Down-regulation of rat organic cation transporter rOCT2 by 5/6 nephrectomy

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Background. In rat kidneys, the organic ion transporters rOCT1, rOCT2, rOAT1 and rOAT3 are considered to mediate the basolateral uptake of various ionic compounds. However, their changes in chronic renal failure (CRF) are poorly understood. The present study examined the renal handling of organic ions and the expression of these transporters under CRF.

Methods. 5/6 Nephrectomized rats were used as the animal model of CRF. Renal handlings of cimetidine and paraaminohippuric acid (PAH) were examined by in vivo experiments. rOAT1, rOAT3, rOCT1 and rOCT2 expressions were determined by Western blotting.

Results. The tubular secretion rates of both PAH and cimetidine were markedly decreased in CRF rats. Although the distribution rates of PAH into the kidney cortex and medulla, and of cimetidine into the kidney cortex were maintained, the distribution rate of cimetidine into the kidney medulla was significantly decreased in CRF rats. The expression level of the rOCT2 protein was markedly depressed in CRF rats, but those of rOCT1, rOAT1 and rOAT3 were maintained. In addition, the plasma concentration of testosterone, a regulator of rOCT2 expression, was significantly reduced by CRF. Both the renal clearance of cimetidine and rOCT2 expression were recovered by the exogenous administration of testosterone in CRF rats.

Conclusions. The levels of urinary excretion of cationic drugs, especially substrates for rOCT2, were reduced under CRF partly due to the reduced expression of rOCT2, and the lowered plasma level of testosterone was suggested to be responsible for the depressed rOCT2 expression in CRF.

Numerous organic anions and cations including endogenous substances, xenobiotics and their metabolites are excreted from the body. The kidney is critical for the elimination of ionic drugs, as well as the liver. The net excretion of drug into urine is defined basically by three processes: glomerular filtration, tubular secretion and reabsorption. The proximal tubular cells play a pivotal role in limiting or preventing toxicity by secreting organic anions and cations actively from the circulation into the urine [1–3]. Recently, renal basolateral types of organic anion transporters, rOAT1 and rOAT3, and organic cation transporters, rOCT1 and rOCT2, have been cloned and characterized [4–7]. These transporters have been suggested to mediate the basolateral entry of various anionic or cationic drugs into the proximal tubular epithelial cells. Therefore, the functional and molecular changes in these organic ion transporters would result in impaired renal excretion of drugs, thereby causing unexpected adverse effects of administered ionic drugs.

As renal ablation results in proteinuria, functional hypertrophy and progressive kidney disease in the rat [8], 5/6 nephrectomized (5/6 Nx) rats have been widely used to study the progression of renal damage. There have been various reports concerning the mechanisms of glomerular dysfunction in remnant nephrons [8, 9]. Recently, renal tubule dysfunction has also been reported in chronic renal failure (CRF). Kwon et al reported that the levels of expression of aquaporin water channels (AQP1, AQP2 and AQP3) were decreased in CRF [10]. In addition, it was reported that there were significant decreases in total kidney levels of proximal tubular sodium transporters such as the type 3 Na+/H+ exchanger (NHE-3) in rats with CRF [11]. Tubular adenosine 5′-ribosephosphate (ATP)-driven multispecific organic anion transporter MRP2 has been shown to be up-regulated in 5/6 Nx rat kidneys [12]. It is generally acknowledged that some ionic drugs should be used in lower dosages or with longer inter-dose intervals to prevent adverse effects in CRF. As circulating organic ions show extensive accumulation into the tubular epithelial cells via OAT1, OAT3, OCT1 or OCT2 [13], information regarding changes in these transporters in CRF would be useful to determine optimal use of drugs in progressive renal failure. In the present study, we examined the renal handling of organic ions and the expression of tubular organic ion transporters in 5/6 Nx rats.

Key words: cimetidine, p-aminohippuric acid, chronic renal failure, renal clearance, organic anion transporter, testosterone, remnant kidney.

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METHODS

Experimental animal

For ablation of the renal mass, male Wistar albino rats (200 to 220 g) were anesthetized with sodium pentobarbital (40 mg/kg) and the kidneys were exposed under aseptic conditions via a ventral abdominal incision. The right kidney was removed, and 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. After surgery, animals were allowed to recover from anesthesia and surgery in cages with free access to water and standard rat chow for two weeks.

To examine the effects of exogenous administration of testosterone on the drug pharmacokinetics and the renal expression of transporters, another batch of animals with or without 5/6 Nx was used. Because Anderson et al reported that the plasma elimination half-life of testosterone after the single administration of the unmodified testosterone and the esterified testosterone-17β-heptanoic acid (testosterone enanthate) to be 2.0 and 51 hours in adult female rats, respectively [14], we chose the latter compound for the following experiments. First, the sham-operated and 5/6 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, 13 days after surgery, respectively. Except for the subcutaneous administration of testosterone every three days, animals were allowed access to water and standard rat chow for two weeks.

Rats were maintained in metabolic cages for 24 hours before the in vivo experiment to determine urine output and urinary levels of creatinine and albumin. The blood urea nitrogen (BUN) was determined by the urease/indophenol method. The levels of creatinine in serum and urine were determined by Jaffé reaction. For measurement, we used assay kits from Wako Pure Chemical Industries (Osaka, Japan). The concentrations of plasma and urinary albumin were measured using an ELISA kit (NEPHRAT II®, Exocell, Inc., Philadelphia, PA, USA). Plasma testosterone and 17β-estradiol levels were measured with an enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI, USA). The animal experiments were performed in accordance with the Guidelines for Animal Experiments of Kyoto University.

Infusion experiment

5/6 Nephrectomized rats and sham-operated controls were anesthetized by intraperitoneal (IP) 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 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, PAH or cimetidine was administered as a bolus via the femoral vein and incorporated into infusion solution as described [15]. The respective loading and maintenance doses of the drugs including 4% mannitol were as follows: 30 μmol/kg and 55 μmol/mL for PAH, and 317 μmol/kg and 21.8 μmol/mL for cimetidine. The infusion rate was 2.2 mL/h using an automatic infusion pump (Natsume Saisakusho, Tokyo, Japan). Mannitol was used to maintain sufficient and constant urine flow rate. After a 30-minute equilibration period, urine samples were collected three times at ten minute 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 PAH and cimetidine and the expression of renal drug transporters. The concentrations of PAH and cimetidine in plasma, urine and renal homogenate were determined by high-performance liquid chromatography (HPLC) [16, 17]. The plasma unbound fraction (fu) of PAH or cimetidine was determined by ultrafiltration using a micropartition system (MRS-1; Amicon, Inc., Beverly, MA, USA), as described earlier [15]. The free fraction of PAH or cimetidine was expressed as the ratio of the concentration in the ultrafiltrate to that in plasma.

Tissue distribution of PAH and cimetidine

[14C]PAH (1.9 GBq/mmol) and [3H]cimetidine (814 GBq/mmol) were obtained from DuPont-New England Nuclear Research Products (Boston, MA, USA). 5/6 Nx and sham rats were anesthetized with sodium pentobarbital. Tracer amounts of [14C]PAH (1 μmol/kg, 20 kBq/mL) or [3H]cimetidine (1.3 nmol/kg, 20 kBq/mL) were administered as a bolus via the catheterized right femoral vein. Blood samples were collected at 0.5, 1, 1.5, 2, 2.5 and 3 minutes from the left femoral artery. The plasma was immediately separated from erythrocytes by centrifugation. Three minutes after injection, several tissue specimens were collected immediately after sacrificing rats [18]. The excised tissues were gently washed, weighed and homogenized in 3 volumes of 0.9% NaCl. Aliquots (100 μL) of blood and tissue homogenates were solubilized in 0.5 mL of NCS2 (Amersham Pharmacia Biotech, Uppsala, Sweden), and the radioactivity was determined in 5 mL of ACS2 (Amersham) by liquid scintillation counting.
Analytical methods

For infusion experiments, pharmacokinetic parameters were calculated using standard procedures for each experimental period. The total plasma clearance (C_{tp}) was calculated by dividing the infusion rate by the steady-state plasma concentration (C_{ps}) at the midpoint of urine collection. Renal clearance (C_{ren}) was obtained by dividing the urinary excretion rates by C_{ps}. The renal clearance of unbound PAH or cimetidine (C_{r.f}) was determined by dividing C_{ren} by the unbound fraction (fu) of PAH or cimetidine, respectively. The glomerular filtration rate (GFR) was assumed to be equal to the C_{ren} of creatinine. The renal secretory clearance of unbound PAH or cimetidine was calculated by subtracting GFR from C_{r.f}. For tissue distribution experiments, the tissue uptake clearance of PAH and cimetidine were calculated by dividing the tissue accumulation at three minutes by the area under the plasma concentration-time curve until three minutes (AUC_{0-3min}).

Polyclonal antibodies against rOAT1 and rOAT3

Polyclonal antibodies were raised against the synthetic peptide corresponding to the intracellular domains near the COOH-terminal of rOAT1 (QQQMMPLQAST QEKNGL) or rOAT3 (SEAEEKSOIIPLKTG), respectively. The peptides were synthesized with cysteine at the NH2-terminal. After obtaining pre-immune serum, rabbit antiserum was raised against the peptide conjugated to keyhole limpet hemocyanin (Calbiochem-Behring, La Jolla, CA, USA). Male New Zealand White rabbits (2.2 to 2.5 kg) were immunized with 1 mL of conjugates (1 mg of the peptide for rOAT1 or rOAT3) emulsified with Freund’s complete adjuvant. Booster injections of the emulsified conjugates were given every two weeks until the antibodies were obtained. After each booster injection, blood was collected and antibody production was determined by enzyme-linked immunosorbent assay (ELISA).

Western blot analysis

The crude plasma membrane fractions were prepared from rat kidneys, as described previously [19]. The crude plasma membrane fractions were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) 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 mmol/L NaCl, 3 mmol/L KCl, 8 mmol/L Na$_2$HPO$_4$, 1 mmol/L KH$_2$PO$_4$, 12 mmol/L K$_2$HPO$_4$, pH 7.5) containing 0.5% Tween 20 (PBS-T), and incubated overnight at 4°C with primary antibody specific for rOAT1, rOAT3, rOCT1 [20], rOCT2 [21], Na$^+$/K$^+$-ATPase (Up-state Biotechnology Inc., Lake Placid, NY, USA), or antibody preabsorbed with the synthetic antigen peptide (10 µg/mL) for rOAT1 or rOAT3. The blots were washed three times with PBS-T, and the bound antibody was detected on X-ray film by enhanced chemiluminescence (ECL) with horseradish peroxidase conjugated secondary antibodies and cyclic diacylhydrazides (Amerham). The relative amounts of the bands in each lane were determined densitometrically using NIH image 1.61 (National Institutes of Health, Bethesda, MD, USA), and the densitometric ratios to each control [Sham or Sham T(–)] were used as the reference and accorded an arbitrary value of 1.0, respectively.

Statistical analysis

All data are expressed as means ± SE. Data of Western blot analysis are expressed in arbitrary units of densitometry/25 µg protein. Comparisons between the sham-operated and 5/6 Nx rats were performed using the unpaired t test. $P < 0.05$ was considered significant.

RESULTS

Renal functional data of 5/6 Nx rats

5/6 Nephrectomized rats showed renal insufficiency. As shown in Table 1, the body weights tended to be lower and 24-hour urine volume was markedly increased in 5/6 Nx rats. The levels of BUN and urinary albumin were significantly increased, and the creatinine clearance and plasma albumin were markedly decreased in 5/6 Nx rats in comparison with sham-operated rats.

<table>
<thead>
<tr>
<th>Table 1. Renal functional data in rats</th>
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<tr>
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<tr>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Body weight g</td>
</tr>
<tr>
<td>Urine volume mL/24 h</td>
</tr>
<tr>
<td>P_{u} mg/dl</td>
</tr>
<tr>
<td>C_{r.f} mL/min/kg</td>
</tr>
<tr>
<td>BUN mg/dl</td>
</tr>
<tr>
<td>P_{u} mg/dl</td>
</tr>
<tr>
<td>U_{a} µg/min/kg</td>
</tr>
</tbody>
</table>

Values are means ± SE of twelve rats. Abbreviations are: P_{u}, plasma creatinine; C_{r.f}, creatinine clearance; BUN, blood urea nitrogen; P_{u}, plasma albumin; U_{a}, urinary albumin; Sham, sham-operated rats; 5/6 Nx, 5/6 nephrectomized rats.

*P < 0.01, significantly different from sham-operated rats
Table 2. Pharmacokinetic parameters of PAH and cimetidine after intravenous infusion in rats

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>5/6 Nx</th>
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<tbody>
<tr>
<td></td>
<td>PAH</td>
<td>Cimetidine</td>
</tr>
<tr>
<td>C_{pss} ( \mu mol/L )</td>
<td>242 ± 21</td>
<td>160 ± 18</td>
</tr>
<tr>
<td>fu</td>
<td>0.86 ± 0.02</td>
<td>0.86 ± 0.05</td>
</tr>
<tr>
<td>C_{tot} mL/min/kg</td>
<td>29.1 ± 2.0</td>
<td>17.2 ± 1.9</td>
</tr>
<tr>
<td>C_{ren} mL/min/kg</td>
<td>29.1 ± 2.1</td>
<td>14.0 ± 1.5</td>
</tr>
<tr>
<td>C_{tot} of unbound fraction mL/min/g kidney</td>
<td>5.4 ± 0.6</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td>Kp</td>
<td>2.0 ± 0.2</td>
<td>2.5 ± 0.2</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SE of six rats. Abbreviations are: C_{pss}, plateau plasma concentration; C_{tot}, total clearance; C_{ren}, renal clearance; fu, plasma unbound fraction; Kp, tissue-to-plasma concentration ratio; PAH, p-aminohippuric acid; Sham, sham-operated rats; 5/6 Nx, 5/6 nephrectomized rats.

\( ^a P < 0.05, ^b P < 0.01 \), significantly different from sham-operated rats.

Fig. 1. Renal secretory clearance of unbound paraaminihippurate (PAH; A) and cimetidine (B). PAH (55 \( \mu mol/mL \)) or cimetidine (21.8 \( \mu mol/mL \)) was infused at a rate of 2.2 mL/h using an automatic infusion pump. The renal secretory clearance of unbound PAH or cimetidine was calculated by subtracting GFR from the renal clearance of unbound PAH or cimetidine. Each column represents the mean ± SE of six rats. \( ^* P < 0.05; ^{**} P < 0.01 \), significantly different from the sham-operated controls. Abbreviations are: Sham, sham-operated rats; 5/6 Nx, 5/6 nephrectomized rats.

Tissue distribution of PAH and cimetidine

The initial PAH and cimetidine uptake were determined in various tissues. The plasma concentrations of \( ^{[14C]} \)PAH and \( ^{[3H]} \)cimetidine to three minutes after intravenous administration are shown in Figure 2. The AUC_{0-3 min} of \( ^{[14C]} \)PAH and \( ^{[3H]} \)cimetidine were 45.9 ± 3.1 and 25.1 ± 1.7 in sham-operated rats, and 102.7 ± 17.7 and 49.8 ± 8.4 in 5/6 Nx rats (mean ± SE, \( N = 6 \)), respectively. Table 3 shows the tissue concentrations and Kp value of \( ^{[14C]} \)PAH and \( ^{[3H]} \)cimetidine in sham-operated and 5/6 Nx rats are illustrated in Figure 3. The Kp value and the tissue uptake clearance indicated that both \( ^{[14C]} \)PAH and \( ^{[3H]} \)cimetidine were markedly accumulated in the kidney. The concentration, Kp values and tissue uptake clearance of PAH in the kidney cortex and medulla were slightly decreased by the 5/6 Nx, but these effects were not significant. Although the concentration and the tissue uptake clearance of cimetidine in the kidney cortex were maintained in 5/6 Nx rats, the Kp value was decreased to 60% of sham-operated control. In contrast, the concentration, Kp value and tissue uptake clearance of cimetidine in the kidney medulla were markedly reduced by 5/6 nephrectomy to 62%, 34% and 39%, respectively. The concentrations of PAH and cimetidine in the small intestine were significantly in-
**Table 3. Tissue distributions of PAH and cimetidine 3 minutes after intravenous bolus administration in rats**

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Sham PAH</th>
<th>Sham Cimetidine</th>
<th>5/6 Nx PAH</th>
<th>5/6 Nx Cimetidine</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Concentration</td>
<td>Kp</td>
<td>Concentration</td>
<td>Kp</td>
</tr>
<tr>
<td>Plasma</td>
<td>µmol/L</td>
<td></td>
<td>µmol/L</td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>2.1 ± 0.1</td>
<td>1</td>
<td>4.8 ± 0.7^b</td>
<td>1</td>
</tr>
<tr>
<td>Brain</td>
<td>0.1 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.2 ± 0.1</td>
<td>0.1 ± 0.0</td>
</tr>
<tr>
<td>Liver</td>
<td>1.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td>0.4 ± 0.0^b</td>
</tr>
<tr>
<td>Small intestine</td>
<td>0.5 ± 0.1</td>
<td>0.2 ± 0.0</td>
<td>0.7 ± 0.1^b</td>
<td>0.2 ± 0.0</td>
</tr>
<tr>
<td>Kidney cortex</td>
<td>28.3 ± 8.5</td>
<td>12.8 ± 3.3</td>
<td>36.3 ± 7.1</td>
<td>7.9 ± 1.7</td>
</tr>
<tr>
<td>Kidney medulla</td>
<td>38.2 ± 11.5</td>
<td>17.5 ± 4.8</td>
<td>36.4 ± 10.6</td>
<td>7.8 ± 2.5</td>
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</table>

Each value represents the mean ± SE of six rats. Abbreviations are: Kp, tissue-to-plasma concentration ratio; PAH, p-aminohippuric acid; Sham, sham-operated rats; 5/6 Nx, 5/6 nephrectomized rats.

^P < 0.05, ^bP < 0.01, significantly different from sham-operated rats.

creased in 5/6 Nx rats, but the Kp values for both drugs were maintained. The Kp value and the tissue uptake clearance of PAH in the liver were decreased to 57% and 70% of those in sham-operated rats, respectively.

**Protein expression of organic ion transporters in 5/6 Nx rats**

To obtain more information about the decreases in renal elimination of PAH and cimetidine in the 5/6 Nx rats in comparison with those in sham-operated controls, we assessed renal organic ion transporters expression at the protein level by Western blotting. As shown in Figure 4A, a primary band for rOAT1 or rOAT3 with a size of 77 kDa (calculated molecular weight of 61 kDa) or 72 kDa (calculated molecular weight of 59 kDa) was detected, respectively. The preabsorption of each antibody with corresponding antigen peptide abolished these positive bands, showing the presence of rOAT1 and rOAT3 proteins in the rat kidney. As illustrated in Figure 4B and C, the protein expression level per 25 µg of renal crude plasma membrane protein fractions of rOCT2 was markedly depressed in the 5/6 Nx rat kidneys (33.3 ± 7.0% of sham-operated rats; P < 0.05, N = 7), whereas there were no significant differences in the expression of rOAT1, rOAT3, rOCT1 or Na+/K+-ATPase between the sham-operated and 5/6 Nx rat kidneys.

**Plasma levels of testosterone and 17β-estradiol in 5/6 Nx rats**

Our previous study found that the renal expression level of rOCT2 was regulated by the plasma concentration of testosterone: higher plasma concentrations of testosterone were associated with higher level of renal expression of rOCT2 protein [21, 22]. To examine whether the decreased expression level of renal rOCT2 was associated with the plasma level of testosterone, we measured the plasma concentrations of testosterone and 17β-estradiol in 5/6 Nx rats in comparison with those in sham-operated controls. As shown in Table 4, the plasma concentration of testosterone in 5/6 Nx rats was significantly decreased in comparison with that in sham-operated controls. In contrast, there was no difference in the plasma concentration of 17β-estradiol between the 5/6 Nx and sham-operated rats.
testosterone was twofold higher in the Sham T(+) rats, threefold lower in the 5/6 Nx T(−) rats and 1.5-fold higher in the 5/6 Nx T(+) rats than that in the Sham T(−) rats, respectively. The values of the urine volume during 24 hours, plasma creatinine (Pc), creatinine clearance (Cc), and BUN values were not affected by the administration of testosterone in the 5/6 Nx rats. However, the body weight loss in the 5/6 Nx rats was relatively ameliorated by the testosterone treatment. In addition, the plasma level of creatinine in the Sham T(+) was significantly lowered than in the Sham T(−) rats. Table 6 showed the pharmacokinetic parameters of cimetidine in infusion experiment after administration of testosterone in rats. Although there were no significant differences in the pharmacokinetic parameters such as Ctp, Ctp, Ctp, Ctp, Ctp, and tubular secretory clearances of cimetidine between the Sham T(−) and the Sham T(+) rats, all these parameters were significantly ameliorated by the administration of testosterone in the CRF rats. In particular, the Ctp value in the 5/6 Nx T(+) rats recovered to almost 80% of that in the sham-operated rats. Additionally, the protein expression level of rOCT2 in 5/6 Nx rats recovered to around the sham-operated rats’ value by the exogenous testosterone treatment (Fig. 5A). Furthermore, the protein expression level per 25 mg renal crude plasma membrane protein fractions of rOCT2 correlated well with the Ctp of cimetidine (Fig. 5B). However, the expression levels of rOAT1, rOAT3, rOCT1 and Na+/K+-ATPase were not affected by the administration of testosterone in the CRF rats as well as the sham-operated rats (Fig. 6).

DISCUSSION

Renal organic anion transporters, rOAT1 and rOAT3, and organic cation transporters, rOCT1 and rOCT2, have been cloned and characterized [4–7]. These transporters are localized in the basolateral membranes of proximal tubules, and have been suggested to mediate basolateral uptake of various ionic compounds. rOAT1 and rOAT3 recognize anionic compounds such as PAH, nonsteroidal antiinflammatory drugs, methotrexate, loop diuretics, acetazolamide, cephalosporins, uric acid and prostaglandin E2 [4, 5, 23–25]. In contrast, rOCT1 and rOCT2 transport cationic compounds such as tetrathyamine, cimetidine, N1-methyl nicotine, guanidine, monoamines and procainamide [20, 26, 27]. Kusuhara et al reported that a H2 blocker cimetidine, which is considered a good substrate for the classical organic cation transport system, was also a substrate for rOAT3 [5]. Although various studies have demonstrated the physiological roles of these transporters, the pathophysiological alterations of these transporters in CRF have not been investigated.

Our previous study found that the expression of the
Fig. 4. Protein expressions of rOAT1, rOAT3, rOCT1, rOCT2 and Na+/K+-ATPase in sham-operated and 5/6 nephrectomized rats. Crude plasma membrane fractions (25 μg) from total kidneys were separated by SDS-PAGE (10%) and blotted onto Immobilon membranes. (A) The antisera (1:1000 dilution) for rOAT1 or rOAT3 was preabsorbed (Preabs) with (+) or without (−) the antigen peptide (10 μg/mL) of rOAT1 or rOAT3, respectively. A horseradish peroxidase-conjugated anti-rabbit IgG antibody was used for detection of bound antibodies, and strips of blots were visualized by chemiluminescence on X-ray film. A 77 kD band for rOAT1 and a 72 kD band for rOAT3 are seen (arrowheads). Blot of the crude plasma membranes isolated from two independent sham-operated rats are shown. (B) Antisera specific for rOAT1, rOAT3, rOCT1, rOCT2 and Na+/K+-ATPase (1:500-1000 dilution) were used as primary antibodies. A horseradish peroxidase-conjugated anti-rabbit (for rOAT1, rOAT3, rOCT1 and rOCT2) or anti-mouse (for Na+/K+-ATPase) IgG antibody was used for detection of bound antibodies, and strips of blots were visualized by chemiluminescence on X-ray film. Representative photographs of three separate results of Western blotting are shown. (C) The protein bands are expressed in densitometry units in sham-operated and 5/6 nephrectomized rats. The values for sham rats were arbitrarily defined as 100%. Each column represents the mean ± SE of the six to seven rats in each group. **P < 0.01, significantly different from sham-operated controls.

Table 4. Plasma levels of testosterone and 17β-estradiol in sham and 5/6 Nx rats

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>5/6 Nx</th>
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<tbody>
<tr>
<td>Testosterone ng/mL</td>
<td>1.43 ± 0.27</td>
<td>0.67 ± 0.17</td>
</tr>
<tr>
<td>17β-Estradiol ng/mL</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.00</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SE of twelve rats. Sham, sham-operated rats; 5/6 Nx, 5/6 nephrectomized rats.  
*P < 0.05, significantly different from sham-operated rats.

luminal organic anion transporters, OAT-K1 and OAT-K2, and the urinary secretion of methotrexate were depressed in rats two weeks after 5/6 nephrectomy, despite the minimal alterations in tubular morphology [28]. There was no significant difference in the basolateral uptake of methotrexate, which is considered to be mediated by rOAT1 and rOAT3, between the sham-operated and 5/6 Nx rat kidneys. In the present study, the tissue uptake clearances of PAH in the kidney cortex and medulla were not significantly altered by 5/6 nephrectomy.
Cs
Kp 2.4
/H11006
Cren of unbound fraction
Cren fu 0.89
/H11006
ng/mL
BUN
mg/dL
Ccr
Pcr
Body weight
g
demonstrated to transport PAH in rat renal brush-border membranes: a potential-sensitive transport system and an anion exchanger [29]. However, the luminal PAH transporters have yet to be identified at the molecular level. Therefore, the luminal PAH transporters may be cloned successfully by comparing the mRNA expression profiles between sham-operated and 5/6 Nx rats.
Cimetidine is a substrate for rOAT3, rOCT1 and rOCT2 [13]. In the present study, the renal clearance of cimetidine was decreased to 19% of that in sham-operated controls (Table 2) and the distribution of cimetidine into the kidney medulla was markedly depressed in the 5/6 nephrectomized rats (Table 3 and Fig. 3B). Western blotting revealed that the expression of rOCT2, but not those of rOAT3 or rOCT1, was significantly decreased in the 5/6 Nx rat kidneys (Fig. 4). The level of rOCT1 mRNA expression in the kidney cortex was higher than that in the kidney medulla, and this message was abundant in the S1 segment of proximal tubule [30]. In contrast, the rOCT2 mRNA expression in the kidney medulla was higher than that in the kidney cortex, and rOCT2 protein was abundant in the proximal tubules of the outer medulla [31]. These results strongly suggested that the rOCT2 localized in the proximal tubules of the outer medulla plays a predominant role in the renal distribution of cimetidine, and would be more sensitive

Table 5. Renal functional data and plasma levels of testosterone after administration of testosterone in rats

<table>
<thead>
<tr>
<th></th>
<th>Sham T(−)</th>
<th>Sham T(+)</th>
<th>5/6 Nx T(−)</th>
<th>5/6 Nx T(+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight g</td>
<td>283 ± 3.6</td>
<td>278 ± 6.8</td>
<td>231 ± 14.26</td>
<td>263 ± 8.9</td>
</tr>
<tr>
<td>Urine volume mL/24 h</td>
<td>16.8 ± 2.1</td>
<td>19.3 ± 2.4</td>
<td>32.0 ± 1.48</td>
<td>34.3 ± 2.34</td>
</tr>
<tr>
<td>Pcr, mg/dL</td>
<td>0.49 ± 0.02</td>
<td>0.41 ± 0.02</td>
<td>1.42 ± 0.29</td>
<td>1.23 ± 0.21</td>
</tr>
<tr>
<td>Ccre, mL/min/kg</td>
<td>4.24 ± 0.61</td>
<td>5.73 ± 0.73</td>
<td>1.51 ± 0.26</td>
<td>1.97 ± 0.29</td>
</tr>
<tr>
<td>BUN mg/dL</td>
<td>18.0 ± 1.3</td>
<td>14.8 ± 1.4</td>
<td>72.4 ± 14.3</td>
<td>62.4 ± 12.4</td>
</tr>
<tr>
<td>Testosterone ng/mL</td>
<td>1.99 ± 0.24</td>
<td>4.52 ± 0.48</td>
<td>0.59 ± 0.15</td>
<td>3.02 ± 0.56</td>
</tr>
</tbody>
</table>

Values are means ± SE of six rats. Pcr, plasma creatinine; Ccre, creatinine clearance; BUN, blood urea nitrogen; Sham T(−), sham-operated rats administrated with vehicle; Sham T(+), sham-operated rats administrated with testosterone, 5/6 Nx T(−), 5/6 nephrectomized rats administrated with vehicle; 5/6 Nx T(+), 5/6 nephrectomized rats administrated with testosterone.

Table 6. Pharmacokinetic parameters of cimetidine in infusion experiment after administration of testosterone in rats

<table>
<thead>
<tr>
<th></th>
<th>Sham T(−)</th>
<th>Sham T(+)</th>
<th>5/6 Nx T(−)</th>
<th>5/6 Nx T(+)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cpe, μmol/L</td>
<td>157 ± 16</td>
<td>165 ± 15</td>
<td>340 ± 18b</td>
<td>268 ± 7b</td>
</tr>
<tr>
<td>fu</td>
<td>0.89 ± 0.02</td>
<td>0.88 ± 0.02</td>
<td>0.85 ± 0.04</td>
<td>0.84 ± 0.04</td>
</tr>
<tr>
<td>Ctot mL/min/kg</td>
<td>18.9 ± 1.6</td>
<td>17.7 ± 1.2</td>
<td>9.0 ± 0.5b</td>
<td>11.4 ± 0.5b</td>
</tr>
<tr>
<td>Cren mL/min/kg</td>
<td>15.2 ± 1.1</td>
<td>14.8 ± 1.0</td>
<td>1.5 ± 0.5b</td>
<td>4.5 ± 0.6d</td>
</tr>
<tr>
<td>Cren of unbound fraction mL/min/g kidney</td>
<td>2.7±0.3</td>
<td>2.7±0.2</td>
<td>0.6±0.1b</td>
<td>2.1±0.2d</td>
</tr>
<tr>
<td>Ccre mL/min/g kidney</td>
<td>2.4±0.3</td>
<td>2.4±0.2</td>
<td>0.5±0.1b</td>
<td>1.8±0.2d</td>
</tr>
<tr>
<td>Kp</td>
<td>2.4 ± 0.3</td>
<td>2.3 ± 0.3</td>
<td>1.0 ± 0.1b</td>
<td>1.6 ± 0.2c</td>
</tr>
<tr>
<td>Cren mL/min/g kidney</td>
<td>2.1±0.2</td>
<td>2.0±0.2</td>
<td>0.1±0.0b</td>
<td>1.3±0.2d</td>
</tr>
</tbody>
</table>

Each value represents the mean ± SE of six rats. Cpe, plateau plasma concentration; Ctot, total clearance; Cren, renal clearance; fu, plasma unbound fraction; Pcr, tissue to plasma concentration ratio; Ccre, renal secretory clearance; sham T(−), sham-operated rats administrated with vehicle; Sham T(+), sham-operated rats administrated with testosterone, 5/6 Nx T(−), 5/6 nephrectomized rats administrated with vehicle; 5/6 Nx T(+), 5/6 nephrectomized rats administrated with testosterone.

(20230h, 20020h) The expression levels of rOAT1 and rOAT3 protein were maintained in the 5/6 Nx rat kidneys (Fig. 4 B, C). These observations and the present results suggested that the basolateral entry of organic anions, especially substrates for rOAT1 and rOAT3, was maintained at least two weeks after nephrectomy. Therefore, the expression of the luminal OAT-K and basolateral OAT should be affected differently in CRF. As highly accumulated anionic drugs such as methotrexate may cause further deterioration of renal failure, the molecular mechanism(s) involved in the differential regulation of OAT-K and OAT expression should be elucidated to prevent drug-induced nephrotoxicity under CRF.

Tubular secretion is a vectorial transcellular transport system consisting of basolateral entry into the epithelial cells and secretion across the brush border membranes. Defects in either of these two processes should influence the tubular secretion of ionic drugs. In the present study, the tubular secretion of PAH was markedly decreased to 44% in CRF rats, despite the maintained distribution rate in the kidney (Figs. 1A and 3A). These results suggested that the limiting step for the urinary excretion of PAH might be the tubular secretion step across the brush border membranes. Two distinct transport systems were demonstrated to transport PAH in rat renal brush-border membranes: a potential-sensitive transport system and an anion exchanger [29]. However, the luminal PAH transporters have yet to be identified at the molecular level. Therefore, the luminal PAH transporters may be cloned successfully by comparing the mRNA expression profiles between sham-operated and 5/6 Nx rats.

Cimetidine is a substrate for rOAT3, rOCT1 and rOCT2 [13]. In the present study, the renal clearance of cimetidine was decreased to 19% of that in sham-operated controls (Table 2) and the distribution of cimetidine into the kidney medulla was markedly depressed in the 5/6 nephrectomized rats (Table 3 and Fig. 3B). Western blotting revealed that the expression of rOCT2, but not those of rOAT3 or rOCT1, was significantly decreased in the 5/6 Nx rat kidneys (Fig. 4). The level of rOCT1 mRNA expression in the kidney cortex was higher than that in the kidney medulla, and this message was abundant in the S1 segment of proximal tubule [30]. In contrast, the rOCT2 mRNA expression in the kidney medulla was higher than that in the kidney cortex, and rOCT2 protein was abundant in the proximal tubules of the outer medulla [31]. These results strongly suggested that the rOCT2 localized in the proximal tubules of the outer medulla plays a predominant role in the renal distribution of cimetidine, and would be more sensitive...
to renal damage than rOAT3 and rOCT1. To our knowledge, this is the first study demonstrating the responsibility of rOCT2 for the urinary excretion of cimetidine in vivo.

Urakami et al demonstrated that the expression of rOCT2, but not of rOCT1 or rOAT1, was associated with the plasma concentration of testosterone [21]. The renal expression level of rOCT2 in male rats was obviously higher than that in females. In addition, the OCT2 expression was up-regulated by treatment with testosterone, but not with estradiol, in rats and cultured Madin-Darby canine kidney cells [22, 32]. In clinical cases, a lower serum testosterone level was reported to be associated with chronic renal failure [33, 34]. Abdel-Gawad et al [35] reported that in 5/6 nephrectomized rats 10 weeks after surgery the plasma concentration of testosterone was decreased to below 30% of that in sham-operated rats. In the present study, plasma concentration of testosterone appeared to be decreased in 5/6 nephrectomized rats two weeks after surgery and the expression level of rOCT2 protein was selectively down-regulated (Table 4 and Fig. 4, respectively). The plasma concentrations of testosterone in male and female rats were reported to be 1.5 ± 0.03 and 0.25 ± 0.02 ng/mL, respectively, and the level of rOCT2 protein expression in female rats was markedly lower than that in male rats [22]. These results suggested that the decreased plasma testosterone was one of the determinants of the down-regulation of rOCT2 expression in 5/6 Nx rats.

Because the plasma concentration of testosterone correlated well with the renal rOCT2 expression [22], we hypothesized that the decreased renal clearance of cimetidine and the depressed expression level of rOCT2 in the CRF rats could be ameliorated by the supplementary treatment of testosterone. As expected, the administration of small amount of testosterone recovered the renal clearance of cimetidine and the rOCT2 expression in the CRF rats to near the level of sham-operated rats (Table 6 and Fig. 5A). Despite of the basolateral localization of rOCT2, the protein expression level of rOCT2 correlated
well with the renal clearance of cimetidine ($r = 0.96$) (Fig. 5B). However, it was confirmed that the protein expression levels of the other basolateral transporters rOAT1, rOAT1, rOAT3 and Na$^{+}$/K$^{+}$-ATPase were independent of the plasma concentration of testosterone (Fig. 6). These results indicated that the plasma concentration of testosterone could be a surrogate marker for predicting the renal clearance of cationic drugs including cimetidine as well as the expression level of rOCT2 in the rats. In addition, the plasma level of testosterone was suggested to specifically induce the expression of rOCT2 among these five basolateral transporters in the kidney.

In the present study, the exogenous testosterone did not induce the expression level of rOCT2 in the Sham T(+) rats comparing with that in the Sham T(−) rats (Tables 5 and 6 and Fig. 5). The high dose treatment of testosterone (10 mg/rat/day for 7 days) resulted in a 100-fold heightened plasma concentration of testosterone and a fourfold induction of the renal mRNA expression level of rOCT2 compared with those values in the vehicle-treated control rats, respectively [22]. Considering the present results and the previous findings, it was reasonable to find no differences in the expression of rOCT2 and the renal handling of cimetidine in the sham-operated rats with or without the small amount of testosterone treatment (0.5 mg/rat/3 days for 2 weeks).

In conclusion, this study demonstrates the functional and molecular alterations of the urinary excretion mechanisms of organic anions and cations by 5/6 nephrectomy. The present results suggest that rOCT2—but not rOCT1 or rOAT2—is a major transporter involved in the renal distribution of cimetidine, and the protein expression of rOCT2 is selectively down-regulated in CRF. The lowered plasma level of testosterone in CRF is responsible for the decreased expression of rOCT2 and urinary excretion of cimetidine.

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APPENDIX

Abbreviations used in this article are: AUC, area under the plasma concentration-time curve; BUN, blood urea nitrogen; $C_{\text{ss}}$, steady state plasma concentration; $C_{\text{cr}}$, renal clearance; $C_{\text{ur}}$, renal clearance of unbound PAH or cimetidine; CRF, chronic renal failure; $C_{\text{tot}}$, total plasma clearance; ELISA, enzyme-linked immunosorbent assay; fu, unbound fraction; GFR, glomerular filtration rate; HPLC, high performance liquid chromatography; Kp, tissue to plasma concentration ratio; Nx, nephrectomy; OAT, organic anion transporter; OCT, organic cation transporter; PAH, paraaminohippurate; T, with (+) or without testosterone enantate.

REFERENCES


