The Sodium-Bicarbonate Cotransporter 1 (NBC1) and Monocarboxylate Transporter 1 (MCT1) in the Rat Kidney are Regulated in Response to Metabolic Acidosis

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Abstract
The present work was designed to study the effect of metabolic acidosis induced by ammonium chloride on the expression of sodium-bicarbonate cotransporter 1 (NBC1) and Monocarboxylate transporter 1 (MCT1) in rat kidney. Following the treatment of NH4Cl, blood pH and bicarbonate concentration were significantly decreased, while significant increase in chloride concentration was developed. Moreover, urine analysis revealed decrease in urine pH and significant increase in ammonia in acidic rats. The protein expression of NBC1 and MCT1 was determined in the rat kidney by immunofluorescence confocal laser-scanning microscopy and Western blotting analyses. In the experimental group, the expression level of NBC1 was significantly increased in the renal proximal tubules, whereas, the expression of MCT1 was significantly decreased. Western blotting data confirmed the immunofluorescence confocal laser-scanning microscopy. In conclusion, metabolic acidosis causes adaptive increases in the basolateral NBC1 in the rat renal proximal tubule that are likely responsible for the increased ability of the proximal tubule to reabsorb filtered bicarbonates. Additionally, acidosis leads to decrease the MCT1 protein expression level in renal proximal tubule that may explain the increase in lactate excretion in the urine of acidic rats due to the reduction in lactate co-transport.

Key words: Monocarboxylate transporter 1, Sodium-bicarbonate cotransporter 1, Ammonium Chloride, Kidney, Rats.

1. INTRODUCTION
Intracellular pH regulation and the whole body acid–base homeostasis are of major importance in both physiological and physiopathological conditions. All cellular and bodily function depends on pH from control of the cell cycle to the muscle contraction (Boron, 2004).

The kidneys have essential roles in maintaining the acid-base balance due to their ability to reabsorb bicarbonate from urine and excrete hydrogen ions into urine (Koeppen, 2009). The renal proximal tubule is the major site of bicarbonate (HCO3−) reabsorption, reclaiming ~80% of the HCO3− filtered by the glomerulus (Boron, 2006). HCO3−, like other ions and nutrients in the blood, is filtered in the kidney at the glomerulus, and then absorbed by transport processes in the renal nephron (Alpern, 2000). Moreover, kidney has an essential role in lactate metabolism. The renal cortex appears to be the major lactate-consuming organ in the body after the liver (Bellomo, 2002).

It has been known that sodium-bicarbonate cotransporter1 (NBC1) and monocarboxylate transporter1 (MCT1) transport acid/base equivalents and coexist in many epithelial cells (Becker et al., 2004). The electrogenic Na+-HCO3− cotransporter (NBC1) carries a net negative charge, consistent with the transport of more than one HCO3− per Na+ (Romero and Boron, 1999; Soleimani and Burnham, 2000). In reabsorptive epithelia (such as kidney proximal tubule cells), NBC is responsible for the reabsorption of bicarbonate from cell to the blood. In kidney proximal tubule, the Na+:HCO3−cotransporter (NBC1) works in efflux mode and has a stoichiometry of 3 HCO3− per Na+ (Alpern, 1990; Romero and Boron, 1999; Soleimani and Burnham, 2000).

MCT1 is one of the transmembrane transporters encoded by proton dependent monocarboxylate transporters (MCTs; SLC16A) gene family. MCT1 facilitate the transport of lactate, pyruvate and β-hydroxybutyrate. The rapid transport of these monocarboxylates across membranes is essential for maintaining cellular homeostasis. One of the important roles of MCT1 is the unidirectional transport of L-lactate (influx or efflux) which depends on the intracellular and extracellular lactate...
concentrations as well as the proton gradient across the membrane. (Adjanto and Philp, 2012).

Metabolic acidosis is a common clinical condition that is characterized by a decrease in blood pH and bicarbonate concentration and is caused by overproduction of an acid or excessive loss of base (Stern, 2004).

The present work was designed to study the effect of metabolic acidosis induced by ammonium chloride on the expression of the acid-base transporters; NBC1 and MCT1 in rat kidney.

2. MATERIALS AND METHODS

2.1. Animals and induction of chronic metabolic acidosis

The experimental protocols were approved by the Ethics Committee for Animal Experiments in the School of Veterinary Medicine, Rakuno Gakuen University. Male Sprague-Dawley rats (210–250 g) were placed in cages and allowed free access to water and normal rat chow. After 72 h of adjustment in cages, rats were divided into two groups: control group (n=10) remained on water, and the treated group (n=10) was switched to a drinking solution containing 280 mM NH₄Cl for 10 days (Good 1990; Ambühl et al., 1996; Amlal et al., 2001). All animals drank *ad libitum* and the volumes ingested were recorded. Only those rats which drank nearly equivalent amounts of water or NH₄Cl were used. After 10 days of treatment, rats were killed under anesthesia, and urine was withdrawn. All animals containing 280 mM NH₄Cl were euthanized by decapitation, and arterial blood was obtained from the abdominal aorta for blood composition analysis. Heparinized rat blood was prepared for analysis. Also serum was collected and frozen until used. The bladder was punctured just after decapsulation, and urine was withdrawn. Blood and urine samples were analyzed to monitor systemic acid-base status. Kidneys were immediately removed, decapsulated, and weighed. Afterwards, the superficial cortex was dissected and stored at -80°C for protein isolation. Kidney samples were also prepared for immunofluorescence confocal laser microscopy as previously described (Kirat et al., 2006).

2.2. Blood Composition and Urine Analyses

Plasma and urinary osmolalities were measured with a Roebelng osmometer (Bioblock, Rungis, France). Heparinized rat blood was collected and analyzed immediately for pH, blood gases, creatinine and electrolytes on a Radiometer ABL 800 FLEX (Radiometer, Copenhagen, Denmark) blood gas analyzer. Plasma samples for HCO₃⁻ were determined by calorimetric assay. Urine pH was measured with a pH meter (Accumet Basic AB15, Fisher Scientific, Hanover Park, IL). Urinary ammonium was measured using the Berthelot protocol (Berthelot, 1859). Urinary creatinine was measured by the Jaffe method (Seaton and Ali, 1984). Urinary Na⁺, K⁺, Ca²⁺, and Cl⁻ were measured by ion chromatography (Metrohm ion chromatograph, Switzerland). Analysis of urinary and serum lactate was performed using a commercially available kit (L-Lactate Assay Kit; Biomedical Research Service Center, NY) according to the manufacturer’s instruction.

2.3. Immunoblot analysis

Plasma membrane was prepared from the renal cortex as previously described (Kirat et al., 2006). The protein concentration was measured, and the membrane proteins were then stored at -20°C until used. Immunoblotting was carried out as previously described (Kirat et al., 2006). Rabbit anti-rat NBC1 polyclonal antibody (AB-3212, 1:1000; EMD Millipore) and chicken anti-rat MCT1 antibody (AB1286; 1:500; EMD Millipore) were used. Proteins were visualized using a secondary antibody conjugated to horseradish peroxidase (donkey anti-rabbit IgG; Pierce or rabbit anti-chicken IgY; Upstate, NY, USA) and an enhanced chemiluminescence detection system (ECL; Amersham International, Buckinghamshire, UK) according to the instructions of the manufacturer. The band antibody was captured on light-sensitive imaging film (Kodak). Negative control blots were probed with the NBC1 or MCT1 antibodies that had been preabsorbed overnight at 4 °C with their corresponding peptide antigen (10 μg/ml). Bands corresponding to NBC1 and MCT1 proteins were normalized to β-actin (ab8227; Abcam, MA, USA) and quantitated by densitometric analysis using Scion Image analysis software (Scion Corporation, Frederick, MD, USA).

2.4. Immunofluorescence confocal laser-scanning microscopy

Rat renal sections (4-micrometer) were subjected to deparaffinization followed by antigen retrieval by heating in sodium citrate buffer (0.01 M, pH 6.0) in a microwave oven for 15 min. The non-specific binding sites were blocked with normal donkey serum (D 9663; Sigma-Aldrich, Inc., MO, USA) for 30 min at room temperature. The sections were then washed and incubated with a mixture of rabbit anti-rat NBC1 antibody (1: 50) and chicken anti-rat MCT1 antibody (1: 200) at 4°C overnight in a humidified chamber. For detection of NBC1, the
sections were incubated with the diluted (1: 50 in PBS) donkey anti- rabbit IgG conjugated to fluorescein isothiocyanate (FITC) (ab6798; Abcam, MA, USA) for 30 min at room temperature; while for MCT1, the sections were incubated with the diluted (1: 50) Alexa Fluor-594-labelled donkey anti- chicken IgY (MBSS39200; MyBioSource Inc., CA, USA) for 30 min at room temperature. Sections were examined under an Olympus Fluoview confocal laser-scanning microscope (Olympus, Tokyo, Japan). For negative controls, sections were probed with the primary antibodies that preincubated with their specific blocking peptides.

2.5. Statistical analyses
The data are expressed as the means ± SE. The significant differences between the values of control and treated group were estimated with the Student’s t test. A value of \( P \leq 0.05 \) or less was considered statistically significant. All data analyses were performed using the VassarStats web site for statistical computation (http://faculty.vassar.edu/lowry/VassarStats.html).

3. RESULTS AND DISCUSSION

3.1. Induction of metabolic acidosis in rats
In the present study, the treatment with NH\(_4\)Cl resulted in the expected hyperchloremic metabolic acidosis and its consequences. Application of NH\(_4\)Cl with drinking water is a well established method to induce metabolic acidosis (Good, 1990; Ambühl et al., 1996; Amlal et al., 2001).

In our study, blood and urine samples were examined to screen the systemic acid-base status and renal acid excretion. The blood and urine data from rats receiving 280 mM NH\(_4\)Cl in drinking water for 10 days in comparison to controls were summarized in Table (1). Metabolic acidosis was developed and confirmed by significant decrease in blood pH and bicarbonate concentration, and a significant increase in chloride concentration in rats treated with NH\(_4\)Cl. Moreover, urine analysis revealed decrease in urine pH and significant increase in ammonia in acidic rats. Plasma and urine osmolalities were significantly elevated only in NH\(_4\)Cl-loaded rats than that of controls. Concentrations of sodium, potassium, and creatinine in rat plasma and urine were not significantly different in both groups (Table 1). Collectively, blood and urine data are consistent with those of (Good, 1990; Ambühl et al., 1996; Menegon et al., 1998; Amlal et al., 2001; Quentin et al., 2004; Nowik et al., 2008) in animals receiving NH\(_4\)Cl in drinking water.

It is well known that chronic metabolic acidosis significantly increases urinary net acid excretion, associated with some adaptive changes in renal tubules that contribute to increase the urinary acidification. Additionally, Good (1990) demonstrated that chronic metabolic acidosis induced by oral NH\(_4\)Cl loading in the drinking water was significantly increased the bicarbonate reabsorption as well as net NH\(_4\) absorption in rat kidney.

All rats survived the acidosis and were clinically healthy up to the day ten of the study. Table (1) revealed a significant lower body weight gain in the NH\(_4\)Cl-loaded rats after ten days compared to the control group. The kidney weight was significantly higher in the acidic rats compared to the control rats (Table 1). These data are consistent with the in vivo studies by Menegon et al., (1998) who demonstrated a significant increase in kidney weight in the acidic rats compared to the control rats. These results are also in agreement with the in vitro studies of proximal tubule cells, which showed that cellular hypertrophy was accompanied by a marked decline in protein degradation but not in cell protein synthesis after the administration of NH\(_4\)Cl (Ling et al., 1996).

3.2. Localization of NBC1 and MCT1 in the rat kidney
Renal proximal tubule is predominantly responsible for the bulk of bicarbonate reabsorption under normal conditions. The mode of HCO\(_3^-\) movement from the proximal tubule cells back into the blood was discovered by Boron and Boulpaep (1983), who demonstrated that this HCO\(_3^-\) absorption process was coupled asymmetrically to Na\(^+\) transport by Na\(^+\)-HCO\(_3^-\)/cotransporter.

In the present study, to examine the localization of NBC1 in the rat kidney, immunostaining of rat kidney sections with NBC1 antibody was performed and the reaction was detected by immunofluorescence confocal laser microscopy as described in the methods section. As shown in Figure (1A, B), we observed a strong fluorescence signal in proximal tubule cells. The staining pattern is typical to the basolateral membrane. No labeling was detected with the NBC1 antibody that was pre-adsorbed overnight with its immunizing peptide (Data not shown). Our images also showed that NBC1 immunoreactivity was consistently absent in all other cortical structures, including the thick ascending limb, cortical collecting duct, glomerulus, vasculature, and interstitium.
Under normal conditions, our data confirmed the previous observations that initially localized by functional studies the NBC1 to the basolateral membrane of the proximal tubule, where it plays a role in mediating electronegative HCO₃⁻ efflux (Boron and Boulaquep, 1983). In rat kidney, the expression of NBC1 is limited and localized to the basolateral membrane of the proximal tubule (Abuladze et al., 1998; Burnham et al., 1998; Schmitt et al., 1999). Moreover, NBC1 localization, based on immunoelectron microscopic studies, is confined to convoluted segments (S1 and S2) but not the S3 segment of the proximal tubule (Maunsbach et al., 2000).

Furthermore, with the use of MCT1 antibody, images of the immunofluorescence confocal laser microscopy confined the cellular localization of MCT1 protein on the basolateral side of the renal proximal tubule of rats (Fig. 1C, D). In kidney, MCT1 was reported in the basolateral membranes of hamster and mouse proximal tubule cells (Garcia et al., 1995 and Becker et al., 2010).

In addition, the results of immunohistochemical studies were further confirmed by Western blotting analysis, which detected the presence of bands for NBC1 and MCT1 proteins at ~130 and 45 kDa, respectively on the immunoblots of membrane proteins isolated from rat kidney cortex (Figs. 2A and 3A). These specific bands were abolished in the negative control blots (data not shown).

### 3.3. Effect of chronic NH₄Cl loading on NBC1 and MCT1 protein abundance in the rat kidney

The renal adaptation to metabolic acidosis involves the regulation of various transport proteins (Nowik et al., 2008). In the present study, confocal imaging and Western blotting analysis were employed to assess the effect of metabolic acidosis on the protein abundance of NBC1 protein expression in rat kidney (Figs. 1 and 2). Our confocal laser scanning images indicated that the number of positively stained proximal tubules for NBC1 protein was higher in NH₄Cl-loaded rats (Fig. 1B). Besides the intensity of NBC1 immunostaining in the renal proximal tubules was stronger in NH₄Cl-loaded rats (Fig. 1B) than that in control group (Fig. 1A). Using Western blot analysis revealed that the protein abundance of NBC1 expression in the kidney was significantly higher in rats after 10 days of NH₄Cl loading in comparison to control group (Fig. 2).

Several studies have examined the effect of metabolic acidosis on fluid and electrolyte reabsorption in various nephron segments. Primary metabolic acidosis, a condition manifested by decreased serum HCO₃⁻ and pH, has been shown to be associated with an increased ability of the renal

### Table 1: Blood and urine parameters obtained from rats receiving 280 mM NH₄Cl in drinking water for 10 days in comparison to controls.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NH₄Cl-loaded</th>
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<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.34 ± 0.03</td>
<td>7.18 ± 0.02*</td>
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<tr>
<td>HCO₃⁻ mM</td>
<td>25.81 ± 0.9</td>
<td>19.53 ± 1.1*</td>
</tr>
<tr>
<td>pCO₂ mmHg</td>
<td>48.7 ± 1.2</td>
<td>51.1 ± 2.1</td>
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<tr>
<td>Na⁺ mM</td>
<td>147.2 ± 0.5</td>
<td>148.3 ± 0.7</td>
</tr>
<tr>
<td>K⁺ mM</td>
<td>4.7 ± 0.1</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>Cl⁻ mM</td>
<td>111.2 ± 0.7</td>
<td>120.6 ± 0.9cc</td>
</tr>
<tr>
<td>Lactate</td>
<td>8.7 ± 0.8</td>
<td>9.7 ± 1.1</td>
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<tr>
<td>Osmolality mM/kg H₂O</td>
<td>288 ± 3</td>
<td>304 ± 2*</td>
</tr>
<tr>
<td><strong>Urine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.62 ± 0.09</td>
<td>5.41 ± 0.07*</td>
</tr>
<tr>
<td>Creatinine mg/dl</td>
<td>58.4 ± 6.2</td>
<td>51.2 ± 3.4</td>
</tr>
<tr>
<td>NH₄ mM/creatinine (mg/dl)</td>
<td>2.3 ±0.18</td>
<td>7.37 ± 0.43**</td>
</tr>
<tr>
<td>Na⁺ mM/creatinine (mg/dl)</td>
<td>1.8 ±0.19</td>
<td>2.16 ± 0.21</td>
</tr>
<tr>
<td>K⁺ mM/creatinine (mg/dl)</td>
<td>3.7 ±0.5</td>
<td>4.68 ±0.6</td>
</tr>
<tr>
<td>Cl⁻ mM/creatinine (mg/dl)</td>
<td>3.2 ±0.71</td>
<td>11.92 ± 0.78**</td>
</tr>
<tr>
<td>Lactate/creatinine</td>
<td>0.09 ± 0.01</td>
<td>0.26 ± 0.03**</td>
</tr>
<tr>
<td>Lactate excretion (mg/24 h)</td>
<td>0.53 ±0.18</td>
<td>1.77 ± 0.34**</td>
</tr>
<tr>
<td>Osmolality mM/kg H₂O</td>
<td>867 ± 132</td>
<td>1698 ± 89**</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>257</td>
<td>239*</td>
</tr>
<tr>
<td>Kidney weight (g)</td>
<td>1.37 ± 0.03</td>
<td>1.61 ± 0.02*</td>
</tr>
</tbody>
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Data are reported as the mean ± SEM for 10 rats per group. Urinary excretion was normalized against creatinine. * p ≤0.05 and ** p ≤0.01 were considered statistically significant different between control and acidic group.
tubules to reabsorb HCO$_3^-$ (Boron 2006; Skelton et al., 2010). Studies which have evaluated the basolateral Na$^+$-HCO$_3^-$ cotransporter in metabolic acidosis suggest that the activity of this transport process was increased (Akiba et al., 1987; Preisig & Alpern, 1988). In this context, Preisig & Alpern (1988) and Soleimani et al. (1992) have shown that the \textit{in vitro} metabolic acidosis causes increase in activity of the basolateral Na$^+$-HCO$_3^-$ cotransporter in the rat and rabbit proximal tubule.

Therefore, the strong expression of the NBC1 protein in the basolateral membrane of the proximal tubule, as detected by NBC1-specific antibodies (Figs. 1 and 2), fully matches the strong functional activity of the Na$^+$-HCO$_3^-$ cotransporter.

Conversely, previous studies by Burnham et al. (1998) and Amlal et al. (2001) have shown that the expression of NBC1 on mRNA and protein remain unchanged in rats placed on 280 mM NH$_4$Cl in the drinking water for 5 days and suggested that increased NBC1 activity in metabolic acidosis involves a post-translational regulatory process.

Concerning the effect of metabolic acidosis on MCT1 expression abundance, Western blotting analyses (Fig. 3A) confirmed the immunofluorescence confocal laser-scanning microscopy data (Fig. 1). The densitometric analysis of the immunoblots (Fig. 3B) demonstrated that MCT1 protein level was significantly reduced in the kidney cortex of rats loaded with NH$_4$Cl for 10 days in drinking water compared with controls. In agreement with our results, a downregulation of MCT1 was observed at the mRNA level in the mouse renal cortex during acidosis (Becker et al., 2010).

Our investigation shows that the urinary excretion of lactate is markedly and consistently increased in NH$_4$Cl-loaded rats (Table 1). This increase is likely to be due to the decrease in MCT1 protein expression level (Fig. 3), which leads to reduced lactate co-transport in the renal proximal tubule of acidotic rats.

**Figure 1: Localization of NBC1 and MCT1 protein in the rat kidney.** Confocal immunofluorescence localization of NBC1 (A; control, B; treated with NH$_4$Cl) and MCT1 (C; control, D; treated with NH$_4$Cl) proteins in the rat kidney. NBC1 protein is detected exclusively in the basolateral membrane of proximal tubule cells. Staining for MCT1 shows distinct distribution of MCT1 protein in the basolateral membranes of the rat proximal tubules. (E and F) Merge images for double staining immunofluorescence of NBC1 (green) and MCT1 (red) showing overlay expression in the same regions. Original magnification: $\times 40$. 
Figure 2: Effect of metabolic acidosis on NBC1 protein abundance in the rat kidney.
(A) Immunoblot of plasma membrane proteins prepared from the rat kidney cortex and probed with the NBC1 antibody as described in Methods. Arrow indicates NBC1 at ~130 kDa. (B) Quantitative densitometric analyses of Western blot for the NBC1 protein abundance in acidotic and control rats. Values are expressed as means ± S.E.M. of three different blots. * p ≤ 0.05 significant different between control and acidotic group.

Figure 3: Effect of metabolic acidosis on MCT1 protein abundance in the rat kidney.
(A) Immunoblot of plasma membrane proteins prepared from the rat kidney cortex and probed with the MCT1 antibody as described in Methods. Arrow indicates MCT1 at ~45 kDa. (B) Quantitative densitometric analyses of Western blot for the MCT1 protein abundance in acidotic and control rats. Values are expressed as means ± S.E.M. of three different blots. ** p ≤ 0.01 significant different between control and acidotic group.

4. CONCLUSION
The physiological response to acidosis is one of the most important roles of the Na⁺/HCO₃⁻ cotransporter. The present study clearly demonstrated that in vivo metabolic acidosis causes adaptive increases in the basolateral NBC1 in the rat proximal tubule of the kidney. These adaptive changes are likely responsible for increased ability of the proximal tubule to reabsorb filtered HCO₃⁻ in systemic acidosis.

5. REFERENCES
Adijanto, J., Philp, N.J. 2012. The SLC16A family of monocarboxylate transporters (MCTs)--physiology and


