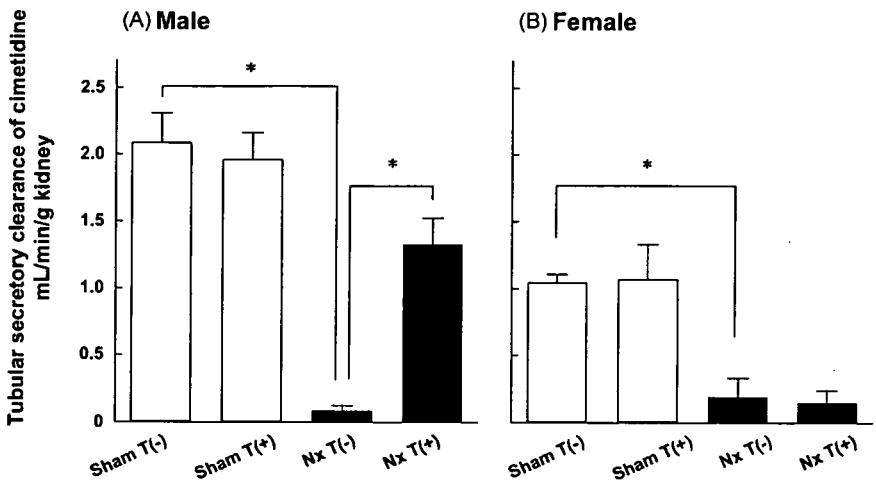

Fig. 1 – (A and B) Renal secretory clearance of unbound cimetidine. Cimetidine (21.8 μmol/mL) was infused at a rate of 2.2 mL/h using an automatic infusion pump. The renal secretory clearance was calculated by substracting GFR from Cr.f. Each column represents the mean ± S.E. for six rats. *Statistically significant difference. Sham T(−), sham-operated rats administered vehicle; Sham T(+), sham-operated rats administered testosterone enanthate; Nx T(−), 5/6 nephrectomized rats administered vehicle; Nx T(+), 5/6 nephrectomized rats administered testosterone enanthate.

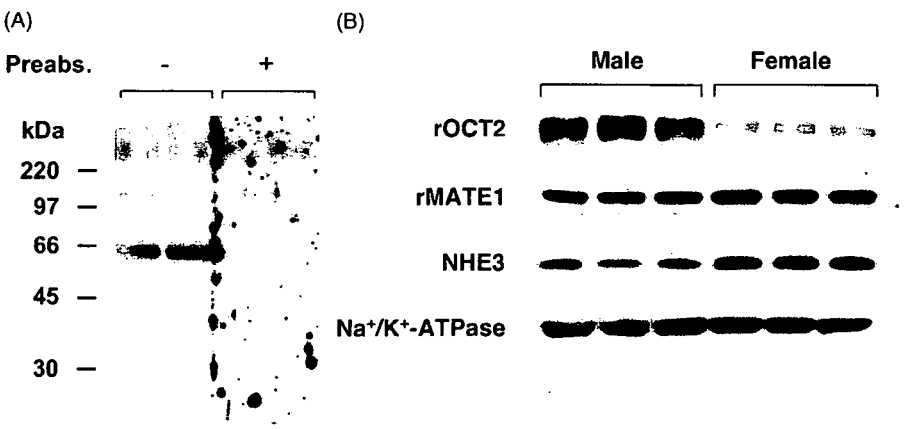

Fig. 2 – Protein expression of rOCT2, rMATE1, NHE3 and Na^+/K^+-ATPase in male and female rats. Crude plasma membrane fractions (25 μg) from total kidneys were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and blotted onto Immobilon® membranes. (A) The antisera (1:1000 dilution) for rMATE1 was preabsorbed (Preab.) with (+) or without (−) antigen peptide (20 μg/mL) of rMATE1. (B) Antisera specific for rOCT2, rMATE1, NHE3 and Na^+/K^+-ATPase (1:1000–10,000 dilution) were used as primary antibodies.

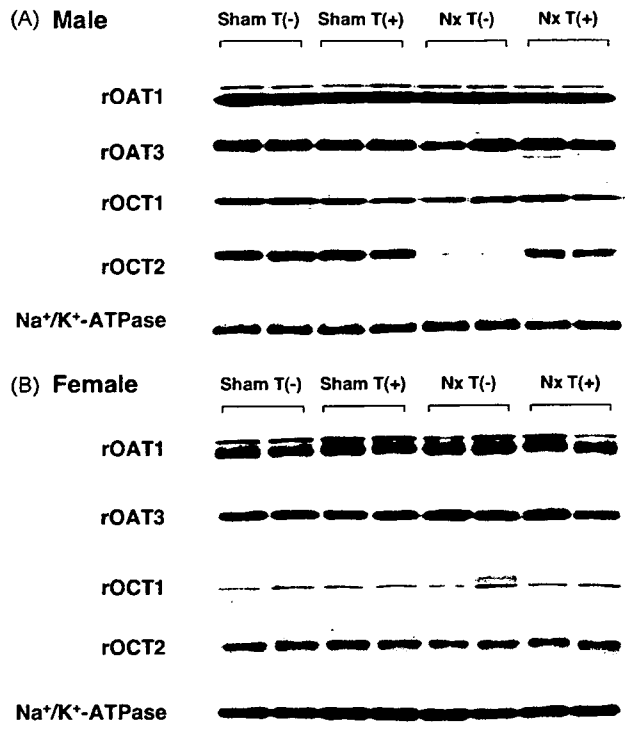

Fig. 3 – Protein expression of organic ion transporters in sham-operated and Nx rats after treatment with testosterone. Crude plasma membrane fractions (25 μg) from total kidneys were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and blotted onto Immobilon® membranes. The expression levels of various transporters in male (A) and female (B) rats. Antisera specific for rOAT1, rOAT3, rOCT1, rOCT2, and Na^+/K^+-ATPase (1:1000–10,000 dilution) were used as primary antibodies. Sham T(−), sham-operated rats administered vehicle; Sham T(+), sham-operated rats administered testosterone enanthate; Nx T(−), 5/6 nephrectomized rats administered vehicle; Nx T(+), 5/6 nephrectomized rats administered testosterone enanthate.

preabsorption of antibody with antigen peptide abolished this band, showing the presence of rMATE1 protein in the rat kidney. The level of rOCT2 in female rats was about 25% of that in male rats (Fig. 2(B)). On the other hand, the expression level of rMATE1 was comparable between sexes. The level of NHE3 was slightly, but not significantly, higher in female rats compared to male rats.

3.4. *Protein expression of basolateral organic ion transporters in Nx rats*

Consistent with our previous findings [11], the protein expression of rOCT2 was markedly depressed in male Nx rats, and recovered to around the control level with the administration of testosterone (T(+)) (Fig. 3(A)). In addition, there was no influence of Nx on the administration of testosterone on the expression levels of rOAT1, rOAT3, rOCT1 and Na^+/K^+-ATPase. In female rats, the levels of the basolateral organic ion transporters, rOAT1, rOAT3, rOCT1 and rOCT2, and Na^+/K^+-ATPase were not affected by Nx with or without the administration of testosterone (Fig. 3(B)).

Although the level of rOCT2 protein correlated well with the Csec of cimetidine in the male rats, no significant correlation was observed in the female rats (Fig. 4).

3.5. *Expressional change and pharmacokinetic significance of luminal rMATE1 and NHE3 in Nx rats*

Next, we examined the expressional change of the luminal H^+/organic cation antiporter rMATE1 and Na^+/H^+ exchanger 3 (NHE3), which generates an inward H^+-gradient at the brush-border membranes. Western blot analysis revealed that the levels of rMATE1 and NHE3 were markedly decreased by Nx in both male and female rats (Fig. 5).

The coefficient of correlation between the Csec of cimetidine and the level of rMATE1 was 0.72 ($P = 0.0037$) in male rats, whereas the data was relatively scattered (Fig. 6(A)). In contrast, the Csec of cimetidine showed better correlation

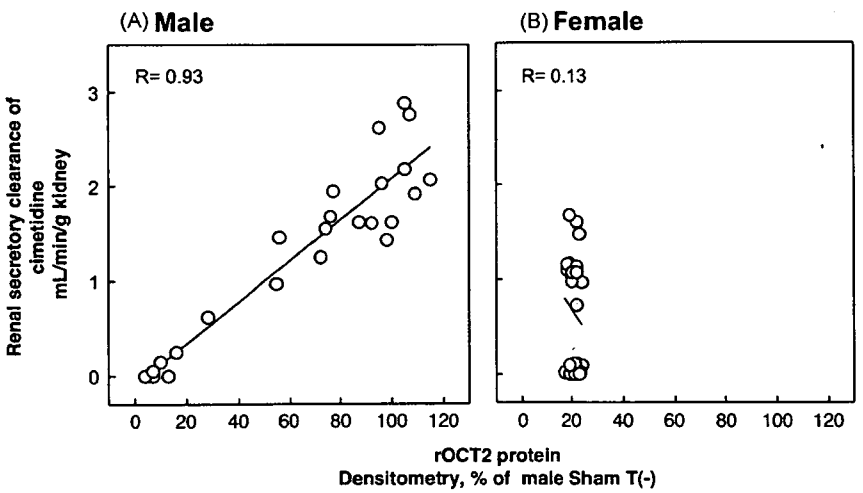

Fig. 4 – Correlation between rOCT2 expression and the renal clearance of cimetidine in rats. Correlation between the rOCT2 protein expression and renal secretory clearance of unbound cimetidine in male (A, open circles) and female rats (B, closed circles). The rOCT2 protein level was determined as outlined in Section 2. Sham T(−), sham-operated rats administered vehicle.

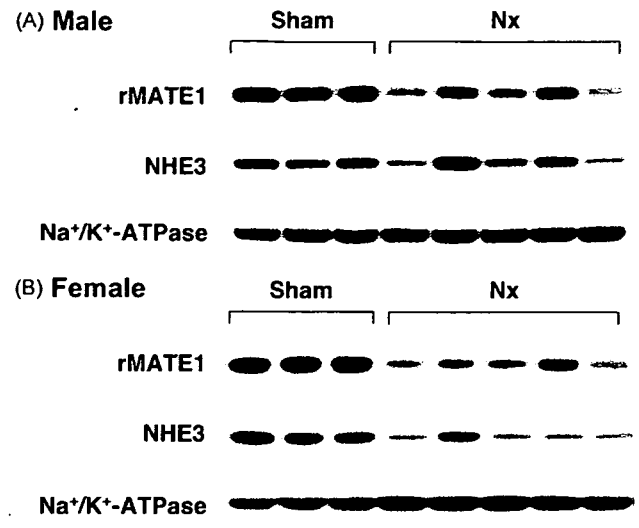

Fig. 5 – (A and B) Protein expression of rMATE1 and NHE3 in sham-operated and Nx rats. Crude plasma membrane fractions (25 μg) from total kidneys were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10%) and blotted onto Immobilon® membranes. Antisera specific for rMATE1, NHE3, and Na^+/K^+-ATPase (1:1000 dilution) were used as primary antibodies. Sham, sham-operated rats; Nx, 5/6 nephrectomized rats.

with the level of rMATE1 protein in female rats ($r = 0.74$, $P = 0.0036$) (Fig. 6(B)).

3.6. *Immunohistochemical analysis of rMATE1 and NHE3*

An immunohistochemical analysis was performed to examine the localization of rMATE1 and NHE3 in female rats. Positive staining for rMATE1 and NHE3 was detected in the brush-border membranes of proximal tubules (Fig. 7(A) and (B)). Both rMATE1 and NHE3 was abundant in the renal cortex. In addition, it seemed that the localization of rMATE1 and NHE3 was not affected by Nx (Fig. 7(C)–(F)).

4. Discussion

Functional changes in renal organic ion transporters are of clinical relevance, particularly to the use of drugs with that are highly toxic and have a narrow therapeutic index. Serious kidney disease, such as chronic renal failure, will influence the renal disposition of organic ions and the expression of drug transporters. Our previous study demonstrated that the mRNA expression levels of OAT-K1 and OAT-K2 were markedly diminished after Nx, and the renal clearance of methotrexate was markedly decreased in Nx rats compared with sham-operated rats [16]. Furthermore, the renal secretion of cimetidine and the level of basolateral rOCT2 was also decreased under chronic renal failure in male rats, and the reduced plasma level of testosterone was considered to cause these phenomena [11]. Although two steps of transmembrane transport were thought to be involved in the vectorial secretion of cationic drugs, there has been no report about the luminal MATE1 in renal disease state. Sex-hormonal levels differ markedly between the genders, and recently transporters such as rOCT2, rOAT2 and oatp1 have been reported to exhibit gender differences in their expression in the rat kidney [9,17,18]. Based on these findings, we hypothesized that female rats would show significantly different changes in the urinary excretion of cimetidine and the expression of organic ion transporters during chronic renal failure, avoiding the influence of testosterone. In the present study, to obtain more information about the pharmacokinetic significance of cationic drug excretion systems focusing on luminal transporter, rMATE1, we have examined the renal excretion of the cationic substrate cimetidine and the expression of organic ion transporters in male and female Nx rats.

Consistent with previous findings, the decreased expression level of the renal rOCT2 and the Csec of cimetidine were recovered by the administration of testosterone in the male Nx

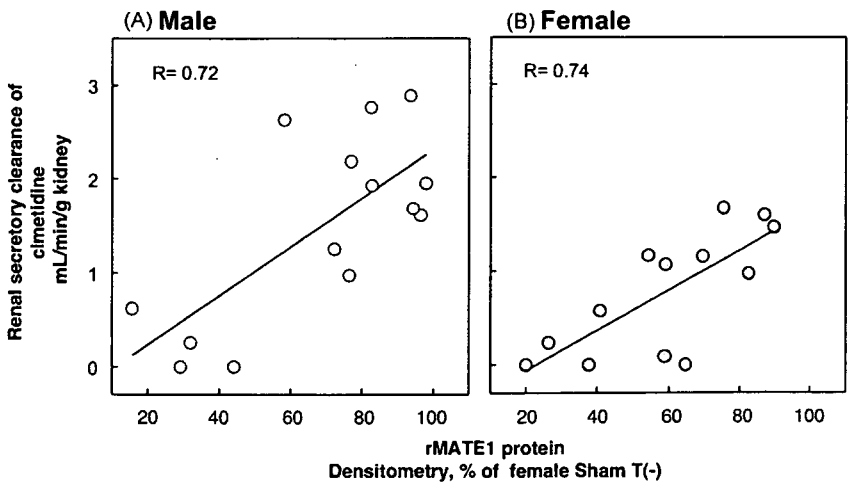

Fig. 6 – Correlation between rMATE1 expression and the renal clearance of cimetidine in rats. Correlation between the rMATE1 protein expression and renal secretory clearance of unbound cimetidine in male (A, open circles) and female rats (B, closed circles). The rMATE1 protein level was determined as outlined in Section 2. Sham T(−), sham-operated rats administered vehicle.

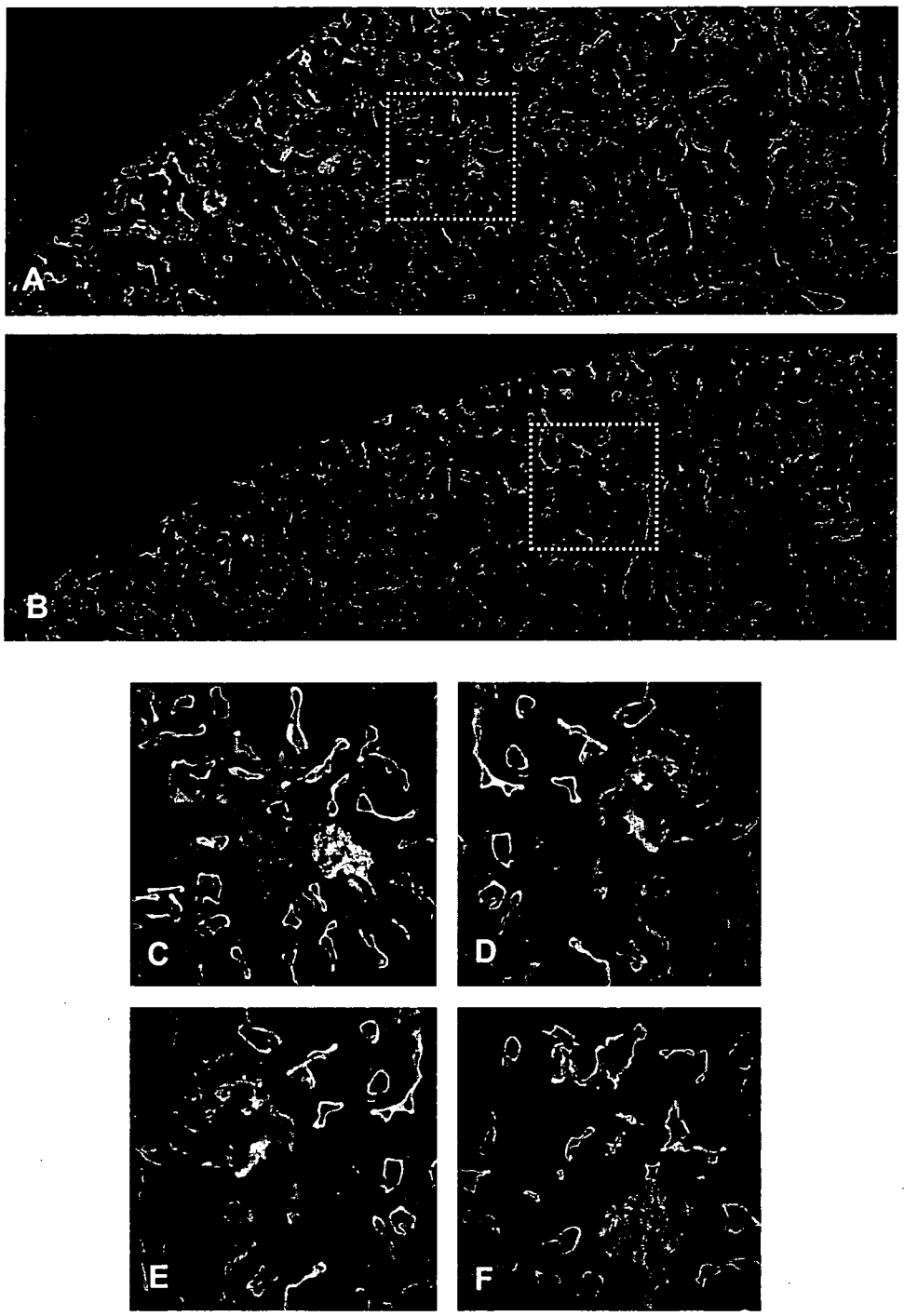

Fig. 7 – Immunohistochemistry of rMATE1 and NHE3 in female sham-operated and Nx rats. The rMATE1 or NHE3 (red), F-actin (green), and DAPI (blue) signals were merged in the same section. The yellow signals consisting of rMATE1 or NHE3 and F-actin were concentrated in the brush-border membranes of proximal tubules. The localization of rMATE1 (A) and NHE3 (B) in sham-operated rats were relatively condensed around the cortex. The luminal localization of rMATE1 (C and D) and NHE3 (E and F) in sham-operated (C and E) and in Nx (D and F) rats were confirmed. Magnification: 150×.

rats (Figs. 1(A) and 3(A)). In the female rats, the renal Csec of cimetidine was about 50% of that in the male rats (Fig. 1(B)). The expression levels of basolateral transporters were not affected by Nx and testosterone. In addition, no correlation was observed between the renal level of rOCT2 and the Csec of cimetidine (Fig. 4(B)). In infusion experiments, tissue to plasma concentration ratio (Kp) was significantly decreased in Nx rats. In Nx rats, the level of the tubular sodium transporters were markedly decreased and the plasma levels of sodium and potassium were significantly changed [19]. This suggested that the difference in the membrane potential at the basolateral side of renal epithelial cells were also changed and

the uptake of cationic drugs in epithelial cells was reduced, though the expressional change of rOCT2 was not observed. Considering these results, only the level of basolateral rOCT2 in the kidney could not explain the entire vectorial secretion of the cationic drugs.

Recently, the luminal H^+/organic cation antiporters, human (h) MATE1, hMATE2-K, rMATE1 and mouse MATE1 have been cloned and characterized [6–8,20]. rMATE1 was mainly expressed in the kidney and placenta, and suggested to be a major contributor to the tubular H^+/organic cation antiport activity in rats [7]. In contrast, hMATE2-K was isolated as a second member of the MATE family in the human kidney, despite no counterpart gene having been identified in rats [8]. Most recently, a platinum agent oxaliplatin was found to be a superior substrate for hMATE2-K rather than hMATE1, and therefore, the extensive tubular secretion of oxaliplatin via hMATE2-K were suggested to be a mechanism behind the lowered renal toxicity of the drug [21].

In the female as well as male rats, the level of rMATE1 was markedly depressed by the Nx (Fig. 5). In contrast to the level of basolateral rOCT2, the level of rMATE1 correlated well with the Csec of cimetidine in the female rats. Considering that there was no significant alteration to the expression of the basolateral transporters (rOAT1, rOAT3, rOCT1 and rOCT2) in the female rats following Nx, the luminal rMATE1 should play a crucial role in the tubular secretion of cimetidine in the female rats.

In the rat renal tubular brush-border membranes, the luminal secretion of cimetidine as well as tetraethyl ammonium was mediated by an electroneutral H^+/organic cation antiport system driven by an inward H^+-gradient, and that H^+-gradient was mainly created by NHE [22–24]. At the brush-border membranes of proximal tubules, the type 3 NHE are considered dominant among the NHE family [25,26]. In the present study, we also examined the expression level and membrane localization of NHE3 as well as rMATE1 in the female rats. The immunohistochemical analysis using antibodies specific for rMATE1 or NHE3 strongly suggested that both transporters cooperatively function on the luminal side of the proximal tubules, especially in the superficial cortex (Fig. 7(A) and (B)), i.e., rMATE1-mediated tubular secretion of cationic compounds could be driven by the NHE3-created inward H^+-gradient.

The expression of NHE3 was depressed after Nx in both the male and female rats (Fig. 5). Kwon et al. [19] showed that the level of the tubular sodium transporters including NHE3 were significantly decreased in Nx rats. Furthermore, they demonstrated that the localization of NHE3 was also found at the brush-border membranes in the Nx rats, which was comparable with our present results. Therefore, the decreased renal tubular secretion of cimetidine was not only due to the decreased in the expression of rMATE1, but also the functional loss of this transporter via a lowered H^+-gradient at the brush-border membrane, caused by the decrease in NHE3.

In conclusion, we have advanced our findings on the renal handling of cationic drugs, demonstrating that the level of luminal rMATE1 was markedly decreased during chronic renal failure, and rMATE1 as well as the basolateral rOCT2 played a crucial role in the vectorial transport of organic cations as detoxicating factors. In addition, the expression of NHE3 was also depressed in Nx rats, and the localization of NHE3 was similar to that of rMATE1, suggesting the expression level of NHE3 might affect rMATE1 activity by creating an inward H^+-gradient.

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Downregulation of organic anion transporters in rat kidney under ischemia/reperfusion-induced qacute renal failure

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The effect of acute renal failure (ARF) induced by ischemia/reperfusion (I/R) of rat kidney on the expression of organic anion transporters (OATs) was examined. The level of serum indoxyl sulfate (IS), a uremic toxin and substrate of OATs in renal tubules, shows a marked increase with the progression of ARF. However, this increase was significantly attenuated by ingestion of cobalt. The level of mRNA and protein of both rOAT1 and rOAT3 were markedly depressed in the ischemic kidney. The uptake of *p*-aminohippuric acid (PAH) and estrone sulfate (ES) by renal slices of ischemic rats was significantly reduced compared to control rats. Renal slices taken from ischemic rats treated with cobalt displayed significantly elevated levels of ES uptake. Cobalt intake did not affect PAH uptake, indicating the functional restoration of rOAT3 but not rOAT1. The expression of Na^+/K^+-ATPase was markedly depressed in the ischemic kidney, suggesting that the inward Na^+ gradient in renal tubular cells had collapsed, thereby reducing the outward gradient of α-ketoglutarate, a driving force of both rOATs. The decreased expression of Na^+/K^+-ATPase was significantly restored by cobalt treatment. Our results suggest that the downregulation of renal rOAT1 and rOAT3 could be responsible for the increase in serum IS level of ischemic rats. Cobalt treatment has a significant protective effect on ischemia-induced ARF, being accompanied by the restoration of rOAT3 and/or Na^+/K^+-ATPase function.

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Together with the liver, the kidney plays a principal role in the excretion of a wide variety of xenobiotics, including drugs and toxins, as well as endogenous compounds. In the renal proximal tubules, several unidirectional solute transport systems facilitate active secretion of a wide range of exogenous and endogenous organic ions into urine.[1–3] Transport proteins for organic anions and cations localized at the apical and basolateral plasma membranes of the proximal tubular cells appear to mediate urinary secretion of endogenous substances and various drugs.[1,4,5] To date, the structure and function of several members of the organic anion transporter (OAT) and organic cation transporter (OCT) gene family (e.g. SLC22A), which mediate trans-epithelial transport of organic ions, have been characterized.[1,4,5] It has been suggested that ATP-dependent primary active transporters, such as multidrug resistance protein/P-glycoprotein and members of the multidrug resistance-associated protein gene family, function as efflux pumps of renal tubular cells to mediate active extrusion of hydrophobic molecules and anionic conjugates.[1,6] Therefore, the functional and molecular variations of these transporters should have an impact on renal clearance of their substrate drugs, causing alteration of pharmacokinetics and/or unexpected adverse side effects of accumulated drugs in the body.

Acute renal failure (ARF) caused by ischemia/reperfusion (I/R) is a crucial clinical issue. Although progress has been made in the diagnosis and treatment of patients with ARF, there is still a high mortality rate associated with this condition.[7–9] I/R-induced ARF is evoked by a complicated interaction between renal hemodynamics, inflammatory cytokines, endothelial, and tubular cell injury.[10] Although the kidney receives about 25% of the cardiac output, the majority goes to the cortex. Therefore even a slight decline in renal blood flow can lead to hypoxic injury of the medullary region.[11] I/R-induced injury to the renal medulla plays a significant role in ARF. In fact, the S3 segment of the proximal tubule in the outer medulla has been shown to be the most susceptible portion of the kidney to I/R-induced ARF.[12] In a recent report, preconditioning of rats with cobalt chloride resulted in improvement of ischemic renal injury.[13]

Cobalt chloride is thought to act by stabilizing hypoxia-inducible factor 1, thereby inducing erythropoietin, glycolytic enzymes, P-glycoprotein, and the glucose transporter.[14–17]

The serum level of the uremic toxin indoxyl sulfate (IS) is markedly elevated in uremic patients[18] and in 5/6 nephrectomized rats,[19] a well-established animal model for chronic renal failure. IS appears to be a substrate for the basolaterally localized transporters OAT1 and OAT3.[20,21] In the 5/6 nephrectomized rats, the renal expression of rOAT1 and rOAT3 was markedly downregulated in chronic renal failure.[22,23] In the clinical situation, patients with renal disease showed a downregulation of hOAT3, which is thought to be responsible for a decreased urinary excretion of cefazolin, an anionic cephalosporin antibiotic.[24] However, there is little information concerning the regulation of renal organic solute transporters in ARF. Such data would be useful for understanding the pharmacokinetic profile of drugs that are excreted mainly into the urine during the treatment of patients with renal impairment.

In this study, we explored the serum levels of IS and the regulation of OATs mediating urinary excretion of endogenous anionic toxins in rats with I/R-induced ARF.

RESULTS

Renal functional data of ischemic rats

Figure 1 summarizes the changes in body weight, blood urea nitrogen (BUN) and serum creatinine (SCr) level of sham-operated rats (control) and I/R-induced ARF rats with and without cobalt chloride in the drinking water. The body weight of both control and ischemic rats decreased until 12 h after sham operation or I/R (Figure 1a). The body weight of control rats recovered to their initial level, whereas ischemic rats showed a further loss in body weight 24 h after ischemia. Body weights of sham rats at 48 h was decreased by cobalt intake. I/R rats also showed a decrease in body weights at 48 h by cobalt intake. The serum BUN level was markedly elevated in ischemic rats from 6 h after I/R, but was significantly depressed by intake of cobalt (Figure 1b). The SCr level also showed a marked increase in the ischemic rats, but was significantly suppressed in the ischemic rats given cobalt (Figure 1c). Cobalt intake had no effect on serum BUN and SCr levels of control rats. Serum Na level decreased in ischemic rats, whereas a significant increase in Na level was observed in rats given cobalt (Table 1). Ischemic rats showed a significant decrease in serum Cl level at 48 h compared to control rats. Inclusion of cobalt in the drinking water resulted in a significant increase in serum Cl levels both in control and ischemic rats, compared with rats given cobalt-free water at 48 h. Although the serum K level increased in ischemic rats, such an increase was partially suppressed upon ingestion of cobalt.

Figure 2 shows the histological alterations of the kidneys of control and I/R-induced ARF rats with or without the cobalt intake. Cobalt intake caused no morphological changes (Figure 2a and b). I/R caused tubular damage, tubular dilation, tubular epithelial injury, debris accumulation, and cast formation, mostly around the outer stripe of the outer medulla (Figure 2c and e). Tubular damage induced by ischemia was substantially reduced by intake of cobalt (Figure 2d and f). Semiquantitative scoring analysis suggested that cobalt intake showed a tendency to protect renal injury in outer medulla of I/R rats (outer stripe, $P=0.131$; inner stripe, $P=0.041$; Figure 2g and h). These

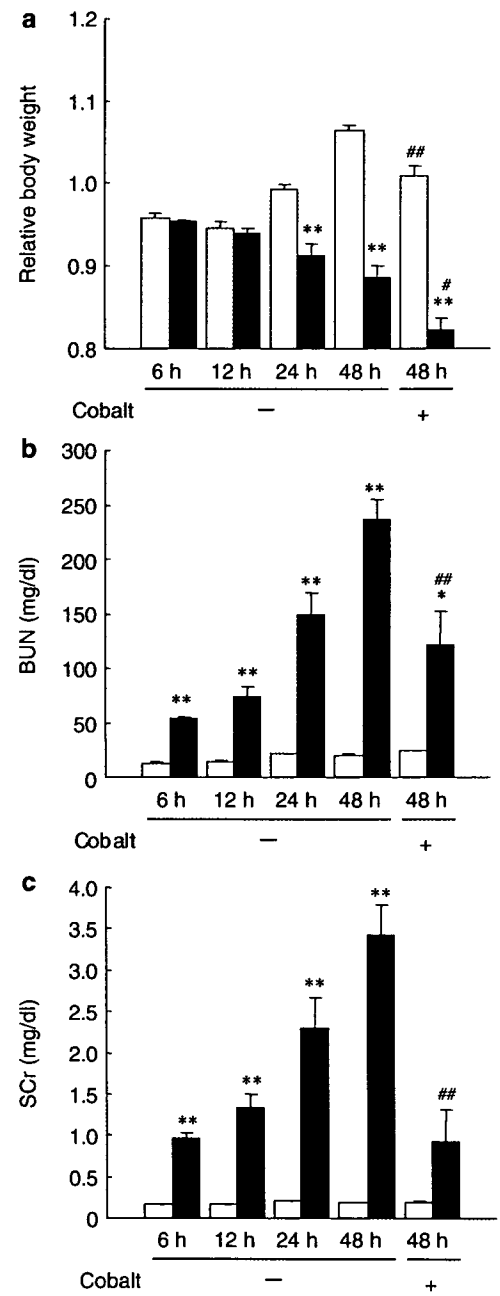

Figure 1 | Changes in body weight and the level of BUN and SCr in control and ischemic rats with/without intake of cobalt. (**a**) Body weight of killing over body weight of operation, (**b**) BUN and (**c**) SCr after sham operation (open column) or I/R injury (closed column). Each column represents the mean ± s.e.m. for three to eight rats. $^{*}P<0.05$, $^{**}P<0.01$, significantly different from control rats at same period. $^{\#}P<0.05$, $^{\#\#}P<0.01$, significantly different from rats without cobalt intake at the same period.

Table 1 | Level of serum electrolytes 48 h after I/R

	Na (mEq/dl)	Cl (mEq/dl)	K (mEq/dl)
Control	142±0	100±1	4.8±0.2
I/R	138±1**	86±1**	7.5±0.9**
Control with cobalt	145±1##	108±1##	4.8±0.3
I/R with cobalt	147±1*#	108±2##	6.3±0.5*

I/R, ischemia/reperfusion.
Each value represents the mean±s.e.m. for seven to eight rats. *$P<0.05$, **$P<0.01$ significantly different from control rats with or without cobalt intake. #$P<0.05$, ##$P<0.01$ significantly different from rats without cobalt intake.

results suggest that cobalt intake ameliorates I/R-induced damage to the renal tubules.

Endogenous IS levels in ischemic rats

As shown in Figure 3a, endogenous IS level was markedly elevated in the ischemic rats compared to that in control rats from 6 h after I/R. The increase in serum IS was significantly suppressed by ingestion of cobalt. These findings suggest that cobalt intake had a partial protective effect against I/R-induced ARF. Cobalt intake had no effect on the IS concentration of control rats. Significant correlation between the level of BUN or SCr with the concentration of serum IS in control and I/R rats was observed (Figure 3b and c), suggesting that IS could be an endogenous marker for evaluating renal dysfunction in ischemic ARF.

mRNA expression of OATs in ischemic rat kidney

The increase in the level of serum IS in ischemic rats suggests that renal handling of the uremic toxin could be disturbed. Thus, we examined whether the expression of OATs mRNA and proteins in the I/R-induced ischemic rat kidney was altered. Figure 4 shows the relative mRNA expression levels of rOAT1 and rOAT3 in the kidney of control and ischemic rats by using the real-time polymerase chain reaction method. Both rOAT1 and rOAT3 mRNA levels were markedly depressed in the ischemic rat kidney (Figure 4a and b). The mRNA levels of rOAT1 and rOAT3 were fractionally higher in the ischemic rat kidney after ingestion of cobalt.

The effect of I/R-induced ARF on mRNA expression of rMdr1, a gene encoding P-glycoprotein located at the brush-border membrane of proximal tubules, was also examined. In contrast to OATs, rMdr1 mRNA expression was transiently stimulated at 6 and 12 h after ischemia and then decreased to the level of control rat kidney (Figure 4c). Cobalt intake had no effect on the expression of rMdr1 mRNA.

Protein expression of OATs in ischemic rat kidney

Changes in the expression of OAT protein 48 h after ischemia was examined by Western blot analyses. As observed for the corresponding mRNA expression, rOAT1 and rOAT3 protein levels were also depressed in the ischemic rat kidney (Figure 5a and b). Cobalt intake had no significant impact on the rOAT1 expression in the kidney of both control and ischemic rats. By contrast, the expression of rOAT3 was significantly restored by ingestion of cobalt. P-glycoprotein level showed no significant difference between the control and the ischemic rats, as found for the rMdr1a mRNA expression. Na^+/K^+-ATPase expression was drastically depressed in the ischemic rat kidney as reported previously,[25,26] but was significantly restored by ingestion of cobalt.

Uptake of PAH and ES by renal slices

To evaluate the functional activity of renal OATs at the basolateral membrane, we measured the accumulation of organic anions, *p*-aminohippuric acid (PAH), and estrone sulfate (ES), in renal slices prepared from control and ischemic rat kidney. As shown in Figure 6a and b, the accumulation of PAH and ES was significantly lower in the renal slices from ischemic rats, compared to renal slices from control rats. Renal uptake clearance of PAH and ES was significantly lower (i.e. reduced to 18 or 48%, respectively) in ischemic rats compared to control rats (Figure 6c and d). This result demonstrates that renal uptake of ES, but not PAH, is significantly restored in slices taken from rats treated with the cobalt-supplemented water. These findings suggest that the function of rOAT3, but not rOAT1, is partially restored by ingestion of cobalt.

DISCUSSION

The mechanisms of ARF involve both vascular and tubular factors.[27] In established ARF, the presence of tubular necrosis upon histological assessment of the kidney is seen in occasional tubular cells.[7] ARF is characterized by tubular dysfunction with impaired sodium and water reabsorption and is associated with the shedding and excretion of proximal tubule brush-border membranes and epithelial tubule cells into the urine.[9] Following I/R, morphological changes occur in the proximal tubules, including loss of polarity, loss of the brush border, and redistribution of integrins and Na^+/K^+-ATPase to the apical membrane.[7,9,28] Because secretion of xenobiotics and endogenous uremic toxins is performed by several transport proteins localized specifically at the basolateral and brush-border membranes of the proximal tubular cells, renal tubular damage will adversely affect excretion of these compounds.

In this study, we have found for the first time that the level of serum IS is markedly elevated in ischemic rats with I/R-induced renal injury. IS is thought to stimulate the progression of chronic renal failure, which is accompanied by glomerular sclerosis.[20] IS appears to induce several genes, including transforming growth factor-β1 and tissue inhibitor of metalloproteinase-1, thereby accelerating the progression of renal sclerosis in subtotal nephrectomized rat kidney.[29,30] The observed increase in the concentration of serum IS in ischemic rats could contribute to acute renal tubular dysfunction. Because >95% of serum IS is bound to albumin, IS is excreted mostly into urine *via* tubular secretion, rather than *via* glomerular filtration.[31] OATs have been reported to mediate IS uptake at the basolateral membrane of renal tubules.[20,21] Our demonstration of elevated IS levels in ischemic rat serum encouraged us

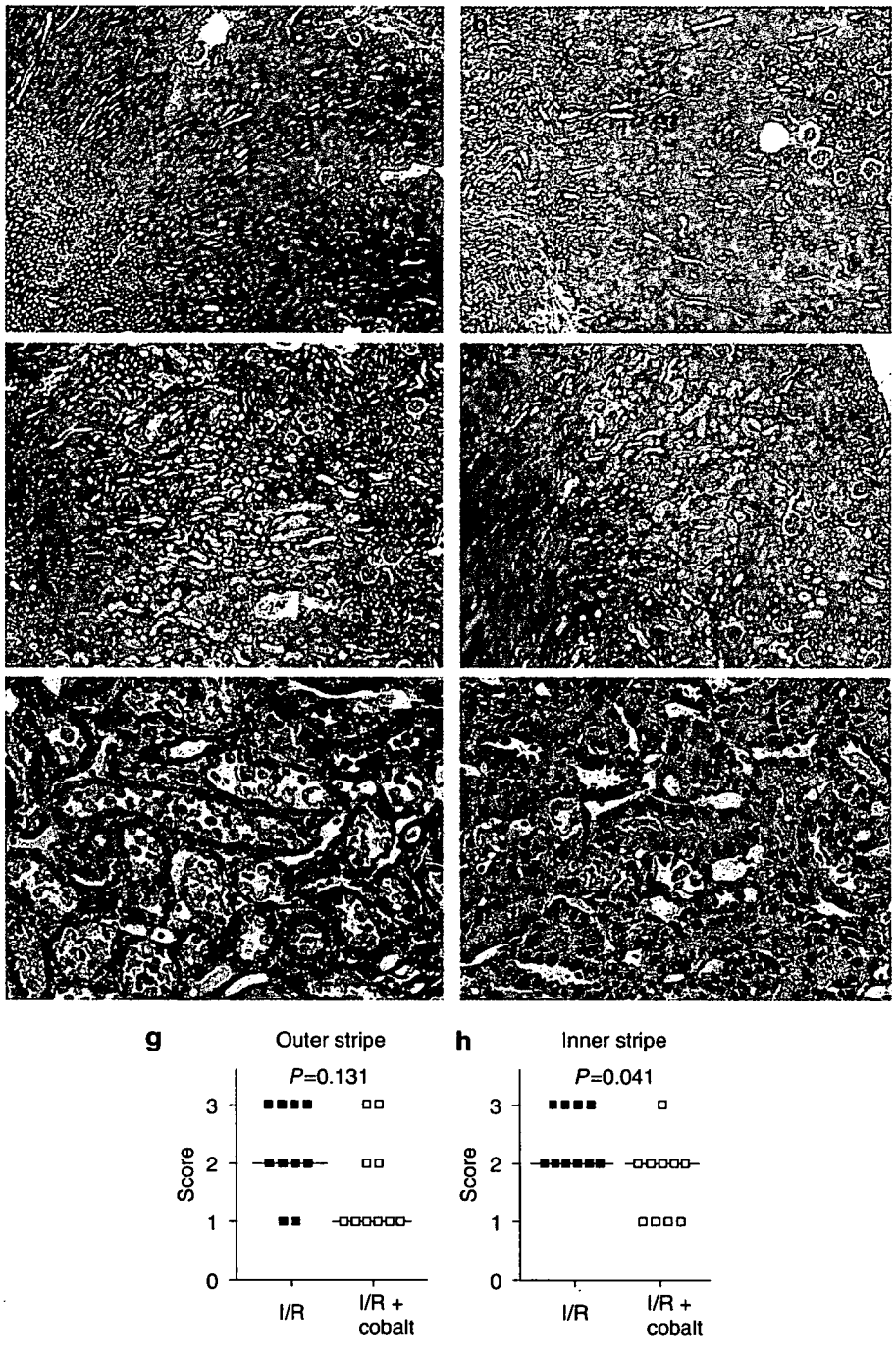

Figure 2 | Histological alteration of the kidneys of control and ischemic rats with or without cobalt intake at 48 h after ischemia. (**a**) Control rats, (**b**) control rats with cobalt intake, (**c**) ischemic rats, (**d**) ischemic rats with cobalt intake, (**e**) outer stripe of ischemic rats, and (**f**) outer stripe of ischemic rats with cobalt intake. (**a–d**) Hematoxylin–eosin staining low magnification; (**e** and **f**) hematoxylin–eosin staining high magnification. Tubular injury of (**g**) outer stripe and (**h**) inner stripe of outer medulla in I/R rats with or without cobalt intake was graded with an arbitrary score of 0–3. The horizontal solid lines indicate the median. Statistical analyses were performed using the Mann–Whitney's test. I/R caused tubular damage to the outer stripe of outer medulla. Cobalt intake reduces tubular damage induced by I/R.

to examine changes in OATs accompanied by I/R of the rat kidney.

We found that the expression of mRNA and protein of both rOAT1 and rOAT3 were downregulated in ischemic rat kidney (Figures 4 and 5). In addition, organic anion transport activity at the basolateral membrane was significantly reduced in the ischemic rat kidney, as demonstrated by the reduction in the accumulation of PAH and ES into renal slices (Figure 6). It was reported that OAT1 mediates renal tubular uptake of PAH and several pharmacological agents, such as methotrexate, β-lactam antibiotics, and non-steroidal anti-inflammatory drugs.[1] OAT3 appears to