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

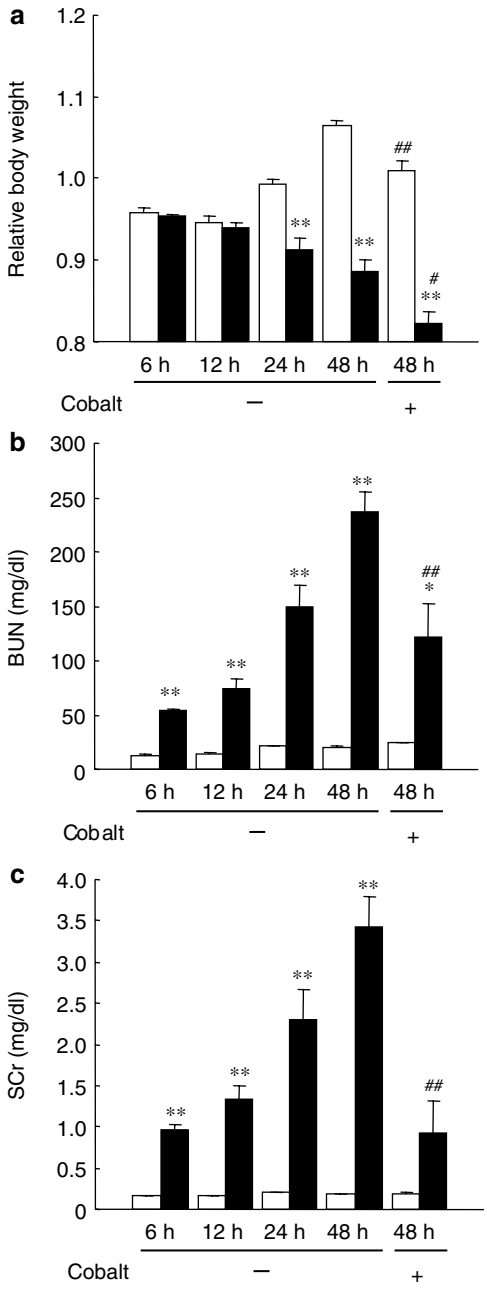

Figure 1 | Changes in body weight and the level of BUN and SCr in control and ischemic rats with/without intake of cobalt. (**a**) Body weight of killing over body weight of operation, (**b**) BUN and (**c**) SCr after sham operation (open column) or I/R injury (closed column). Each column represents 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

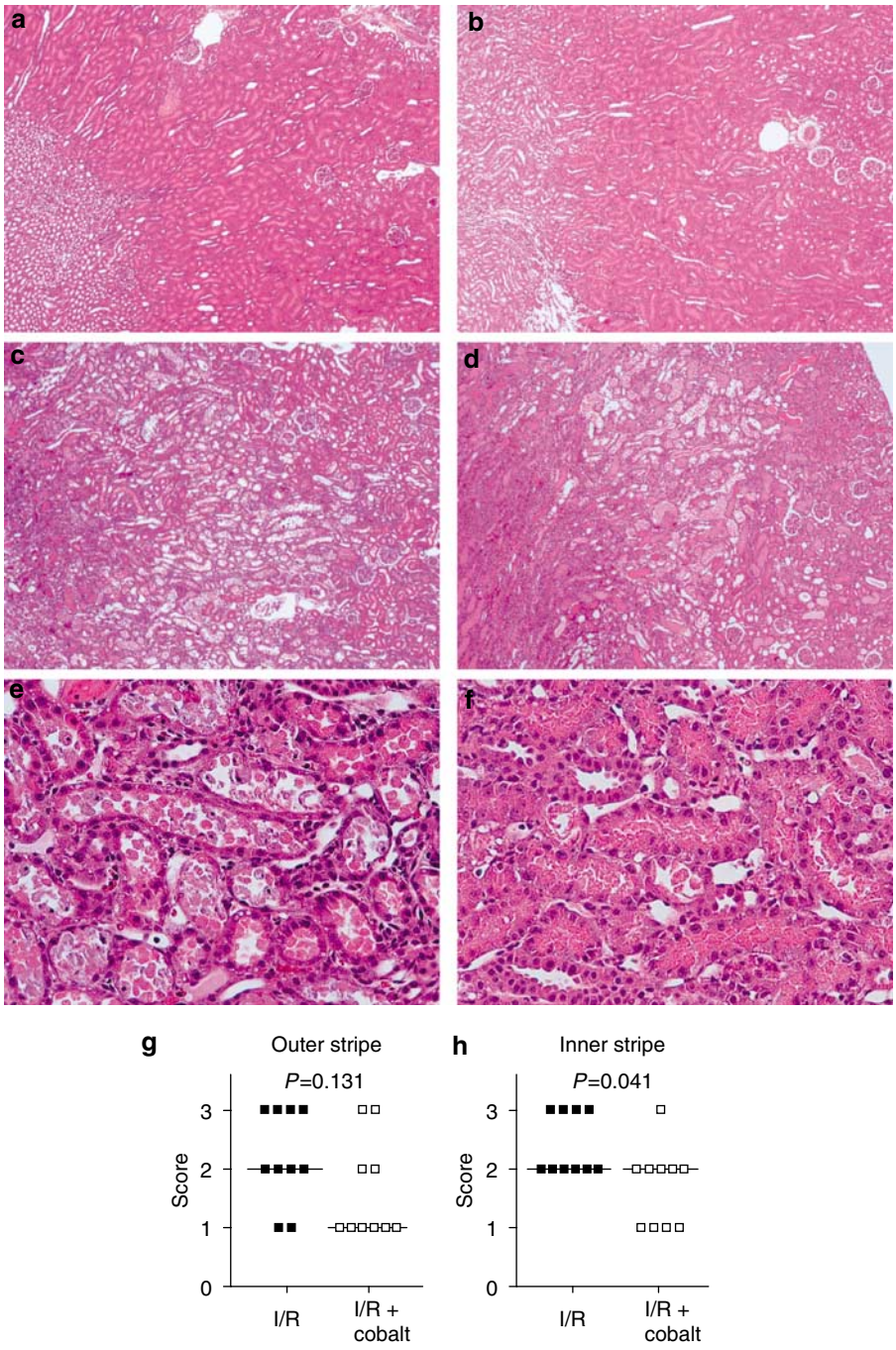

Figure 2 | Histological alteration of the kidneys of control and ischemic rats with or without cobalt intake at 48 h after ischemia. (**a**) Control rats, (**b**) control rats with cobalt intake, (**c**) ischemic rats, (**d**) ischemic rats with cobalt intake, (**e**) outer stripe of ischemic rats, and (**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

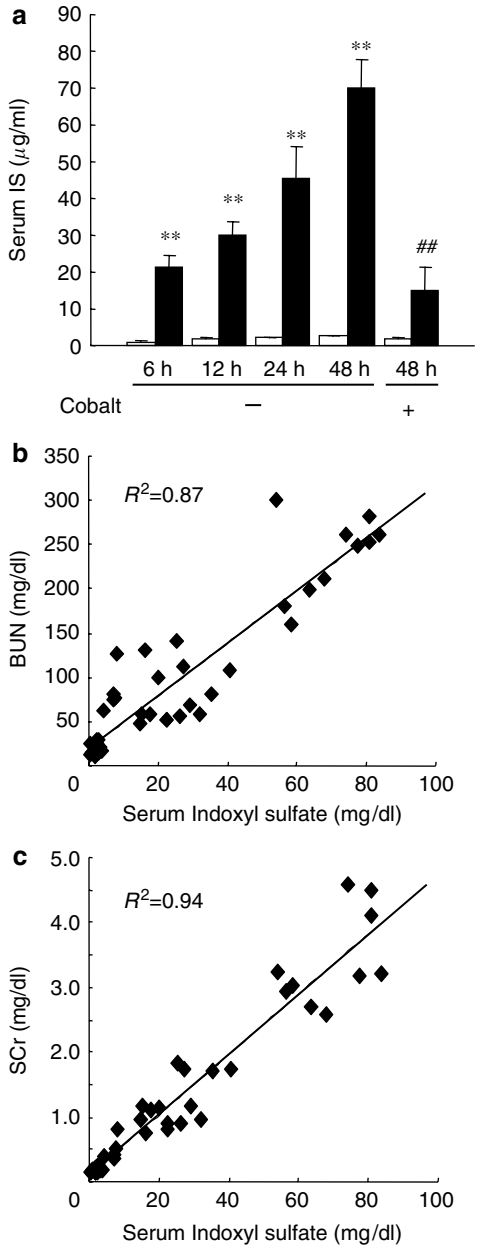

Figure 3 | Serum IS concentration in control and ischemic rats with and without cobalt intake. (**a**) High-performance liquid chromatography analysis of serum to determine the concentration of IS after sham operation (open column) or I/R injury (closed column). Each column represents the mean ± s.e.m. for three to eight rats. $^{**}P<0.01$, significantly different from control rats at the same period. $^{\#\#}P<0.01$, significantly different from rats without cobalt intake over the same period. Correlation between (**b**) BUN or (**c**) SCr and serum IS concentration.

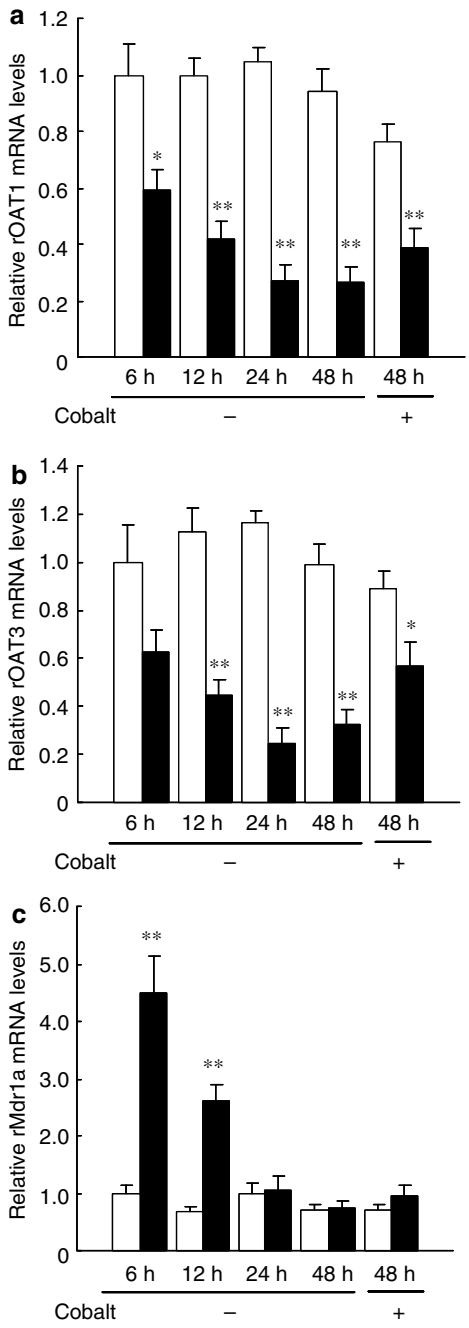

Figure 4 | mRNA expression of organic anion transporters and rMdr1a in ischemic rat kidney. Relative mRNA expression levels of (**a**) rOAT1, (**b**) rOAT3 and (**c**) rMdr1a in control (open column) and ischemic rats (closed column) with and without cobalt intake. The expression level in the control at 6 h was set at 1.0. 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 the same period.

efficiently mediate ES transport with high affinity in addition to transporting several anionic compounds such as PAH, methotrexate, prostaglandin E_2, and cyclic adenosine mono phosphate.[1] Therefore, downregulation of both rOAT1 and rOAT3 should account for the decrease in PAH and ES uptake at the basolateral membrane of tubular cells in ischemic rat kidney. Intriguingly, we also observed a significant recovery in the accumulation of ES, but not PAH, by renal slices derived from I/R-induced rats given water supplemented with cobalt. *In vitro* studies have shown that the contribution of rOAT1 and rOAT3 to the uptake of IS is 38 and 62%, respectively.[32] Therefore, the decrease in serum IS levels in ischemic rats treated

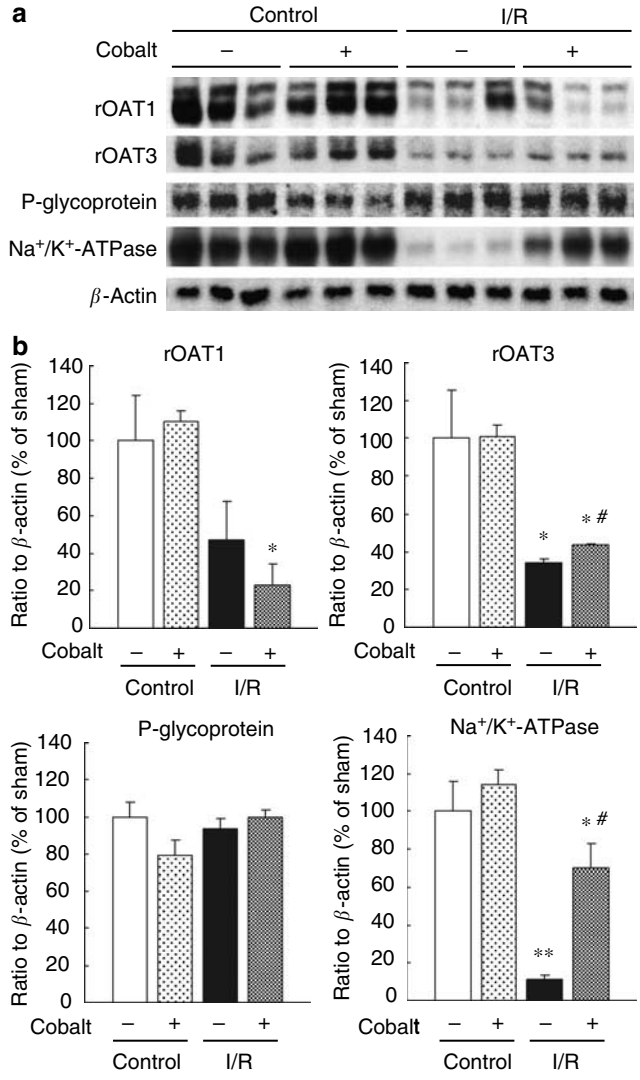

Figure 5 | Immunoblots of kidneys from control and ischemic rats with or without cobalt intake 48 h after ischemia. (**a**) Antiserum specific for rOAT1, rOAT3, P-glycoprotein, Na^+/K^+-ATPase, and β-actin were used as primary antibodies. (**b**) The ratio of rOAT1, rOAT3, P-glycoprotein, and Na^+/K^+-ATPase density to β-actin density. The values for control rats without cobalt intake were arbitrarily defined as 100%. Each bar represents the mean ± s.e.m. for three rats in each group. $^*P<0.05$, $^{**}P<0.01$, significantly different from control rats with or without cobalt intake; $^{\#}P<0.05$, significantly different from ischemic rats without cobalt intake.

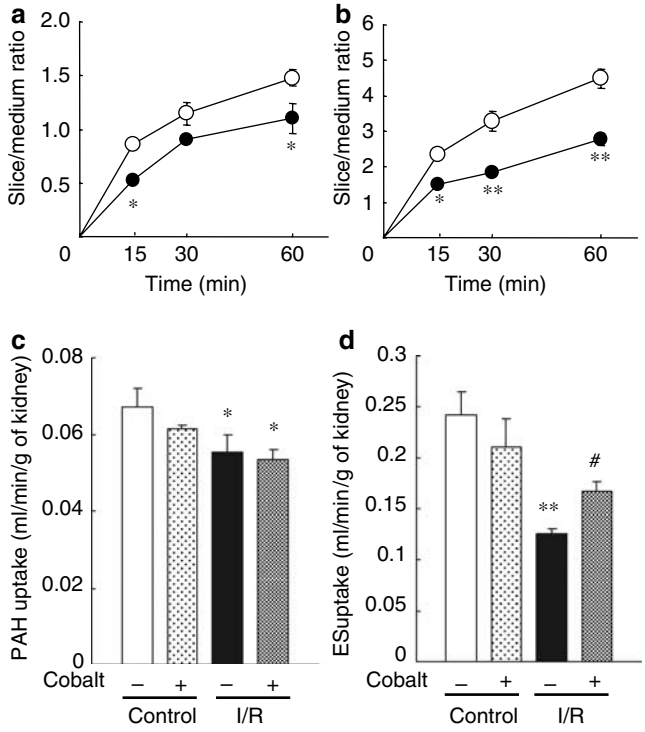

Figure 6 | Renal uptake of PAH and ES in control and ischemic rats with or without cobalt intake. (**a**) PAH and (**b**) ES accumulation by renal slices of control and ischemic rats at 48 h after ischemia. Renal slices from control (○) and ischemic rats (●) were incubated at 37°C in incubation buffer containing 5 μM [^{14}C]PAH or 5 μM [^{3}H]ES, for the period indicated. D-[^{3}H]Mannitol or [^{14}C]mannitol was used to estimate the extracellular trapping and nonspecific uptake of [^{14}C]PAH or [^{3}H]ES, respectively. Each point represents the mean ± s.e.m. for three to five slices. $^*P<0.05$, $^{**}P<0.01$, significantly different from control rats at same period. The renal uptake clearance of (**c**) PAH and (**d**) ES in control and ischemic rats with or without cobalt intake at 48 h after ischemia. Renal slices from rats were incubated at 37°C in incubation buffer containing 5 μM [^{14}C]PAH or 5 μM [^{3}H]ES, for 60 min. $^*P<0.05$, $^{**}P<0.01$, significantly different from control rats with or without cobalt intake; $^{\#}P<0.05$, significantly different from ischemic rats without cobalt intake.

cobalt might be the result of partially restored rOAT3 function and/or expression.

Alternatively, the driving force of rOAT1 and rOAT3 activity could be depressed in renal tubular cells of I/R kidney. rOAT1 and rOAT3 are known to mediate exchange of anionic substrates with dicarboxylic acids, including α-ketoglutarate, where the concentration in the cytoplasm is much higher than in the serum, producing an outward-directed driving force for influx of anionic substrates.[33,34] Na^+/K^+-ATPase actively pumps Na^+ out of the renal proximal tubule cells sustaining an inwardly directed Na^+ gradient.[35] The inward movement of Na^+ drives the uptake of α-ketoglutarate by Na^+/dicarboxylate cotransporter 3.[35] The depression of Na^+/K^+-ATPase expression in the ischemic rat kidney eliminates the inward Na^+ gradient, thereby decreasing uptake of α-ketoglutarate by Na^+/dicarboxylate cotransporter 3. The protein level of Na^+/K^+-ATPase in the ischemic rat kidney was significantly restored to the expression level found in the control rat kidney by ingestion of cobalt. In ischemic renal tubules, the driving force may not be fully maintained, thereby resulting in reduced uptake of IS *via* rOAT3. The preventive effect of cobalt intake on the level of serum IS could involve restoration of the driving force for the rOAT1 and rOAT3 transporters. Cobalt intake might block the production of factors that decrease the driving force for these transporters. Alternatively, the activity of the efflux transporter(s) for IS at the apical membrane of renal tubules, which is reduced by I/R, might be restored by ingestion of cobalt. The precise mechanism involved in the relationship between the levels of

serum IS and the expression levels of OATs and the I/R-induced downregulation of these transporters should be further studied.

It was reported that the level of P-glycoprotein increased in rat kidney after glycerol-induced ARF.[36] In this study, we found that renal rMdr1 mRNA showed a transient upregulation during I/R of the rat kidney. To our knowledge, this is the first report indicating upregulation of rMdr1 in the kidney after I/R injury. Mdr1 is one of the known genes transcriptionally regulated by hypoxia-inducible factor 1 under hypoxic conditions.[17] Therefore, it is suggested that hypoxia-inducible factor 1 induced by ischemia stimulates the transient induction of rMdr1 gene. However, there was no significant difference in the P-glycoprotein level between the control and the ischemic rats at 48 h after ischemia, as found in the rMdr1 mRNA expression. P-glycoprotein may be upregulated during the early stage of ischemia, thereby preventing tubular cell derangement by secreting cytotoxic substances produced in ischemic kidney.

In conclusion, we report that I/R of rat kidney induces the serum accumulation of IS, which could be caused by the downregulation of renal rOAT1 and rOAT3, accompanied by decreased transport activities. Although ingestion of cobalt ameliorates the progression of renal dysfunction, as evident from the decrease in the level of BUN, SCr, and IS, it has no significant effect on the expression of rOAT1 and rOAT3. ES uptake in renal slices of ischemic rats suggests that ingestion of cobalt causes a partial restoration of transport activity of rOAT3, but not rOAT1. This study has increased our understanding the mechanism of regulation and pathophysiological implications of OATs of renal tubules as well as the renal handling of anionic drugs and endogenous toxins under ischemic ARF conditions.

MATERIALS AND METHODS

Materials

Cobalt chloride was obtained from Wako Pure Chemical Industries, Ltd (Osaka, Japan). [6,7-^{3}H(N)]-ES ammonium salt (2120 GBq/mmol), D-[1-^{3}H(N)]-mannitol (525.4 GBq/mmol), *p*-[Glycyl-^{14}C]-aminohippuric acid (1.95 GBq/mmol) and D-[1-^{14}C]-mannitol (1.89 GBq/mmol) were obtained from PerkinElmer Life and Analytical Sciences (Boston, MA, USA). IS was obtained from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals used were of the highest purity available.

Experimental animals

Male Sprague–Dawley rats, initially weighing 150–180 g (Crea Japan Inc., Tokyo, Japan), were housed in a standard animal maintenance facility at constant temperature (21–23°C), humidity (50–70%), and a 12:12 h light/dark cycle for at least 1 week before the day of the experiment. All animal experiments were conducted according to the guidelines of Kumamoto University for the care and use of laboratory animals. Rats in the control group were given tap water, whereas rats in the cobalt-intake group were given water containing 2 mM cobalt chloride 24 h before the induction of ischemic injury to kill.

Rats were anesthetized using sodium pentobarbital (50 mg/kg intraperitoneally), and placed on a heating plate (39°C) to maintain a constant temperature. The kidneys were exposed via midline abdominal incisions. Renal ischemia was induced by vascular clamps (AS ONE, Osaka, Japan) over both pedicles for 30 min. After the clamps were released, the incision was closed in two layers with 3–0 sutures. Sham animals (control) underwent anesthesia, laparotomy, and renal pedicle dissection only. All animals received warm saline solution instilled in the peritoneal cavity during the surgical procedure, and were then allowed to recover with *ad libitum* access to food and water. Animals were killed under surgical anesthesia at 6, 12, 24, and 48 h after experimental intervention. Blood samples were collected for measurement of SCr, BUN, sodium (Na), chloride (Cl), and potassium (K) in serum. A 50 μl aliquot of serum was added directly to 100 μl of methanol. After centrifugation at $3000 \times g$ for 10 min, the supernatant was assayed by high-performance liquid chromatography. For histological assessment, kidney samples were fixed in 4% buffered formaldehyde. Paraffin sections of the excised kidney were stained with hematoxylin–eosin reagent. For semiquantitative analysis of morphologic change, 10 high-magnification fields (0.0675 mm^2) of outer stripe and inner stripe of outer medulla in rats were randomly selected. Then, tubular injury was graded with an arbitrary score of 0–3: 0, the absence of necrosis; 1, mild; 2, moderate; and 3, severe. All quantification was performed in a blinded manner.

High-performance liquid chromatography determination of IS

The high-performance liquid chromatography system consisted of a Shimadzu LC-10ADVP pump and a Shimadzu RF-10AXL fluorescence spectrophotometer. A column of LiChrosorb RP-18 (Cica Merck, Tokyo, Japan) was used as the stationary phase and the mobile phase consisted of acetate buffer (0.2 M, pH 4.5). The flow rate was 1.0 ml/min. IS was detected by means of a fluorescence monitor (excitation 280 nm, emission 375 nm).

mRNA isolation and cDNA synthesis

For mRNA extraction, MagNA Pure LC mRNA Isolation Kit II (Roche Diagnostics, Basel, Switzerland) was used according to the instruction manual. In this automated process, the tissue samples were dissolved in a buffer containing a chaotropic salt and an RNase inactivator. The samples were homogenized with Lysing Matrix D (Qbiogene Inc., Morgan Irvine, CA, USA) and FastPrep (Qbiogene Inc.) at an oscillation speed of 4.5 for 30 s. The 3′-poly (A^+) from the released mRNA hybridizes to the added biotin-labeled oligo(dT). This complex is immobilized onto the surface of streptavidin-coated magnetic beads. After a DNase digestion step, unbound substances were removed by several washing steps, and purified mRNA was eluted (elution volume, 50 μl) with a low-salt buffer. cDNA was synthesized by using High-Capacity cDNA Archive Kit (Applied Biosystems Inc., Foster City, CA, USA). Briefly, 50 μl of mRNA substrate, 10 μl of reverse transcription (RT) buffer, 4 μl of dNTP mixture, 10 μl of RT random primers, 5 μl of MultiScribe reverse transcriptase, and 21 μl of nuclease-free water were used for cDNA synthesis. After each reverse transcription, cDNA was stored at −30°C.

Real-time polymerase chain reaction

We performed a TaqMan quantitative real-time RT polymerase chain reaction using ABI PRISM 7900 sequence to determine the expression level of rOAT1, rOAT3, rMdr1a and eukaryotic

18S ribosomal RNA (18 S rRNA). TaqMan 18S rRNA control reagents, the primer sets and products of TaqMan Gene Expression Assays were purchased from Applied Biosystems Inc. as follows: rOAT1, Rn00568143_m1; rOAT3, Rn00580082_m1; rMdr1a, Rn00591394_m1; and 18S rRNA, 4319413E.

Western blot analysis

Kidneys were homogenized in a homogenization buffer comprising 230 mM sucrose, 5 mM Tris-HCl, pH 7.5, 2 mM ethylenediaminetetraacetic acid, 0.1 mM phenylmethanesulfonyl fluoride, 1 mg/ml leupeptin, and 1 mg/ml pepstatin A. After measurement of the protein content using a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA), each sample was mixed in a loading buffer (2% sodium dodecyl sulfate, 125 mM Tris-HCl, 20% glycerol, 5% 2-mercaptoethanol) and heated at 100°C for 2 min. The sample were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA, USA) by semi-dry electroblotting. The blots were blocked overnight at 4°C with 2% ECL advance Blocking agents (GE Healthcare Bio-sciences Corp., Piscataway, NJ, USA) in Tris-buffered saline containing 0.3% Tween 20, and incubated 1 h at room temperature with primary antibody specific for rOAT1, rOAT3, P-gp (C219 monoclonal antibody, Signet Laboratories, Dedham, MA, USA), Na^+/K^+-ATPase α-1 subunit (Upstate Biotechnology Inc., Lake Placid, NY, USA) or β-actin (Sigma Chemical Co.). The blots were washed with Tris-buffered saline containing 0.3% Tween 20 and incubated with the secondary antibody (horseradish peroxidase-linked antirabbit immunoglobulin F $(ab)_2$ or horseradish peroxidase-linked anti-mouse immunoglobulin F$(ab)_2$, GE Healthcare Bio-sciences Corp.) for 1 h at room temperature. Immunoblots were visualized with an ECL system (ECL Advance Western Blotting Detection Kit, GE Healthcare Bio-sciences Corp.). The relative amount of each band was determined densitometrically using Densitograph Imaging Software (ATTO Corporation, Tokyo, Japan). Densitometric ratios to control rats were used as the reference and accorded an arbitrary value of 100.

Uptake by rat renal slices

Uptake studies by using isolated rat renal slices were carried out as described in a previous report.[37] Briefly, slices of whole kidney from control and ischemic rats with or without the cobalt intake were stored in ice-cold oxygenated incubation buffer composed of 120 mM NaCl, 16.2 mM KCl, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM NaH_2PO_4/Na_2HPO_4, pH7.5. Renal slices were randomly selected and placed for incubation in flasks containing 6 ml of the incubation buffer with [^{14}C]PAH (5 μM, 0.37 kBq/ml) or [^{3}H]ES (5 μM, 1.85 kBq/ml). The uptake of these compounds was carried out at 37°C under an atmosphere of 100% oxygen. [^{3}H]Mannitol (5 μM, 1.85 kBq/ml) was used to calculate the extracellular trapping and nonspecific uptake of [^{14}C]PAH as well as to evaluate the viability of slices. [^{14}C]Mannitol (5 μM, 0.37 kBq/ml) was used for [^{3}H]ES. After incubation for a specified period, the incubation buffer containing radiolabeled compounds was rapidly removed from the flask, washed twice with 5 ml of ice-cold phosphate buffered saline, blotted on filter paper, weighed, and solubilized in 0.5 ml of NCSII (GE Healthcare Bio-sciences Corp.). The amount of radioactivity was then determined in a liquid scintillation counter after adding 5 ml of OCS (GE Healthcare Bio-sciences Corp.).

Statistical analysis

Data were analyzed statistically by analysis of variance and Scheffe's multiple comparison test, unpaired *t*-test or Mann–Whitney's test. A *P*-value of less than 0.05 was considered statistically significant.

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