Metabolic acidosis has dual effects on sodium handling by rat kidney

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Faroqui, Somia, Sulaiman Sheriff, and Hassane Amlal. Metabolic acidosis has dual effects on sodium handling by rat kidney. Am J Physiol Renal Physiol 291:F322–F331, 2006.—Chronic metabolic acidosis (CMA) is associated with decreased NaCl reabsorption in the proximal tubule (PT). However, the effect of CMA on Na+ transport in the distal tubule (DT) and collecting duct (CD) is poorly understood. Rats were placed in metabolic cages and had access to water (control), 0.28 M NH4Cl, or 0.28 M KCl solutions in a pair-feeding protocol for 5 days (5d). Metabolic acidosis developed within 24 h in NH4Cl-, but not in KCl-loaded rats. Interestingly, NH4Cl- but not KCl-loaded rats exhibited a significant natriuresis after 24 h of treatment. Urinary Na+ excretion increased from 1.94 to 2.97 meq/24 h (P < 0.001) and returned to below baseline level (1.67 meq/l) after 5d of CMA. The protein abundance of the cortical Na-CI cotransporter (NCC) remained unchanged at 24 h, but increased significantly (P < 0.01) after 5d of CMA. The protein abundance of α-, β-, and γ-subunits of the epithelial Na+ channel (ENaC) in the cortex decreased sharply during the first 24 h and then returned to baseline levels after 5d of CMA. Interestingly, Sgk1 expression decreased after 24 h (~31%, P < 0.05) and then returned to baseline after 5d of CMA. Nedd4–2 expression was not altered during CMA. CMA increased serum aldosterone levels by 54% and increased the expression of aldosterone synthase by 134% after 5d of CMA. In conclusion, metabolic acidosis has dual effects on urinary Na+ excretion. The early natriuresis results from decreased Na+ reabsorption in the PT and Sgk1-related decreased ENaC activity in the DT and CD. Aldosterone-induced upregulation of NCC, Sgk1, and ENaC likely contributes to the antinatriuretic phase of metabolic acidosis. This adaptation prevents Na+ wasting and volume depletion during chronic acid insult.

THE KIDNEY PLAYS AN IMPORTANT role in the regulation and maintenance of systemic acid-base balance. In response to an acid insult, the kidney undergoes several adaptive changes in its function and appropriately increases acid excretion in the urine (13). This leads to the correction of metabolic acidosis and thus prevents the development of its adverse effects, such as osteoporosis, muscle wasting, and nephrolithiasis. The majority of these adaptive changes occur in the proximal tubule, which exhibits an increase in H+ secretion/bicarbonate reabsorption (20, 31), stimulation of ammoniagenesis and bicarbonate generation (35), and a reduction in the reabsorption of inorganic phosphate and sulfate (2, 27), major components of titratable acidity, which contribute to increased acid secretion in the collecting duct. Interestingly, despite these adaptive and appropriate changes, the proximal tubule exhibits a significant reduction in salt and water transport in response to metabolic acidosis (23, 30, 36). Studies have demonstrated that metabolic acidosis is associated with the downregulation of organic anion pathways that are coupled to apical Na+/H+ exchanger NHE3, such as the Cl-/formate/oxalate/HCO3 exchange (CFEX or PAT1) (36), Na+-dependent inorganic sulfate (Na-Si) cotransporter (27), and Na+/phosphate cotransporter NaPi-2 (2). The downregulation of these transport pathways could contribute to decreased NaCl and water reabsorption in the proximal tubule of acid-loaded animals. It should be noted, however, that a good amount of filtered Na+ is still reabsorbed in the proximal tubule through the upregulation of NHE3. A defect in salt and water reabsorption in the proximal tubule results in an increase in the fluid delivery to Henle’s loop, the distal tubule, and the collecting duct system. Recently, we demonstrated that despite the increased delivery of water out of the proximal tubule, there was no impairment in the overall water handling by the kidney. The collecting duct system compensates for decreased water reabsorption in the proximal tubule during metabolic acidosis. Indeed, metabolic acidosis increased water reabsorption in the collecting duct system through a vasopressin-mediated upregulation of the apical water channel AQP2 (4). These studies did not examine the effects of metabolic acidosis on renal handling of Na+.

Na+ delivered out of the proximal tubule is handled by several highly specialized pathways along the thick ascending limb (TAL), distal tubule, and in the collecting duct system (28). Na+/H+ exchanger NHE3 and the Na+-K+-2Cl– cotransporter (NKCC2 or BSC1) are the apical limiting barrier for Na+ reabsorption in the TAL (28). NHE3 (11) and NKCC2 (5, 15) have been shown to be appropriately upregulated in response to acid loading and contribute to the correction of metabolic acidosis by reabsorbing bicarbonate and excreting ammonium, respectively. The distal tubule and cortical collecting duct play an important role in the fine-tuning of Na+ balance. In these segments, the fine regulation of Na+ excretion is crucial to the control and maintenance of extracellular fluid volume and blood pressure. This regulation is mediated through the action of aldosterone via its effects on the thiazide-sensitive Na-CI cotransporter (TSC or NCC) in the distal tubule (16) and on the amiloride-sensitive Na+ channel in the cortical collecting duct (9, 25).

Thus the purpose of the present studies is to determine the time course effects of metabolic acidosis on urinary Na+ excretion and correlate its pattern with the expression of the key apical Na+-dependent transporters along the distal tubule and collecting duct. Metabolic acidosis was induced using the established protocol of NH4Cl loading, and rats were killed at 24 h or 5 days after NH4Cl loading. The circulating levels of adrenal steroids and the expression pattern of the key enzymes involved in their synthesis were studied. In additional experiments, the effects of NH4Cl loading were compared with those of KCl loading in a pair-feeding protocol.
METHODS

Animal Treatment

The experimental protocols used in the present study were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati.

Protocol 1. Sprague-Dawley rats (200–250 g) were placed in metabolic cages and allowed free access to regular rat chow and distilled water. After a period of adjustment of 3 days in metabolic cages, the rats were divided into two groups. In the first group of rats, metabolic acidosis was induced using the classic protocol of NH4Cl (280 mM) loading added to the drinking water. The second group remained on food and water and served as a control. In our previous studies (4), we observed that switching rats to an NH4Cl solution is associated with a transient decrease (−25%) in fluid intake, which then returned to baseline level within 72 h of the treatment (4). Hence, the access of control rats to water was limited to the same amount of fluid taken by NH4Cl-loaded rats, mostly during days 1–3 of the experiment (pair-feeding protocol).

Protocol 2. In this experiment, another set of Sprague-Dawley rats was placed in metabolic cages with free access to food and distilled water for 72 h. The rats were then divided into two groups. The first group was subjected to KCl loading (280 mM) added to the distilled water, and the second group remained on distilled water (control). The access of both control and KCl-loaded rats to their respective drinking fluid was limited to the same volume of NH4Cl solution (pair-watering) taken by NH4Cl-loaded animals in protocol 1 (see Fig. 2).

The animals were maintained in a temperature-controlled room regulated on a 12:12-h light-dark cycle for the duration of the experiment. Rats were given free access to food and free or restricted access (as needed) to drinking fluid. Metabolic cages were sanitized, and urine was collected under mineral oil on a daily basis. Food access (as needed) to drinking fluid. Metabolic cages were sanitized, and the access of control rats to water was limited to the same amount of fluid taken by NH4Cl-loaded rats, mostly during days 1–3 of the experiment (pair-feeding protocol).

Blood Composition and Urine Analyses

Serum electrolytes, total CO2, creatinine, and blood urea nitrogen levels were measured using commercial services (LabOne of Ohio, Cincinnati, OH). Creatinine was measured in the urine using the sodium picrate method and as described by the kit provider [Pointe Scientific, Canton, MI (www.pointescientific.com)]. Na+ concentration in the urine was measured using a dual-channel flame photometer [model 2655–10, Cole-Parmer Instrument, Vernon Hills, IL (www.coleparmer.com)].

Measurement of Circulating Levels of Adrenal Steroids

Another set of rats was placed in metabolic cages and treated identically as described above. Rats were subjected to control or NH4Cl loading for 5 days and decapitated at the end of the treatment. Blood was collected and used to measure the serum levels of corticosterone and aldosterone with radioimmunoassay kits (ICN Biomedicals, Costa Mesa, CA) and as previously done in our laboratory (37).

Total RNA Isolation and Northern Hybridization

Total cellular RNA was extracted from the renal cortex, inner stripe of outer medulla, and inner medulla by the method of Chomczynski and Sacchi (7) and as previously done in our laboratory (4, 37). In brief, 0.5–1 g of tissue was homogenized at room temperature in 5–10 ml of Tri Reagent (Molecular Research Center, Cincinnati, OH). Total RNA was extracted by phenol/chloroform and precipitated by isopropanol. RNA was then quantitated by spectrophotometry and stored at −80°C. Total RNA samples (30 μg/lane) were fractionated on a 1.2% agarose-formaldehyde gel and transferred to Magna NT nylon membranes using 10× sodium chloride-sodium phosphate-EDTA (SSPE) as a transfer buffer. Membranes were cross-linked by ultraviolet light and baked for 1 h. Hybridization was performed according to Church and Gilbert (8) and as previously described (4, 37). The probes used for various genes were generated by RT-PCR. The identity of the PCR products was confirmed by sequencing using commercial services (DNA Core, University of Cincinnati, Cincinnati, OH).

Preparation of Membrane Fractions

A total cellular fraction containing plasma membrane and intracellular vesicles was prepared from cortex, as previously described (4, 37). Briefly, the kidney cortex samples were homogenized with a polytron homogenizer in an ice-cold solution (250 mM sucrose, 10 mM triethanolamine, pH 7.60) containing protease inhibitors (0.1 mg/ml phenazine methosulfate, 1 μg/ml leupeptin). The homogenate was centrifuged at low speed (1,000 g) for 10 min at 4°C to remove cell debris and nuclei. The supernatant was spun at 150,000 g for 90 min at 4°C. The pellet containing plasma membrane fractions and intracellular vesicles was suspended in the isolation solution containing protease inhibitors. The total protein concentration was measured, and the membrane fractions were solubilized at 65°C in Laemmli buffer for 20 min and stored at −20°C.

Electrophoresis and Immunoblotting of Proteins

Semiquantitative immunoblotting experiments were carried out, as previously described (4, 37). Briefly, the solubilized membrane proteins were size fractionated on 10% polyacrylamide minigels (Novex, San Diego, CA) under denaturing conditions. Using a Bio-Rad transfer apparatus (Bio-Rad Laboratories, Hercules, CA), the proteins were electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked with 5% milk proteins and then probed with affinity-purified anti-TSC or anti-ENaC subunits (α, β, and γ). The secondary antibody was a donkey anti-rabbit IgG conjugated to horseradish peroxidase (Pierce). The site of antigen–antibody complexation on the nitrocellulose membranes was visualized using the chemiluminescence method (SuperSignal Substrate, Pierce) and captured on light-sensitive imaging film (Kodak). Bands corresponding to AQP1 and AQP2 proteins were quantitated by densitometric analysis using UN-SCAN-IT gel software (Silk Scientific, Orem, UT) and were expressed as percentage of control. The equity in protein loading in all blots was verified by gel staining using Coomassie brilliant blue R-250 (Bio-Rad Laboratories).

Materials

[32P]dCTP was purchased from PerkinElmer (Boston, MA). Nitrocellulose filters and other chemicals were purchased from Sigma (St. Louis, MO). A High Prime DNA labeling kit was purchased from Roche Diagnostics (Indianapolis, IN). α-ENaC-subunit antibody and α-ENaC immunizing peptide were purchased from Affinity Bioreagents (Golden, CO). β- and γ-ENaC-subunit antibodies and their respective immunizing peptides were purchased from Alpha Diagnostic International (San Antonio, TX). TSC antibody was purchased from Chemicon International (Temecula, CA).

Statistical Analysis

Semiquantification of Northern hybridization band densities was determined by densitometry using ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA). Semiquantification of immunoblot band densities was determined by densitometry using a scanner (ScanJet ADF, Hewlett Packard) and UN-SCAN-IT gel software (Silk Scientific). Data are expressed as percentage of control. Results are pre-
sented as means ± SE. Statistical significance between experimental groups was determined by Student’s t-test or one-way ANOVA as needed. P < 0.05 was considered significant.

RESULTS

Blood Electrolyte Composition in NH₄Cl-Loaded Rats

Blood chemistry data shown in Table 1 indicate that rats subjected to NH₄Cl loading exhibit a significant increase in blood urea nitrogen, hyperchloremia, and hypophosphatemia, compared with control rats. In addition, NH₄Cl-loaded rats developed metabolic acidosis, as indicated by a significant drop in serum bicarbonate concentration within the first 24 h of NH₄Cl loading, which remained low for the duration of the experiment compared with control rats (Table 1). These results are qualitatively similar to those previously reported by our laboratory (4).

Urinary Na⁺ Excretion, Serum Bicarbonate Concentration, and Creatinine Clearance in NH₄Cl-Loaded Rats

The results depicted in Fig. 1 indicate a significant natriuresis during the first 24 h of NH₄Cl loading, as shown by a significant increase in urinary Na⁺ excretion (from 1.94 ± 0.01 at baseline to 2.97 ± 0.08 meq/24 h after 24 h of NH₄Cl loading) (Fig. 1, A). A slight but significant decrease in food intake was observed (Fig. 1, B). No significant changes in urine volume and creatinine clearance were observed (Fig. 1, C and D).

Table 1. Blood composition of rats subjected to control (water) or 280 mM NH₄Cl loading to induce metabolic acidosis and euthanized after 24 h or 5 days

<table>
<thead>
<tr>
<th></th>
<th>Glucose, mg/dl</th>
<th>BUN, mg/dl</th>
<th>Cr, mg/dl</th>
<th>Na⁺, meq/l</th>
<th>K⁺, meq/l</th>
<th>Cl⁻, meq/l</th>
<th>CO₂, meq/l</th>
<th>P₃, mg/dl</th>
<th>Ca²⁺, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>161 ± 7.9</td>
<td>18 ± 0.51</td>
<td>0.3 ± 0</td>
<td>143 ± 0.7</td>
<td>5.14 ± 0.14</td>
<td>98 ± 0.37</td>
<td>24 ± 0.7</td>
<td>9.84 ± 0.4</td>
<td>10.16 ± 0.07</td>
</tr>
<tr>
<td>Acidosis (24 h)</td>
<td>132 ± 5.4</td>
<td>28 ± 1.47</td>
<td>0.3 ± 0</td>
<td>142 ± 0.3</td>
<td>5.16 ± 0.13</td>
<td>103 ± 0.58</td>
<td>18 ± 1.0</td>
<td>8.86 ± 0.23</td>
<td>10.34 ± 0.09</td>
</tr>
<tr>
<td>P</td>
<td>0.01</td>
<td>0.0001</td>
<td>NS</td>
<td>NS</td>
<td>0.0001</td>
<td>NS</td>
<td>0.003</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Acidosis (5 days)</td>
<td>157 ± 8.9</td>
<td>27 ± 1.11</td>
<td>0.4 ± 0.05</td>
<td>141 ± 0.63</td>
<td>5.12 ± 0.08</td>
<td>101 ± 0.73</td>
<td>18 ± 0.7</td>
<td>8.38 ± 0.27</td>
<td>9.74 ± 0.17</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>0.0001</td>
<td>NS</td>
<td>NS</td>
<td>0.006</td>
<td>NS</td>
<td>0.0006</td>
<td>0.016</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 rats in control, 6 rats in 24 h of acidosis, and 7 rats in 5 days of acidosis. NS, not significant; BUN, blood urea nitrogen; Cr, creatinine.

Fig. 1. Effects of NH₄Cl loading on Na⁺ excretion, food intake, creatinine clearance, and serum bicarbonate concentration. Thirteen rats were placed in metabolic cages with free access to food and water. At time 0, all rats (n = 13) were switched to a drinking solution containing 280 mM NH₄Cl with access to rat chow. After 24 h, 6 rats were killed, and the remaining 7 rats were euthanized after 5 days of NH₄Cl loading. Control rats (n = 6) were fed rat chow and water in metabolic cages for the duration of the experiment as described in METHODS. A: urinary Na⁺ excretion (**P < 0.002 vs. control, *P < 0.03 vs. control at 24 and 72 h, respectively). B: food intake (*P < 0.001 vs. control and **P < 0.0001 vs. control at 24 and 48 h, respectively). C: creatinine clearance. D: urine volume. NH₄Cl loading is associated with dual effects on urinary Na⁺ excretion, a slight, but significant, decrease in food intake, and no significant changes in urine volume and creatinine clearance.

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sis, we examined the effect of KCl loading on Na+ serum bicarbonate concentration within the first 24 h of NH4Cl loading (Fig. 1). *P < 0.002 vs. baseline for both KCl- and NH4Cl-loaded rats; n = 8 rats before and n = 4 rats after 24 h for KCl loading; n = 13 rats before and n = 7 rats after 24 h of NH4Cl loading.

In control animals, urinary Na+ excretion dropped below baseline level for the duration of the treatment (1.84 ± 0.12 meq/24 h on the last day of treatment, P < 0.005, n = 4 rats, Fig. 3A) and remained significantly lower than baseline for the duration of the treatment (1.84 ± 0.12 meq/24 h on the last day of treatment, P < 0.005, n = 4 rats, Fig. 3A). This decrease in Na+ excretion correlates with the reduction in food intake, which decreased significantly within the first 24 h of KCl loading (from 2.30 ± 0.09, n = 8 rats, to 1.75 ± 0.07 meq/24 h, P < 0.005, n = 4 rats, Fig. 3A) and remained significantly lower than baseline up to the last day of treatment (18 ± 0.34 g, P < 0.0003, n = 4 rats, Fig. 3B).

Effects of Metabolic Acidosis on Na+-Dependent Transporters in the Distal Tubule and Collecting Duct

Expression of NCC or TSC in response to metabolic acidosis. The protein abundance of NCC was examined by Western blotting in membrane fractions harvested from the cortex of control and acid-loaded rats. The results depicted in Fig. 4 indicate that metabolic acidosis did not significantly alter the expression of TSC at 24 h (136 ± 19 vs. 100 ± 22% in control, P > 0.05, n = 6 rats/group, Fig. 4) but did significantly increase its abundance after 5 days of treatment (to 240 ± 12%, P < 0.002 vs. control, n = 7 rats/group, Fig. 4). A parallel Coomassie blue-stained gel indicates the equity of the protein loading between the different groups (Fig. 4A, bottom).

Table 2. Blood composition of rats subjected to control (water) or 280 mM KCl loading and euthanized after 24 h or 5 days

<table>
<thead>
<tr>
<th>Glucose, mg/dl</th>
<th>BUN, mg/dl</th>
<th>Cr, mg/dl</th>
<th>Na+, meq/l</th>
<th>K+, meq/l</th>
<th>Cl-, meq/l</th>
<th>CO32-, meq/l</th>
<th>P+, mg/dl</th>
<th>Ca++, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>141±14</td>
<td>18±0.5</td>
<td>0.42±0.05</td>
<td>141±0.4</td>
<td>5.62±0.29</td>
<td>99±0.55</td>
<td>23±0.4</td>
<td>7.54±0.29</td>
</tr>
<tr>
<td>KCl (24 h)</td>
<td>223±10</td>
<td>23±0.8</td>
<td>0.48±0.03</td>
<td>139±1.1</td>
<td>5.35±0.45</td>
<td>100±1.11</td>
<td>22±0.63</td>
<td>7.85±0.24</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>KCl (5 days)</td>
<td>102±5.8</td>
<td>30±1.0</td>
<td>0.35±0.03</td>
<td>143±1.1</td>
<td>6.1±0.13</td>
<td>98±0.85</td>
<td>21±1.03</td>
<td>6.83±0.23</td>
</tr>
<tr>
<td>P</td>
<td>NS</td>
<td>0.001</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 rats in each group.

In the next experiments, we measured the effects of KCl loading on urinary Na+ excretion, food intake, and on serum bicarbonate concentration. As shown in Fig. 3, urinary Na+ excretion decreased significantly after 48 h of KCl loading (from 2.30 ± 0.09, n = 8 rats, to 1.75 ± 0.07 meq/24 h, P < 0.005, n = 4 rats, Fig. 3A) and remained significantly lower than baseline for the duration of the treatment (1.84 ± 0.12 meq/24 h on the last day of treatment, P < 0.005, n = 4 rats, Fig. 3A). This decrease in Na+ excretion correlates with the reduction in food intake, which decreased significantly within the first 24 h of KCl loading (from 23 ± 0.63 to 17 ± 0.94 g, P < 0.002, n = 8 rats, Fig. 3B) and remained significantly lower than baseline up to the last day of the experiment (18 ± 0.34 g, P < 0.0003, n = 4 rats, Fig. 3B).

Urinary Na+ Excretion and Food Intake in KCl-Loaded Rats

To determine whether the initial natriuresis is related to chloride loading or to NH4+-loading-induced metabolic acidosis, we examined the effect of KCl loading on Na+ excretion in a pair-feeding protocol, as described in METHODS. The results depicted in Fig. 2 show the time course of drinking fluid by rats having free access to a 280 mM NH4Cl solution or those with restricted access to a 280 mM KCl solution. The data indicate that rats in both groups consumed the same amount of fluid during any given time of the experiment (Fig. 2). It should be noted that the controls for both NH4Cl- and KCl-loaded rats also had restricted access to distilled water (data not shown).

The blood chemistry analysis shown in Table 2 indicates that rats subjected to KCl loading did not show any major electrolyte or acid-base abnormalities, except for transient hyperglycemia at 24 h (P < 0.01, n = 4 rats) and a sustained increase in blood urea nitrogen levels (Table 2) compared with control rats. In contrast, a slight drop in blood glucose level was observed during the first 24 h of NH4Cl-loaded vs. control animals (Table 1).
levels of all three subunits of ENaC (α, β, and γ) using immunoblotting experiments. The results shown in Fig. 5 indicate that the protein abundance of all ENaC subunits is significantly reduced during the first 24 h of acid loading, compared with control (Fig. 5, \( n = 6 \) rats/group). Interestingly, a rebound increase in the protein abundance of all three ENaC subunits occurred after 5 days of acid loading (Fig. 5, \( n = 7 \) rats). During this recovery period, the protein levels of ENaC subunits increased to, or slightly above, their baseline expression levels. This picture of changes in ENaC protein abundance correlates inversely with the profile of urinary Na\(^+\) excretion in metabolic acidosis shown in Fig. 1A. Figure 5B demonstrates the specificity of the antibodies used to detect ENaC subunits. As shown, the bands corresponding to α-, β-, and γ-subunits of ENaC are completely ablated when the antibodies were preadsorbed overnight with an excess of immunizing peptides (Fig. 5B).

In additional experiments, we examined the mRNA expression levels of ENaC subunits in the kidney cortex of control and acid-loaded rats. Northern hybridization experiments shown in Fig. 6 indicate that the mRNA expression levels of all ENaC subunits did not change during the first 24 h of acidosis compared with control (Fig. 6, \( n = 6–7 \) rats/group).

**Effects of Metabolic Acidosis on the Expression of Sgk1 and Nedd4–2**

Sgk1 and Nedd4–2 play an important role in the regulation of the activity and expression of ENaC (14, 24). In the next set of experiments, we examined the expression levels of Sgk1 and Nedd4–2 in the kidney cortex of control and acid-loaded animals. Northern hybridization experiments depicted in Fig. 7 indicate a significant decrease in Sgk1 mRNA (from 100 ± 10 in control to 69 ± 2.9%, \( P < 0.05 \), \( n = 6 \) rats/group, Fig. 7, A and C) during the first 24 h of acid loading. This was followed by a significant rebound increase in Sgk1 mRNA to a near baseline level after 5 days of acidosis (93 ± 3.2%, \( P < 0.01 \) compared with 24 h, \( n = 7 \) rats/group, Fig. 7, A and B). This regulation of Sgk1 expression is likely specific to NH\(_4\)Cl-induced acidosis, as its expression was actually increased during the initial 24 h of KCl loading (from 100 ± 7% in

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**Fig. 3.** Effects of KCl loading on Na\(^+\) excretion and food intake. Twelve rats were placed in metabolic cages and had a restricted access to KCl solution (\( n = 8 \) rats) or water (control; \( n = 4 \) rats) as described in METHODS. The animals were monitored daily for urinary Na\(^+\) excretion (A) and food intake (B) for the duration of the experiment. Rats subjected to KCl loading were divided into 2 groups. One group (\( n = 4 \)) was terminated after 24 h, and the other group was euthanized after 5 days of KCl treatment. Control rats (\( n = 4 \)) were killed after 5 days of treatment. KCl loading is associated with a slight, but significant, decrease in urinary Na\(^+\) excretion (*\( P < 0.005 \) vs. control; **\( P < 0.05 \) vs. control), which correlates with a significant reduction in food intake (*\( P < 0.002 \) vs. control; **\( P < 0.0003 \) vs. control).

**Fig. 4.** Expression of thiazide-sensitive Na-Cl cotransporter (TSC or NCC) protein and gel staining of cortical membrane proteins. Membrane proteins were isolated from kidney cortex harvested from control or acid-loaded rats for 24 h or 5 days and used for Western blotting. A: representative immunoblot of TSC protein expression (~160 kDa) in the kidney cortex (top) and corresponding gel stained with Coomassie blue for the control of protein loading (bottom). B: densitometric analysis showing the mean values of TSC band in 2 immunoblots loaded with different samples. As shown, the protein abundance of TSC did not change at 24 h (\( n = 6, \ P > 0.05 \)) but increased significantly after 5 days (\( n = 7, \ P < 0.002 \)) of acid loading, compared with control rats (\( n = 6 \)). NS, not significant.
control, \( n = 3 \) rats, to 143 ± 12%, \( n = 4 \) rats, \( P < 0.04 \), Fig. 7, B and C). Sgk1 mRNA returned to a baseline level by day 5 of KCl loading (111 ± 12%, \( P > 0.05 \) vs. control, \( n = 3 \) rats, Fig. 7, B and C).

Last, the Northern hybridization experiment depicted in Fig. 7D shows the mRNA expression levels of Nedd4–2, which remained unchanged during the entire period of acid loading (Fig. 7, C and D, \( P > 0.05 \) vs. control, \( n = 3–4 \) rats/group).

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**Fig. 5.** Protein abundance of epithelial \( \text{Na}^+ \) channel (ENaC) subunits in the kidney cortex. The same membrane proteins used for TSC abundance were loaded and probed with \( \alpha \) (–97 kDa), \( \beta \) (–98 kDa), and \( \gamma \) (–98 kDa)-subunits of ENaC antibodies. A: representative immunoblots showing the abundance of 3 ENaC subunits in control, after 24 h, and after 5 days of acidosis. B: average of the densitometry analysis of each ENaC subunit band obtained from 2 separate blots with different protein samples. C: protein abundance of all 3 ENaC subunits is significantly reduced during the first 24 h of acidosis and then returned toward and/or slightly above control levels after 5 days of acid loading (\( n = 6–7 \) rats/group). *\( P < 0.0001 \) vs. control. #\( P < 0.05 \) vs. control.

**Fig. 6.** mRNA expression of ENaC subunits in the kidney cortex. A: representative Northern hybridization of \( \alpha \), \( \beta \)-, and \( \gamma \)-subunits of ENaC in the kidney cortex of rats subjected to control or acid loading. 28S rRNA was used as a constitutive gene for the control of the equity of RNA loading into Northern gels (blot not shown). B: corresponding densitometric analysis showing the mean of ENaC subunits mRNA-to-28S rRNA ratio. Metabolic acidosis did not affect the mRNA expression levels of ENaC subunits. Thirty micrograms of total RNA from different rats were loaded per lane; \( n = 6–7 \) rats in each group.
**Signaling Hormones Involved in the Regulation of NCC and ENaC in Response to Metabolic Acidosis**

Adrenal steroids, mainly aldosterone and glucocorticoid, play an important role in the regulation of Na⁺ homeostasis, extracellular fluid balance, and blood pressure. Moreover, both of these hormones have been shown to be involved in the renal acid excretion in response to metabolic acidosis (6, 11, 19). Hence, the following experiments were carried out to examine the effects of metabolic acidosis on the synthesis and secretion of aldosterone and corticosterone (a major circulating glucocorticoid in rat).

**Serum aldosterone levels and expression of aldosterone synthase in the adrenal gland.** Radioimmunoassay studies shown in Fig. 8A indicate that metabolic acidosis did not alter the circulating levels of aldosterone during the first 24 h of acid loading (99 ± 29 vs. 110 ± 15 pg/ml in control, n = 6 rats in group, P > 0.05, Fig. 8A). However, a significant increase in serum aldosterone levels was observed after 5 days of acid loading (282 ± 22 pg/ml, P < 0.0001, n = 6 rats/group, Fig. 8A). These results correlate with the expression levels of aldosterone synthase or CYP11B2, a key enzyme in aldosterone synthesis in the adrenal gland. This is shown by a Northern hybridization experiment depicted in Fig. 8B, which indicates that the expression of CYP11B2 mRNA did not change during the first 24 h (P > 0.05 vs. control) but increased significantly by the end of acid-loading treatment (from 100 ± 13 in control to 234 ± 16% in acid-loaded rats, P < 0.0001 vs. control, Fig. 8B).

**Serum corticosterone levels and expression of 11β-hydroxylase in the adrenal gland.** Radioimmunoassay experiments shown in Fig. 9A indicate that the circulating levels of corticosterone did not change during the entire period of acid loading (125 ± 13, 99 ± 23, and 162 ± 26 ng/ml in control, 24 h, and after 5 days of acid loading, respectively, P > 0.05, n = 6 rats/group, Fig. 9A). These data correlate with the mRNA expression levels of 11β-hydroxylase or CYP11B1, a key enzyme in glucocorticoid synthesis in the adrenal gland. Indeed, the Northern hybridization experiment depicted in Fig. 9B indicates that the expression of CYP11B1 mRNA did not change during the entire period of metabolic acidosis (P > 0.05 vs. control, Fig. 9B).

**DISCUSSION**

We have demonstrated for the first time that NH₄Cl loading-induced metabolic acidosis is associated with dual effects on Na⁺ handling by the rat kidney. Significant natriuresis occurred within the first 24 h of acid loading, followed by a sharp decline in urinary Na⁺ excretion to a lower level (less than baseline) for the duration of the treatment (Fig. 1A). The transient natriuresis is not the result of increased Na⁺ intake, as food intake is actually decreased in response to acid loading (Fig. 1B). Moreover, the early increase in Na⁺ excretion did not result from an increase in urine volume (Fig. 1C) or changes in filtered load, as creatinine clearance remained unchanged during the entire period of acid loading (Fig. 1D).

In support of this, inulin clearance studies demonstrated that NH₄Cl loading of rats for 7 days did not affect GFR (29). NH₄Cl loading is widely used to induce metabolic acidosis in both humans and experimental animals. Hence, the use of NH₄Cl in our experiments raises a question as to whether early natriuresis is a consequence of chloride loading with subsequent changes in extracellular fluid volume, or rather results from 100 ± 13 in control to 234 ± 16% in acid-loaded rats, P < 0.0001 vs. control, Fig. 8B).
from NH₄Cl-induced metabolic acidosis. The pair-feeding studies with KCl loading indicate the absence of natriuresis in KCl-loaded rats despite the fact that these animals consumed the same amount of chloride taken by NH₄Cl-loaded animals (Fig. 2). In addition, the transient natriuresis correlates with the development of metabolic acidosis in NH₄Cl-loaded rats (Table 1), which is absent in KCl-loaded animals (Table 2). These data clearly indicate that the early natriuresis induced by

Fig. 8. Aldosterone synthesis and secretion during acid loading. A: serum aldosterone was measured in control and acid-loaded rats by radioimmunoassay. The circulating levels of aldosterone increased significantly after 5 days of metabolic acidosis (+182%, P < 0.001 vs. control, n = 6 rats/group). B: representative Northern hybridization of CYP11B2 (aldosterone synthase) mRNA in the adrenal gland of NH₄Cl-loaded rat (top) and corresponding densitometric analysis showing the mean of CYP11B2 mRNA-to-28S rRNA ratio (bottom). CYP11B2 expression increased significantly in response to 5 days of metabolic acidosis (+134%, P < 0.0001). Fifteen micrograms of total RNA isolated from pooled adrenal glands were harvested from 6–7 rats/group and loaded into 2 separate gels (n = 3–4 lanes loaded with different RNA in each group).

Fig. 9. Corticosterone synthesis and secretion during metabolic acidosis. A: corticosterone level was measured in the serum of control and acid-loaded rats by radioimmunoassay. The circulating levels of corticosterone did not change significantly during the entire acid-loading period. B: representative Northern hybridization of CYP11B1 (11β-hydroxylase) mRNA in the adrenal gland of control and acid-loaded rat (top) and corresponding densitometric analysis showing the mean of CYP11B1 mRNA-to-28S rRNA ratio (bottom). CYP11B1 mRNA expression remained unchanged during acid-loading treatment. Fifteen micrograms of total RNA isolated from pooled adrenal glands were harvested from 6–7 rats/group and loaded into 2 separate gels (n = 3–4 lanes loaded with different RNA in each group).
NH₄Cl loading is likely independent of changes in extracellular fluid volume secondary to the chloride loading and is rather related to NH₄⁺ loading-induced metabolic acidosis. The results further suggest that the early natriuresis is likely of renal origin and results from impairment in renal tubular transport of Na⁺.

In the TAL and distal nephron, apical Na⁺ uptake is mediated through the apical NKCC2 and NHE3 in the TAL, and via NCC and ENaC in the distal tubule and cortical collecting duct, respectively (28). Previous studies have demonstrated that chronic NH₄Cl loading-induced metabolic acidosis is associated with an increase in both the activity and the expression of NKCC2 (5, 15) and NHE3 (21) in the medullary TAL. Moreover, the upregulation of NKCC2 was observed as early as 3 h after acid loading in rats (5). This suggests that NKCC2 has no role in the early natriuresis observed during the first 24 h of acid loading. The present studies indicate that 24 h of acid loading are associated with a significant reduction in the protein abundance of α-, β-, and γ-subunits of ENaC (Fig. 5) with no effect on NCC protein expression (Fig. 4). This effect is mediated via a posttranscriptional mechanism, as the mRNA expression levels of ENaC subunits remained unchanged during metabolic acidosis (Fig. 6). Hence, it is likely that early natriuresis of metabolic acidosis results from the downregulation of ENaC subunits in the cortical collecting duct, along with decreased expression and activities of apical Na⁺-dependent transporters in the proximal tubule (2, 27, 36). It should be noted that after 5 days of metabolic acidosis, the expression of NCC protein increased significantly (Fig. 4) and the protein abundance of ENaC subunits returned to baseline levels (Fig. 5). The upregulation of these transporters inversely correlates with decreased urinary Na⁺ excretion after 48 h and during the entire period of acid loading (Fig. 1A). This indicates that the upregulation of apical Na⁺ transporters in the TAL (NKCC2 and NHE3), in the distal tubule (NCC), and in the collecting duct (ENaC) is an adaptive response, which compensates for decreased Na⁺ reabsorption in the proximal tubule and prevents Na⁺ wasting during chronic metabolic acidosis. In addition, we have demonstrated that metabolic acidosis is associated with an increase in water reabsorption in the collecting duct through a vasopressin-induced AQP2 water channel in rat kidney (4). Hence, these adaptive processes could play an important role in the maintenance of Na⁺ homeostasis and water balance when an acid insult occurs.

Several studies have examined the effects of NH₄Cl loading on the expression of Na⁺ transporters along the nephron (17, 18) and showed results that conflict with our present studies. In these reports (17, 18), NH₄Cl loading failed to increase NKCC2 expression and caused a significant downregulation of NCC and both β- and γ-subunits of ENaC (18). In these studies, however, NH₄Cl was provided in the diet for 7 days. Hence, the discrepancy between these findings and our results, as well as those for NKCC2 (5, 15), is likely due to a difference in the model of NH₄Cl loading. Moreover, NH₄Cl loading in the drinking water is associated with increased vasopressin synthesis and secretion (4). This hormone could contribute to the upregulation of NKCC2 in this model of metabolic acidosis. The acid-base status and the time course of Na⁺ excretion were not determined in NH₄Cl-fed rats (17, 18). Interestingly, in NH₄Cl-fed rats, the protein abundance of NHE3 increased in the outer medulla but not in the cortex (17), whereas, in rats loaded with NH₄Cl in the drinking water, NHE3 protein abundance increased in both the cortex (1, 22, 38) and medulla (5). The expression and activities of NKCC2 (5, 15) and NHE3 (3) are increased in response to in vitro acidosis (acidic pH), and the fact that the expression of these transporters did not change in NH₄Cl-fed rats likely suggests that the animals may not have developed a significant metabolic acidosis.

The mechanism underlying the dual effect of metabolic acidosis on renal Na⁺ handling is only partially elucidated in the present study. The pioneer work of several investigators has led to a better understanding of the structure and molecular and functional regulation of ENaC (12, 14, 24, 34). ENaC activity and cell surface expression are under the control of several accessory proteins including Sgk1 and Nedd4–2 (14, 24). Sgk1 is also known as an aldosterone- and glucocorticoid-induced kinase and plays an important role in the regulation of both the expression and the activity of ENaC by these hormones. Nedd4–2 is an ubiquitin-protein ligase, which controls the surface expression and the activity of ENaC. The binding of active Sgk1 with Nedd4–2 inhibits the ubiquitination of ENaC and thus increases the surface expression and Na⁺ transport activity of ENaC. In the absence or decreased expression of Sgk1, Nedd4–2 is activated and leads to the retrieval of ENaC from the cell surface and an increase in its endosomal degradation (12, 14, 24). Hence, with regard to early (during the first 24 h) natriuresis with acid loading, our results indicate that 24 h of NH₄Cl loading did not affect the expression of Nedd4 but significantly reduced the expression of Sgk1 (Fig. 7, A and B). This effect correlates with the downregulation of ENaC subunits and the increase in urinary Na⁺ excretion during the first 24 h of NH₄Cl loading. The downregulation of Sgk1 is independent of chloride loading or changes in serum levels of aldosterone (Fig. 8) or corticosterone (Fig. 9) and is likely related to the development of metabolic acidosis induced by NH₄⁺ loading (Fig. 7). It should be noted that the interpretation of these changes in Sgk1 expression is limited by its widespread distribution in a variety of nephron segments. Whether acidic pH per se regulates the expression of Sgk1 in the nephron segments where ENaC is expressed (i.e., distal tubule and collecting duct) remains to be investigated.

With respect to the antinatriuretic phase (after 48 h) of NH₄Cl loading, our results indicate a coordinated upregulation of Sgk1 and ENaC subunits, along with increased expression of NCC at the end of the acid-loading treatment. This correlates with increased synthesis (Fig. 8B) and secretion (Fig. 8A) of aldosterone with no changes in serum corticosterone levels (Fig. 9). The increase in aldosterone levels during metabolic acidosis has been shown by other investigators (32, 33) and is likely independent of increased renin expression or activity (4). Our results did not show an increase in corticosterone synthesis or secretion (Fig. 9), but they do not exclude an increase in the sensitivity of the renal tubule to the existing glucocorticoids in response to metabolic acidosis. The increase in aldosterone activity could contribute to the upregulation of NCC during metabolic acidosis. Moreover, the increase in aldosterone levels is concomitant to an increase in the synthesis and secretion of vasopressin during metabolic acidosis (4), and both of these hormones are known to upregulate the expression and activity of ENaC (10, 25) in the kidney.

In conclusion, metabolic acidosis has dual effects on Na⁺ excretion by the kidney. The early natriuresis results from coordinated downregulation of Sgk1 and ENaC subunits ex-
pression in the kidney. The antinatriuresis phase is associated with a rebound increase in the expression of both Sgk1 and ENaC subunits, along with an upregulation of NCC protein in the distal tubule. These changes are likely triggered by an increase in aldosterone levels. The enhanced Na\(^+\) transport in the distal nephron compensates for a decrease in its reabsorption in the proximal tubule and thus prevents Na\(^+\) wasting and volume depletion during chronic metabolic acidosis.

**REFERENCES**


