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RESEARCH PAPER

Effects of Metabolic Acidosis on Expression Levels of Renal Drug Transporters

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ABSTRACT

Purpose In the renal proximal tubular cells, various transporters play important roles in the secretion and reabsorption of drugs. When metabolic acidosis is induced, a number of adaptive changes occur in the kidney. The purpose of this study was to clarify the changes of drug transporters under the acidosis and the effects of these changes on urinary drug excretion.

Methods Wistar/ST rats were given 1.5% NH_4Cl in tap water for 48 h to induce the acidosis. Pharmacokinetics of PSP or metformin was evaluated. In addition, expression levels of drug transporters were examined by Western Blotting.

Results The renal clearance of PSP was markedly decreased, whereas the creatinine clearance and renal clearance of metformin were unchanged. Furthermore, Western blots indicated that the protein expression level of organic anion transporter (OAT) 3 was decreased. In contrast to OAT3 levels, OAT1 and organic cation transporter (OCT) 2 levels were unaffected. An immunohistochemical analysis showed that the OAT3 protein in the proximal tubules was localized in the basolateral membrane both of the normal and the acidosis rats.

Conclusion The decrease of renal excretion of anionic drugs during metabolic acidosis might be partly due to a reduction in the level of OAT3 protein.

KEY WORDS acidosis · anion · kidney · secretion · transporter

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INTRODUCTION

The kidney plays an important role in the urinary excretion of endogenous and exogenous organic ions, including drugs, toxins, and endogenous metabolites. In the renal proximal tubules, various kinds of membrane transporters mediate the secretion of a wide range of organic ions (1–3). Organic anion transporter (OAT) 1 (SLC22A6) and OAT3 (SLC22A8) on the basolateral membrane are involved in the uptake of anionic drugs into epithelial cells (4,5), and apical types of ATP-dependent primary active transporters, such as multidrug resistance-associated proteins (MRP) 2 (ABCC2) and MRP4 (ABCC4), play important roles in the secretion of anionic compounds (6). OCT1 (SLC22A1) and OCT2 (SLC22A2) mediate the basolateral uptake of organic cations in rats (7–9). Recently, an apical H^+/organic cation antiporter, multidrug and toxin extrusion 1 (MATE1), has been identified as responsible for the final step in the secretion of organic cations in the kidney (10,11). Alterations in the function and/or expression levels of transporters would affect the renal excretion of xenobiotics (12–15).

The kidney also plays a major role in systemic acid-base homeostasis. Metabolic acidosis is a common clinical condition characterized by decreases in blood pH and bicarbonate concentrations. Various factors cause the acidosis, including the excessive production or ingestion of acid. Under acidotic conditions, a number of adaptive changes occur in the kidney (16,17), acting in concert to reduce the acid load and restore the systemic acid-base balance. For example, during metabolic acidosis, the secretion of H^+ increases by the activation of the apical membrane Na^+/H^+ antiporter NHE3 (18,19). Moreover, expression levels of system N glutamine transporter (SNAT) 3 (SLC38A3) also increased in the basolateral membrane of

renal proximal tubules (20,21). As a result, increased secretion of ammonium ions from the proximal tubules into urine and of bicarbonate ions into the blood makes to restore the acid-base balance. It is possible that the tubular drug transport system also changes under acidotic condition. However, the effects of acidosis on urinary drug excretion have not been investigated.

The purpose of this study was to elucidate the changes to renal drug transporters under acidosis and to evaluate the effects of these changes on renal drug excretion.

MATERIALS AND METHODS

Materials

NH_4Cl was purchased from Wako Pure Chemical Industries (Osaka, Japan), phenolsulfonphthalein (PSP) from Nacalai Tesque (Kyoto, Japan), metformin from Sigma-Aldrich (St. Louis, MO, USA). The antibodies for OAT1, OAT3, OCT1, OCT2 and MATE1 were constructed previously in our laboratory (22–24). The antibodies for MRP2 and MRP4 were obtained from Sigma-Aldrich, Na^+/K^+-ATPase from Upstate Biotechnology Inc. (Lake Placid, NY, USA), and NHE3 from Chemicon International Inc. (Temecula, CA, USA). All other chemicals used were of the highest purity available.

Animals

Animals were treated in accordance with regulations of the Institutional Animal Use and Care Committee of Kyoto University. To induce metabolic acidosis, Male Wistar/ST rats (7 weeks old) were given 1.5% NH_4Cl in tap water *ad lib*. for 2 days. The controls received regular tap water *ad lib*. All rats were pair-fed normal chow. For the identification of metabolic acidosis, rats were anesthetized, and blood gas was analyzed with heparinized arterial blood using an i-STAT Portable Clinical Analyzer from Fuso Pharmaceutical Industries Ltd. (Osaka, Japan).

In Vivo Clearance Study

Rats were anesthetized with an intraperitoneal administration of 50 mg/kg pentobarbital sodium. Catheters were inserted into the right femoral artery and the left femoral vein with polyethylene tubes (SP-31; Natsume Seisakusho, Tokyo, Japan) filled with heparin solution (100 U/mL) for blood sampling and drug administration, respectively. Urine was collected from urinary bladder catheterized with PE-50 tube (Intramedic PE-50; Becton Dickinson and Co., Parsippany, NJ, USA). Thereafter, PSP or metformin was administered as a bolus via the femoral vein and incorporated into infusion solution as described previously (25). Loading doses were 10 mg/kg and 1 mg/kg for PSP and metformin, respectively. Maintenance doses were as follows: PSP 2.58 mg/mL and mannitol 50 mg/mL for the PSP solution, and metformin 0.21 mg/mL and mannitol 60 mg/mL for the metformin solution. The infusion was conducted at a rate of 2.2 mL/h using an automatic infusion pump (Natsume Seisakusho, Tokyo, Japan). Mannitol was used to maintain sufficient and constant urine flow rate. After stabilizing for 20 min, urine samples were collected three times at 20-min intervals for PSP and 10-min intervals for metformin. 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, both kidneys were harvested, immediately frozen in liquid nitrogen, and stored at −80°C until protein extraction. The concentrations of PSP in plasma and urine were determined spectrophotometrically at 560 nm after appropriate dilution with 1 M NaOH. The concentrations of metformin in plasma and urine were measured by high-performance liquid chromatography (25). The levels of creatinine in plasma and urine were determined by the Jaffe reaction, using an Assay kit (Creatinine test Wako) from Wako Pure Chemical Industries (Osaka, Japan). Pharmacokinetic parameters were calculated using standard procedures for each experimental period. Total plasma clearance (CLtot) was calculated by dividing the infusion rate by the steady-state plasma concentration (Cpss) at the midpoint of urine collection. Renal clearance (CLren) was obtained by dividing the urinary excretion rates by Cpss. The glomerular filtration rate (GFR) was assumed to be equal to the CLren of creatinine.

Western Blots

The crude membrane fractions were prepared from rat kidneys, as described previously (26). The fractions were separated on 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE; Bio-Rad Laboratories, Hercules, CA) and transferred electrophoretically to polyvinylidene fluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA). The blots were blocked with 5% skim milk in Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl, pH 7.5) containing 0.3% Tween-20 (TBS-T) for 3 h at room temperature and then incubated with the primary antibodies for OAT1, OAT3, OCT1, OCT2, MRP2, MRP4, MATE1, NHE3 and Na^+/K^+-ATPase overnight at 4°C. The blots were washed three times with TBS-T, and the bound antibodies were detected on X-ray film by enhanced chemiluminescence (ECL) with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody and cyclic diacylhydrazides (Amersham Pharmacia Biotech, Uppsala, Sweden). The density of signals was evaluated

using Image J 1.38 software (National Institutes of Health, Bethesda, MD).

Immunohistochemical Analysis

The rats were anesthetized, and the kidneys were perfused with 4% paraformaldehyde as described previously (14). After the perfusion, the sections were cut 5 μm thick, blocked with 5% bovine serum albumin (BSA) in PBS for 3 h, and incubated with the primary antibody against OAT3 (1:100) at 4°C for 12 h. Sections were then washed three times with PBS and incubated with Cy3-labeled donkey anti-rabbit IgG (GALTAG Laboratory, San Francisco, CA, USA), and Alexa Phalloidin-488 (Molecular Probe, Eugene, OR, USA) at room temperature for 1 h. These sections were viewed under a fluorescence microscope (BX-50-FLA, Olympus, Tokyo, Japan) at ×-100 or ×-400 magnification.

Determination of Alpha-Ketoglutarate (α-KG) Levels in Renal Cortex

The intracellular concentration of α-KG was determined by a fluorometric method (27,28). The renal cortex was homogenized with 4 volumes of ice-cold 3% perchloric acid. After centrifugation (13,000 rpm, 2 min, 4°C), the supernatant was transferred into a micro tube and neutralized with NaOH. The amount of extracted α-KG in the neutralized buffer was measured with an enzymatic analysis. The conversion of NADH to NAD^+ was determined fluorometrically (Excitation, 355 nm; Emission, 460 nm) at 37°C with Mithras LB 940 (Berthold Technologies, Osaka, Japan).

Statistical Analysis

Data are expressed as the mean ± SEM. Differences were compared with an unpaired Student's *t-test*, and a value of $P<0.05$ was considered significant.

RESULTS

Acid-Base Status of Animals

Rats were given 1.5% NH_4Cl in tap water for 48 h to induce metabolic acidosis. The controls received tap water only. Blood gas parameters are listed in Table I. Blood pH, HCO_3^- concentrations and base excess (BE) were decreased, whereas blood urea nitrogen (BUN) levels and blood chloride concentrations were increased in the rats subjected to NH_4Cl loading. The treatment with NH_4Cl was confirmed to induce metabolic acidosis.

Table I Arterial Blood Gas Analysis of Normal and Acid-Loading Rats

	Normal	Acidosis
Glucose (mg/dL)	176.8 ± 3.8	181.6 ± 7.4
BUN (mg/dL)	11.2 ± 0.8	20.4 ± 1.6***
Na^+ (mM)	139.6 ± 1.5	141.4 ± 0.9
K^+ (mM)	3.8 ± 0.1	3.7 ± 0.1
Cl^- (mM)	103.0 ± 0.9	108.6 ± 1.0**
TCO_2 (mM)	29.0 ± 0.6	23.4 ± 1.2
Anion Gap (mM)	12.4 ± 0.6	14.8 ± 0.6
Hematocrit (% PCV)	41.6 ± 0.7	47.0 ± 0.5
Hemoglobin (g/dL)	14.1 ± 0.2	15.9 ± 0.2
pH	7.40 ± 0.02	7.22 ± 0.03**
PCO_2 (mmHg)	48.9 ± 2.4	53.7 ± 4.4
HCO_3^- (mM)	27.5 ± 0.6	21.6 ± 1.1**
Base Excess (mM)	2.0 ± 0.7	−6.2 ± 1.2***

Summary of the result of the arterial blood gas analysis of normal and 1.5% NH_4Cl-treated rats. All rats received standard rodent chow. Each value represents the mean ± SEM for 5 rats. pCO_2, partial pressure of CO_2; BE, base excess. ** $p<0.01$, *** $p<0.001$, significantly different from normal rats.

Pharmacokinetics of PSP and Metformin in Rats with Metabolic Acidosis

Fig. 1 shows the plasma concentration of PSP and metformin. During the experiments, plasma concentrations of both drugs were attained to steady-state. Table II shows the pharmacokinetic parameters of PSP after the intravenous infusion in normal and rats with metabolic acidosis. The steady-state plasma concentration (Cpss) of PSP was markedly elevated compared with control values. The total clearance (CLtot) and renal clearance (CLren) of PSP were significantly decreased. The Cpss of metformin was also higher than in the control rats (Table III). The CLtot of metformin was markedly decreased, while the CLren of metformin was unchanged. In addition, the plasma creatinine level was not significantly affected (normal rats: 0.59 ± 0.04 mg/dL; metabolic acidosis rats: 0.54 ± 0.02 mg/dL).

Protein Expression Levels of Renal Tubule Drug Transporters

The expression level of OAT3 protein decreased significantly under acidosis compared with that of normal rats (Fig. 2b), whereas the expression levels of OAT1, MRP2 and MRP4 were normal (Fig. 2a, c and d). While the expression level of OCT2 was unchanged in metabolic acidosis rats, expression level of OCT1 was decreased and that of MATE1 was increased (Fig. 3a, b and c). The expression level of Na^+/K^+-ATPase, which is well known as the basolateral transporter, was not affected (Fig. 3d). These

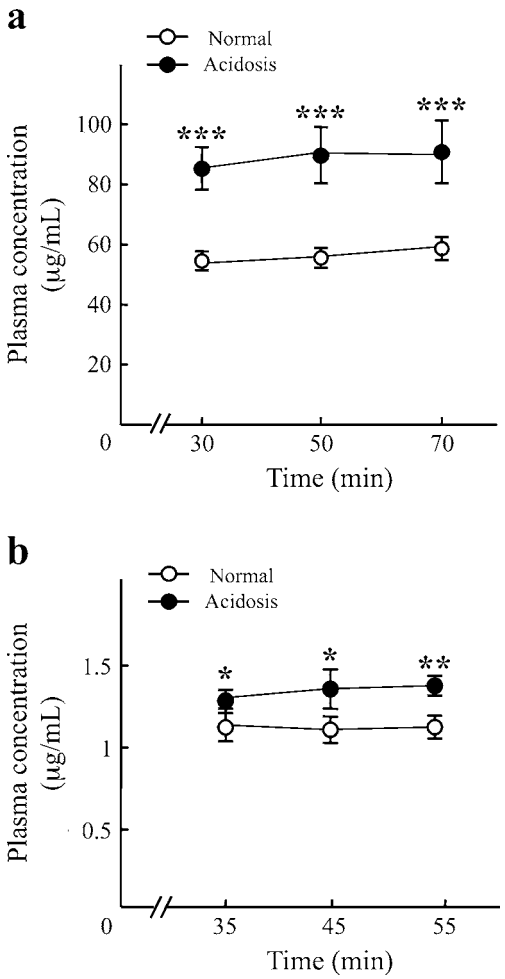

Fig. 1 Plasma concentration profile of PSP (**a**) and Metformin (**b**) in normal and metabolic acidosis rats. PSP at 5.67 mg/h or metformin at 0.462 mg/h was intravenously infused after a loading dose of PSP at 10 mg/kg or metformin 1 mg/kg. The infusion rate was 2.2 mL/h. Blood samples were collected at the time points indicated. Each point represents the mean ± SEM of 4~5 rats. $^{*}P<0.05$; $^{**}p<0.01$; $^{***}p<0.001$, significantly different from normal rats.

Table II Pharmacokinetic Parameters of PSP in Normal and Metabolic Acidosis Rats at the Steady-State

	Normal	Acidosis
Body weight (g)	240 ± 6	216 ± 1**
Urine volume (mL)	0.46 ± 0.02	0.32 ± 0.03***
Cpss (μg/mL)	56.44 ± 1.71	87.76 ± 6.97***
Cuss (μg/mL)	1981 ± 118	1838 ± 145
CLtot (mL/min/kg)	7.11 ± 0.29	5.86 ± 0.30**
CLren (mL/min/kg)	3.34 ± 0.18	1.56 ± 0.19***

PSP was infused intravenously at 5.67 mg/h. Each value represents the mean ± SEM for 5 rats. Cpss, steady-state plasma concentration; Cuss, steady-state urine concentration; CLtot, total clearance; CLren, renal clearance. $^{**}P<0.01$, $^{***}P<0.001$, significantly different from normal rats.

Table III Pharmacokinetic Analysis of Metformin and Plasma Creatinine Levels in Normal and Metabolic Acidosis Rats at the Steady-State

	Normal	Acidosis
Body weight (g)	230 ± 3	217 ± 2*
Urine volume (mL)	0.20 ± 0.01	0.25 ± 0.00**
Cpss (μg/mL)	1.11 ± 0.04	1.32 ± 0.04**
Cuss (μg/mL)	305 ± 13	280 ± 12
CLtot (mL/min/kg)	30.87 ± 0.51	27.19 ± 0.80**
CLren (mL/min/kg)	24.63 ± 1.17	24.28 ± 1.27
Pcr (mg/dL)	0.59 ± 0.04	0.54 ± 0.02
Ccr (mL/min/kg)	4.17 ± 0.22	4.76 ± 0.17

Metformin was infused inravenously at 0.462 mg/h. Each value represents the mean ± SEM of 5 rats. Cpss, steady-state plasma concentration; Cuss, steady-state urine concentration; CLtot, total clearance; CLren, renal clearance; Pcr, plasma creatinine; Ccr, creatinine clearance. $^{*}P<0.05$, $^{**}P<0.01$, significantly different from normal rats.

results indicate that the change of OAT3 is not nonspecific alteration. Consistent with previous reports (18,19), the renal expression level of NHE3, as positive control, was significantly increased in the rats with metabolic acidosis (Fig. 3e).

Immunohistochemical Analysis of OAT3

PSP has been used widely as a substrate for the organic anion transport system and is reported to have high affinity for OAT3 and low affinity for OAT1 (12). It was hypothesized that decreased renal excretion of PSP during metabolic acidosis could be caused by the decreased expression of OAT3 in the kidney. Alternatively, a change in the localization of OAT3 could affect the secretion of PSP. As shown in Fig. 4, the OAT3 protein was expressed in the basolateral membrane of proximal cells, and its localization did not differ between the normal and acidotic rat kidney. These findings suggested that metabolic acidosis affected the protein expression level of OAT3, but not its localization.

Effect of Metabolic Acidosis on the Intracellular α-KG Level

It was reported that α-KG was necessary for increased glutamine synthesis during metabolic acidosis (29). The outward gradient of α-KG is considered a driving force for the basolateral uptake of organic anion in renal proximal tubules (30). Therefore, intracellular α-KG may influence the excretion of organic anions in renal proximal tubules. It was reported that OAT3 exchanged intracellular α-KG for an extracellular organic anion. The α-KG level in the renal cortex was lower in the acidotic rats than normal rats, but not significantly (Fig. 5).

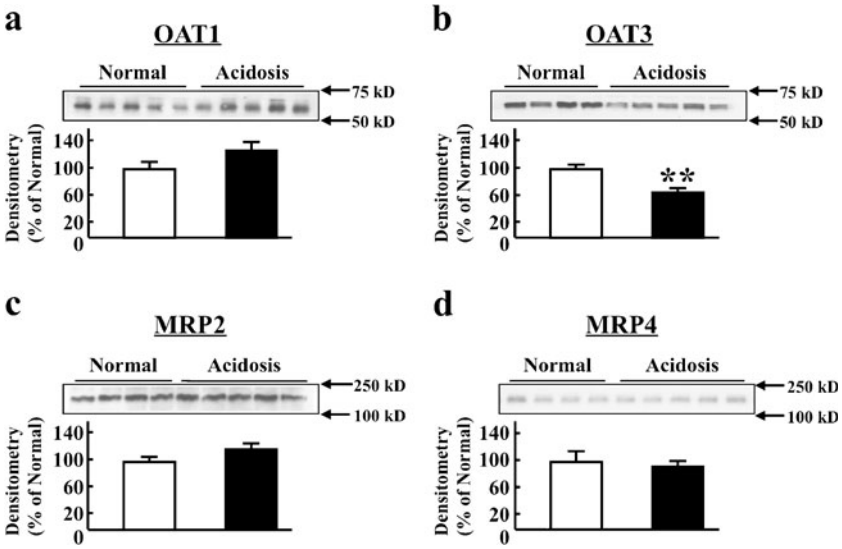

Fig. 2 Western blot analysis of renal crude plasma membrane with antibodies for OAT1 (**a**), OAT3 (**b**), MRP2 (**c**), and MRP4 (**d**) in normal and metabolic acidosis rats. Upper, representative results of Western blot analysis; lower, densitometric analysis. Open columns, normal rats; closed columns, metabolic acidosis rats. The values for normal rats were arbitrarily defined as 100%. Each column represents the mean ± SEM of 4~5 rats. $^{**}P < 0.01$, significantly different from normal rats.

DISCUSSION

In this study, we first investigated the expression levels of renal drug transporters under acidosis in the rats. It was found that the level of OAT3 protein was decreased, and the decrease of OAT3 partly affected the urinary excretion of PSP. These findings should be important information to clarify the urinary excretion of drugs and xenobiotics under acidosis.

The renal secretion of various drugs is mediated by drug transporters in the tubular epithelial cells, and the alteration in the expression levels of these transporter may affect excretion of the drug via the kidney. During metabolic acidosis, a number of adaptive changes occur in the kidney. For example, the apical membrane transporter NHE3 (18,19) and system N glutamine transporter SNAT3 were highly up-regulated in their expression (20,21). Indeed,

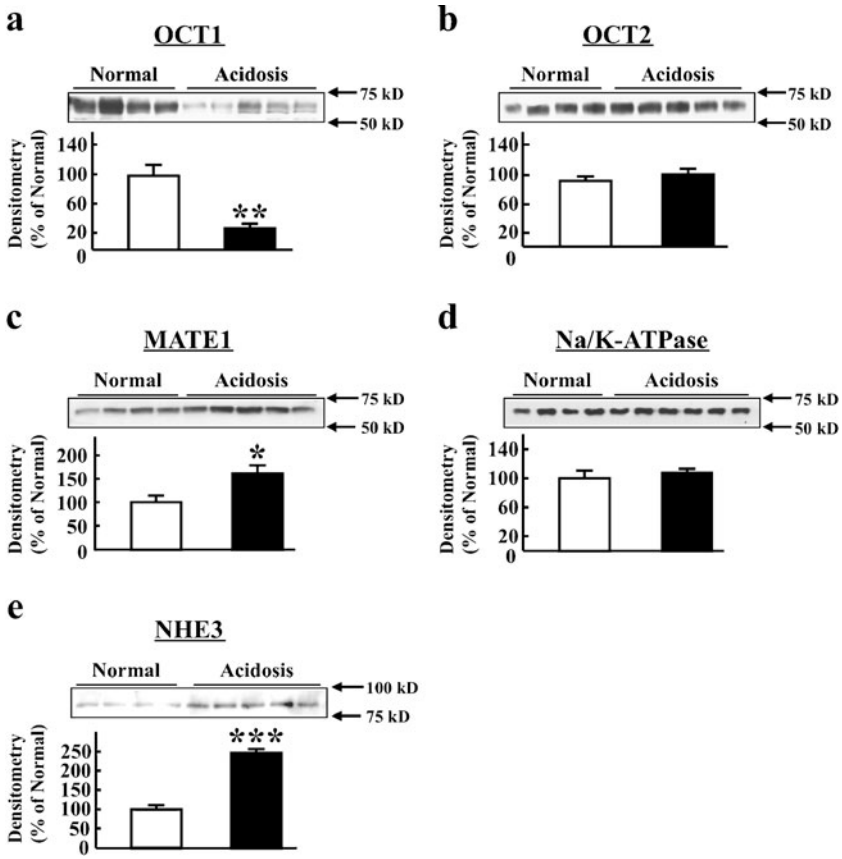

Fig. 3 Western blot analysis of renal crude plasma membrane with antibodies for OCT1 (**a**), OCT2 (**b**), MATE1 (**c**), and Na^+/K^+-ATPase (**d**) with positive control NHE3 (**e**) in normal and metabolic acidosis rats. Upper, representative results of Western blot analysis; lower, densitometric analysis. Open columns, normal rats; closed columns, metabolic acidosis rats. The values for normal rats were arbitrarily defined as 100%. Each column represents the mean ± SEM of 4~5 rats. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ significantly different from normal rats.

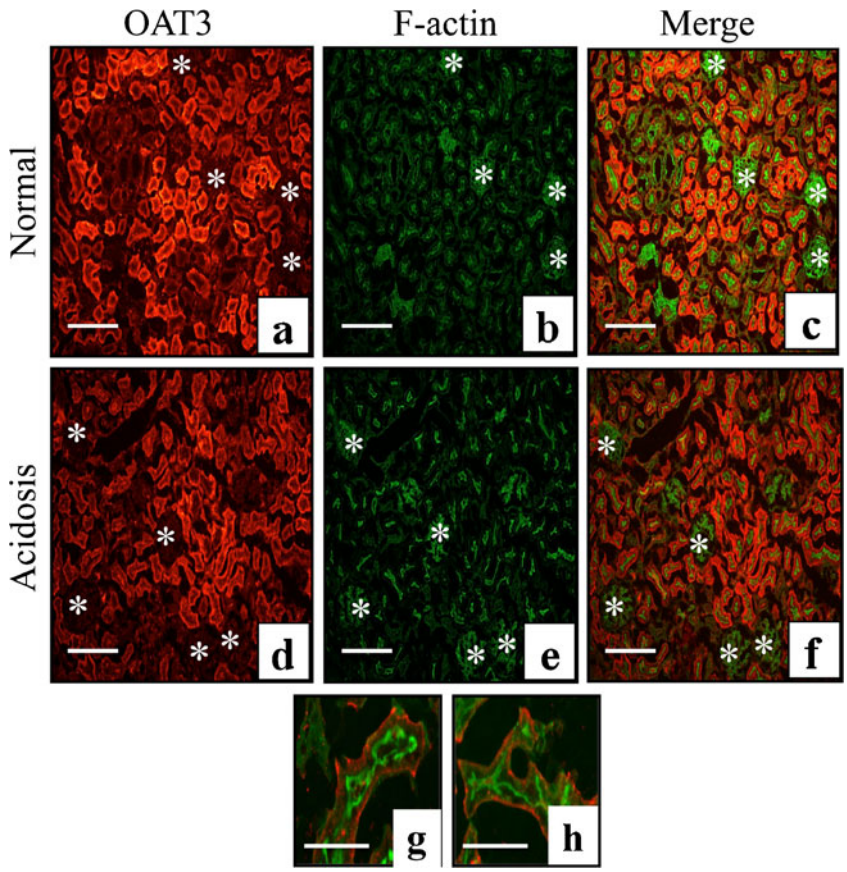

Fig. 4 Immunohistochemistry of OAT3 in the kidney cortex of normal (**a**, **b**, **c** and **g**) and metabolic acidosis rats (**d**, **e**, **f** and **h**). Red (**a**, **d**), OAT3; green (**b**, **e**), F-actin; merge (**c**, **f**, **g**, **h**), the OAT3 (*red*) and F-actin (*green*) signals were merged in the same section. *, Glomerulus. **a**–**f**, ×100 magnification, scale bar 300 μm; **g** and **h**, ×400 magnification, scale bar 100 μm.

NHE3 was increased in our study. However, no information is available about the functional and molecular changes to renal drug transporters and their effects on the renal excretion of drugs during metabolic acidosis. In the present study, we examined the pharmacokinetics of an anionic drug, PSP, and a cationic drug, metformin, and the expression levels of tubular drug transporters under acidotic conditions.

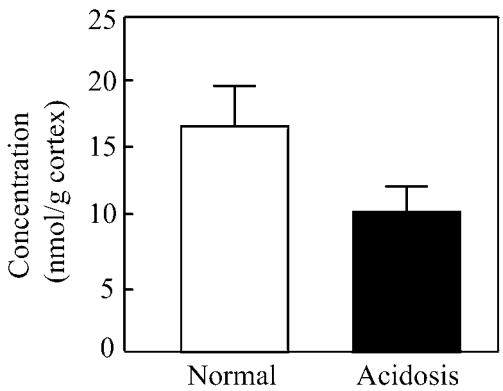

Fig. 5 Effects of metabolic acidosis on the intracellular α-KG levels in renal cortex. The concentration of α-KG was determined by fluorimetric methods (excitation wavelength: 355 nm; fluorescence wavelength: 460 nm; temperature: 37°C; lamp energy: 7000). Data are expressed as the mean ± SEM of 5 rats.

Plasma creatinine and blood urea nitrogen (BUN) levels are commonly used for testing renal function. In this study, the level of creatinine was not changed in the acid-loaded rats (control: 0.59±0.04; metabolic acidosis: 0.54± 0.02 mg/dL), suggesting that the GFR was not affected by metabolic acidosis. However, the BUN level was increased, likely as a result of the metabolism of NH_4Cl (Table I). Orally administered NH_4Cl can be metabolized by enzymes of the urea cycle in hepatocytes, causing an increase in the metabolism of urea (31). Therefore, the increase in BUN was not considered indicative of a change in renal function.

PSP is widely used clinically for testing renal function. Blood PSP binds to the protein over 80%. While PSP scarcely passes through the glomerulus, a large fraction of PSP is secreted via proximal tubules. Moreover, PSP, as well as PAH, is recognized by organic anion transporter and more effectively transported by OAT3 than by OAT1 from the blood circulation into tubular epithelial cells (12). In the present study, the total and renal clearance of organic anion PSP were markedly decreased in metabolic acidosis rats (Table II). The Western blots showed the protein expression level of OAT3 was decreased in acid-loaded rats (Fig. 2b). OAT3 protein levels were decreased about 40%, and renal excretion of PSP was decreased

about 50%. It seems that the decrease of PSP excretion is comparable to decrease of OAT3 protein. MRP2 and MRP4 are reported as the apical membrane transporters of renal proximal tubules. However, apical MRP2 and MRP4 levels were not changed by treatment with NH_4Cl. Therefore, changes of PSP secretion seem to be not related to MRP2 and MRP4. In addition, renal α-KG levels were not significantly affected by acidosis (Fig. 5). Therefore, the decrease in the renal tubular secretion of PSP was considered to be related to the reduction of OAT3 protein.

Metformin is mainly excreted in the urine as an unchanged form (32). The renal clearance is much higher than the glomerular filtration rate in rats. The secretion of metformin is mediated by OCT1 and OCT2 on the basolateral side of proximal cells (25,33). On the apical side, metformin is transported by MATE1 from proximal cells to urine (34,35). In this study, both the renal clearance of metformin and the protein expression level of OCT2 were unchanged in the rats with metabolic acidosis (Table III, Fig. 3b). In contrast to the level of OCT2, the amount of OCT1 protein was reduced, and that of MATE1 protein was increased under acidotic conditions (Fig. 3a and c). It seemed that the renal clearance of metformin was not affected by the changes to OCT1 and/or MATE1 levels. Previously, it was reported that the mRNA and protein expression levels of OCT2 were markedly higher than the OCT1 in rat and human proximal cells (25,36), and it was suggested that the OCT2 plays a major role in the pharmacokinetics of metformin. According to our findings, the renal excretion of organic cations was not affected by metabolic acidosis, although the OCT1 protein level was decreased.

The non-renal clearance of metformin was decreased under acidosis condition. Metformin is considered to be taken up into liver by OCT1. Though OCT1 protein levels in the liver were not measured, it was possible that changes of hepatic transporters were involved in the change of metformin clearance.

In the rat kidney, several SLCOs were expressed in the proximal tubules. For example, oatp1a1 was expressed at brush-border membrane (37) and SLCO4C1 at basolateral membrane (38). It was considered that oatp1a1 mediated reabsorption of organic anion and that SLCO4C1 mediated renal uptake of the drugs. However, contributions of these transporters for renal secretion of PSP have not been known. In addition, oatp1a3v1 and oatp1a3v2 (OAT-K1 and OAT-K2) were expressed at rat brush-border membrane (39). The changes of these transporters levels under the acidosis should be considered in future studies.

CONCLUSION

In conclusion, this study suggests the renal excretion of anionic drugs decreases during metabolic acidosis and that the decrease is at least partly due to a reduction in the level of OAT3 protein.

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