Long-term regulation of renal Na-dependent cotransporters and ENaC: response to altered acid-base intake

GHEUN-HO KIM, STEPHEN W. MARTIN, PATRICIA FERNÁNDEZ-LLAMA, SHYAMA MASILAMANI, RANDALL K. PACKER, AND MARK A. KNEPPER

Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892-1603; and Department of Biological Sciences, George Washington University, Washington, District of Columbia 20052


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Kim, Gheun-Ho, Stephen W. Martin, Patricia Fernández-Llama, Shyama Masilamani, Randall K. Packer, and Mark A. Knepper. Long-term regulation of renal Na-dependent cotransporters and ENaC: response to altered acid-base intake. Am J Physiol Renal Physiol 279: F459–F467, 2000.—Increased systemic acid intake is associated with an increase in apical Na/H exchange in the renal proximal tubule mediated by the type 3 Na/H exchanger (NHE3). Because NHE3 mediates both proton secretion and Na absorption, increased NHE3 activity could inappropriately perturb Na balance unless there are compensatory changes in Na handling. In this study, we use semiquantitative immunoblotting of rat kidneys to investigate whether acid loading is associated with compensatory decreases in the abundance of renal tubule Na transporters other than NHE3. Long-term (i.e., 7-day) acid loading with NH4Cl produced large decreases in the abundances of the thiazide-sensitive Na-Cl cotransporter (TSC/NCC) of the distal convoluted tubule and both the β- and γ-subunits of the amiloride-sensitive epithelial Na channel (ENaC) of the collecting duct. In addition, the renal cortical abundance of the proximal type 2 Na-dependent phosphate transporter (NaPi-2) was markedly decreased. In contrast, abundances of the bumetanide-sensitive Na-K-2Cl cotransporter of the thick ascending limb and the α-subunit of ENaC were unchanged. A similar profile of changes was seen with short-term (16-h) acid loading. Long-term (7-day) base loading with NaHCO3 resulted in the opposite pattern of response with marked increases in the abundances of the β- and γ-subunits of ENaC and NaPi-2. These adaptations may play critical roles in the maintenance of Na balance when acid-base balance occurs.

sodium-proton exchange; sodium-potassium-2 chloride cotransport; distal convoluted tubule; collecting duct; amiloride-sensitive epithelial sodium channel

IN THE RENAL PROXIMAL TUBULE, the apical type 3 Na+/H+ exchanger (NHE3) mediates both proton secretion and Na absorption (4). Coupling between proton secretion and Na absorption creates a potential conflict when acid-base balance and Na balance must be regulated independently. Chronic metabolic acidosis is associated with increased apical plasma membrane Na+/H+ exchange activity (15, 46) and increased abundance of NHE3 in brush-border membrane fractions of renal cortex (1, 50). The increase in NHE3 activity would predict a nonhomeostatic increase in proximal Na absorption, a prediction supported by some studies of animal models of chronic metabolic acidosis (32, 44) but not others (14, 33) (see DISCUSSION).

Compensation for increased NHE3-mediated proximal Na absorption in metabolic acidosis could occur through downregulation of other Na transport processes at the level of the proximal tubule or in renal tubule segments downstream from the proximal tubule. Evidence for proximal compensation was obtained by Ambuhl et al. (2), who demonstrated that chronic metabolic acidosis is associated with a marked suppression of Na-phosphate cotransport activity and a decrease in the abundance of the type 2 Na-phosphate cotransporter (NaPi-2) in proximal tubule brush-border membranes. Thus increased NHE3-mediated Na uptake into proximal tubule cells in metabolic acidosis could be partially compensated for by a fall in Na uptake via NaPi-2. Another type of proximal compensation was demonstrated by Wang et al. (48), who showed metabolic acidosis causes a marked suppression of organic anion-stimulated NaCl absorption (presumably due to suppression of formate-chloride and/or oxalate-chloride exchange activity), a response that would be predicted to shift proximal tubule Na transport from a NaCl absorption mode to a NaHCO3 absorption mode.

Hypothetically, another way that Na balance could be maintained despite the increase of proximal Na/H exchange activity in chronic metabolic acidosis would be through downregulation of Na transporters and channels responsible for Na absorption at more distal sites along the renal tubule. Distinct diuretic-sensitive, apically located Na transporters and channels have been identified (initially physiologically and then by molecular cloning) that mediate the apical components of virtually all of the Na reabsorption that occurs beyond the proximal tubule. The cloned transporters

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are the bumetanide-sensitive Na-K-2Cl cotransporter (25, 39) (BSC-1 or NKCC2) in the thick ascending limb, the thiazide-sensitive Na-CI cotransporter (25) (TSC or NCC) in the distal convoluted tubule, and the amiloride-sensitive Na channel (11, 12) (ENaC) in the connecting tubule and collecting duct. The cloning of these transporters has permitted us to make rabbit polyclonal antibodies to them (20, 30, 31, 35), yielding a valuable set of tools for the study of integrative renal physiology at a molecular level. We use these antibodies here to investigate whether changes in acid-base intake in rats alters the abundance of one or more distal Na transporter or Na channel proteins in a manner that would help to maintain Na balance when proximal Na/H exchange activity is regulated to maintain acid-base balance.

METHODS

Animals and experimental protocol. Male Sprague-Dawley rats (Taconic Farms, Germantown, NY), weighing between 180 and 220 g, were placed in metabolism cages 3 days before the beginning of the study. Control and treated rats were chosen randomly, and all were provided with a gelled agar (1%) diet, modified from an approach originally designed by Bouby and colleagues (8). By using this gelled diet, a daily, fixed amount of water (37 ml/220 g body wt (BW)−1·day−1) and regular rat chow (15 g diet containing 7.2 mmol NaHCO3 for 7 days. The control diet was identical except for the absence of added NaHCO3. Detailed characterization of the physiologic response of the rats to the NH4Cl-loading and NaCO3-loading protocols has been previously reported by this laboratory (9).

Polyclonal antibodies. Affinity-purified, peptide-derived rabbit polyclonal antibodies to Na transporters, Na channels, and water channels were used for immunoblotting. The initial characterization of antibodies to NHE3 (24, 29), BSC-1/NKCC2 (30), TSC/NCC (31), the three ENaC subunits (α-, β-, and γ-ENaC) (35), aquaporin-1 (45), and aquaporin-2 (16) were described previously.

Preparation of kidney tissue for immunoblotting. Rats were killed by decapitation, and kidneys were rapidly removed and placed in chilled isolation solution containing 250 mM sucrose, 10 mM triethanolamine (Calbiochem, La Jolla, CA), 1 μg/ml leupeptin (Bachem, Torrance, CA), and 0.1 mg/ml phenylmethylsulfonyl fluoride (US Biochemical, Toledo, OH) and titrated to pH 7.6. Then, the kidneys were sliced longitudinally, slightly off center, and the cortex, outer medulla, and inner medulla were quickly separated. Tissue samples were homogenized in ice-cold isolation solution by using a tissue homogenizer (Omini 1000 fitted with a microsawtooth generator; Omini International, Warrenton, VA). After homogenization, protein concentration was measured by using the Pierce bicinechonic acid protein assay reagent kit (Pierce, Rockford, IL). Samples were then solubilized at 60°C for 15 min in Laemmli sample buffer.

To prepare cortical membrane fractions for anti-NaPi-2 antibody characterization, the cortical homogenate was initially centrifuged at 1,000 g for 10 min. Then, the supernatant was centrifuged at 17,000 g for 20 min. The pellet was resuspended in chilled isolation solution (17,000-g pellet), and the supernatant was centrifuged at 200,000 g for 1 h. The 200,000-g pellet pellet was resuspended in chilled isolation solution.

Electrophoresis and immunoblotting of proteins. SDS-PAGE was done by using 7.5% polyacrylamide minigels to assess BSC-1 or TSC protein abundance. Ten percent polyacrylamide minigels were used for NHE3, NaPi-2, or ENaC protein, and 12% polyacrylamide minigels were used for aquaporins. In all cases, to confirm equality of loading among lanes, electrophoresis was initially run for the entire set of samples in a given experiment on a single 12% polyacrylamide-SDS gel, which was then stained with Coomassie blue.

Selected bands from these gels were analyzed by densitometry (Molecular Dynamics, San Jose, CA) to provide quantitative assessment of loading. These loading gels established that subsequent immunoblots (loaded identically) were uniformly loaded.

Proteins were transferred electrophoretically from gels to nitrocellulose membranes. After being blocked with 5 g/dl nonfat dry milk, proteins were probed overnight at 4°C with the desired antibody at the following IgG concentrations (in μg/ml): 0.40 for NHE3, 0.54 for NaPi-2, 0.12 for BSC-1, 0.20 for TSC, 0.09 for α-ENaC, 0.25 for β-ENaC, 0.27 for γ-ENaC, 0.23 for aquaporin-1, and 0.12 for aquaporin-2. The antibodies were prepared in an antibody diluent containing 150 mM NaCl, 50 mM Na phosphate, 10 mg/dl Na azide, 50 mg/dl Tween-20, and 1 g/dl bovine serum albumin (pH 7.5). The secondary antibody was donkey anti-rabbit IgG conjugated to horseradish peroxidase (Pierce no. 31458) used at a concentration of 0.16 μg/ml. Sites of antibody-antigen reaction were visualized by using luminol-based enhanced chemiluminescence (LumiGLO, Kirkegaard and Perry Laboratories, Gaithersburg, MD) before exposure to X-ray film (Kodak no. 165–1579 Scientific Imaging Film).

Production and characterization of antibody to NaPi-2. For development of the peptide-derived polyclonal antibody to NaPi-2, a 25-amino acid synthetic peptide corresponding to amino acids 614–637 of rat NaPi-2 (with an added NH2-terminal cysteine) was produced by standard solid-phase peptide synthesis techniques (sequence: NH2-CLEELP-PATPSPRALPAHMNATRL-COOH), on the basis of the se-
quence reported by Magagnin et al. (34). Analysis using the BLAST computer program showed no significant overlap of the immunizing peptide with any other known eukaryotic protein. The peptide was purified by HPLC and was conjugated to maleimide-activated keyhole limpet hemocyanin via covalent linkage to the \textit{NH}$_2$-terminal cysteine. Two rabbits were immunized with this conjugate by using a combination of Freund’s complete and incomplete adjuvants. One of these antisera (L697) was used for the present studies after affinity purification on a column made with the same synthetic peptide used for immunizations (immobilization kit no. 2, Pierce).

The specificity of the affinity-purified anti-NaPi-2 antibody was assessed by immunoblotting using whole homogenates from rat renal cortex, outer medulla, and inner medulla as well as membrane fractions (17,000- and 200,000-g pellet) from cortex (Fig. 1). The antibody recognized a broad major band of molecular mass at \~85 kDa, which was noted only in the cortex. In addition, weaker bands were detected at \~40 and 170 kDa. The former has been identified as a physiological cleavage product of NaPi-2 (6), whereas the latter is likely to be a dimer of NaPi-2. Differential centrifugation revealed that the NaPi-2 labeling is associated with membrane fractions. The NaPi-2 abundance is enriched in membrane fractions relative to whole homogenate. All these bands were fully ablated when the anti-NaPi-2 antibody was preadsorbed with an excess (1 mg) of the immunizing peptide (Fig. 1).

Statistical analysis. Relative quantification of immunoblot band densities was carried out by densitometry by using a laser scanner (Molecular Dynamics) and ImageQuaNT software (Molecular Dynamics). Results were presented as means \pm SE. Statistical significance of the effects of various treatments on transporter expression was assessed by using unpaired \textit{t}-tests when SDs were the same or by Welch \textit{t}-test between means.

### Results

#### Effects of chronic NH\textsubscript{4}Cl loading

To test the effect of chronic NH\textsubscript{4}Cl loading on the abundances of Na transporter and Na channel proteins expressed along the renal tubule, immunoblots were run by using cortical and outer medullary homogenates from six rats receiving 7.2 mmol·220 g BW$^{-1}$·day$^{-1}$ of NH\textsubscript{4}Cl for 7 days and from six control rats. Table 1 shows data obtained from analysis of the final 24-h urine collections. As expected because of the matched-feeding approach used, urine volumes were not different between the two groups. NH\textsubscript{4}Cl-loaded rats had a lower urine pH and a higher urinary ammonium excretion compared with control animals. There was no significant difference in urinary Na excretion between NH\textsubscript{4}Cl-loaded rats and control animals, reflecting equal Na intakes and indicating that the rats were in steady state with respect to Na excretion. Urine potassium excretion was not different between the two groups, but urine chloride excretion was substantially higher in NH\textsubscript{4}Cl-loaded rats than in control animals, reflecting the higher chloride intake in NH\textsubscript{4}Cl-loaded rats. NH\textsubscript{4}Cl-loaded rats showed a marked increase in urinary calcium excretion and urinary phosphate excretion compared with control animals. In addition, NH\textsubscript{4}Cl-loaded rats excreted more urea and the urinary osmolality was increased, reflecting the expected increase in solute excretion in the same amount of water.

NH\textsubscript{4}Cl-loaded rats showed comparable concentrations of serum aldosterone (0.89 \pm 0.42 nmol/l, \(n = 6\)) to those in control animals (0.76 \pm 0.30 nmol/l, \(n = 6\)). In a separate set of identically treated rats, the plasma vasopressin concentration was 5.0 \pm 1.2 pg/ml (\(n = 6\)) with NH\textsubscript{4}Cl loading and 2.1 \pm 0.7 pg/ml (\(n = 6\)) in control rats (\(P = 0.06\)).

### Table 1. Urine data from chronic NH\textsubscript{4}Cl loading study

<table>
<thead>
<tr>
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<th>Control (n = 6)</th>
<th>NH\textsubscript{4}Cl-Loaded (n = 6)</th>
</tr>
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<tbody>
<tr>
<td>Urine volume, ml/day</td>
<td>17.4 \pm 0.7</td>
<td>18.0 \pm 1.8</td>
</tr>
<tr>
<td>pH</td>
<td>7.33 \pm 0.05</td>
<td>7.23 \pm 0.05</td>
</tr>
<tr>
<td>Na\textsuperscript{+}, mmol/day</td>
<td>6.58 \pm 0.05</td>
<td>5.57 \pm 0.03</td>
</tr>
<tr>
<td>K\textsuperscript{+}, mmol/day</td>
<td>1.16 \pm 0.05</td>
<td>1.39 \pm 0.09</td>
</tr>
<tr>
<td>Cl\textsuperscript{−}, mmol/day</td>
<td>2.12 \pm 0.05</td>
<td>2.24 \pm 0.17</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}, mmol/day</td>
<td>0.03 \pm 0.00</td>
<td>0.11 \pm 0.02</td>
</tr>
<tr>
<td>PO\textsubscript{4}\textsuperscript{3−}, mmol/day</td>
<td>0.54 \pm 0.04</td>
<td>1.02 \pm 0.08</td>
</tr>
<tr>
<td>Urea, mmol/day</td>
<td>6.3 \pm 0.4</td>
<td>9.6 \pm 0.5</td>
</tr>
<tr>
<td>Osmolality, mosmol/kgH$_2$O</td>
<td>853 \pm 26</td>
<td>1,589 \pm 122</td>
</tr>
<tr>
<td>Creatinine, mg/day</td>
<td>7.54 \pm 0.36</td>
<td>7.41 \pm 0.36</td>
</tr>
</tbody>
</table>

Values are means \pm SE. \(n\), No. of rats. \(*P < 0.01, \#P < 0.001\).
proteins showed differential regulation in response to chronic NH₄Cl loading. In addition, there were no changes in renal cortical abundances of NaPi-2 and NHE3. Figure 4 (top) shows the effect of NH₄Cl loading on NaPi-2 abundance in cortical homogenates. Consistent with previous observations (2), the abundance of NaPi-2 protein in the cortex was significantly decreased in NH₄Cl-loaded rats compared with antibodies to distal Na transporters (BSC1/NKCC2, TSC/NCC, and the three subunits of ENaC). Each lane was loaded with a sample from a different rat. There was no significant difference in the abundance of BSC-1/NKCC2 protein between the two groups (normalized band densities: NH₄Cl-loaded, 91 ± 12%; control, 100 ± 7%). However, the abundance of TSC/NCC protein was strongly decreased in NH₄Cl-loaded rats relative to control animals (normalized band densities: NH₄Cl-loaded, 47 ± 5%; control, 100 ± 8%, P < 0.0005). The individual ENaC subunit proteins showed differential regulation in response to chronic NH₄Cl loading. The renal cortical abundance of α-ENaC protein was not significantly altered (normalized band densities: NH₄Cl-loaded, 85 ± 8%; control, 100 ± 6%) However, there were marked decreases in the abundances of β-ENaC (normalized band densities: NH₄Cl-loaded, 36 ± 4%; control, 100 ± 8%, P < 0.0001) and γ-ENaC (normalized band densities: NH₄Cl-loaded, 49 ± 5%; control, 100 ± 8%, P < 0.0005) protein in response to chronic NH₄Cl loading. In addition, there were no changes in the renal cortical abundances of aquaporin-1 (NH₄Cl-loaded, 101 ± 9%; control, 100 ± 11%) and aquaporin-2 (NH₄Cl-loaded, 109 ± 21%; control, 100 ± 8%; blots not shown).

Figure 3 shows immunoblots of renal outer medullary homogenates from NH₄Cl-loaded and control rats, probed with antibodies to the bumetanide-sensitive Na-K-2Cl cotransporter (present in medullary thick ascending limb) as well as all three subunits of ENaC (present in outer medullary collecting ducts). The abundance of outer medullary BSC-1/NKCC2 was not changed (normalized band densities: NH₄Cl-loaded, 92 ± 3%; control, 100 ± 8%), as in the cortex. The immunoblots for the ENaC subunit proteins from outer medulla revealed the same pattern of response as in the cortex. Specifically, the abundance of α-ENaC protein was unchanged (normalized band densities: NH₄Cl-loaded, 111 ± 13%; Control, 100 ± 13%), whereas the abundances of the other two subunits were markedly decreased. For the β-ENaC, the normalized band densities were 47 ± 16% for NH₄Cl-loaded and 100 ± 16% for control, P < 0.05. For the γ-ENaC the normalized band densities were 30 ± 10% for NH₄Cl-loaded and 100 ± 9% for control, P < 0.0005.

Figure 4 shows immunoblots from the same experiments for NaPi-2 and NHE3. Figure 4 (top) shows the effect of NH₄Cl loading on NaPi-2 abundance in cortical homogenates. Consistent with previous observations (2), the abundance of NaPi-2 protein in the cortex was significantly decreased in NH₄Cl-loaded rats compared with controls.
pared with control animals (normalized band densities: NH₄Cl-loaded, 53 ± 7%; control, 100 ± 14%, P < 0.05). This finding presumably accounts for the observed increase in phosphate excretion in NH₄Cl-loaded rats (Table 1). Interestingly, as shown in Fig. 4 (middle), there was no demonstrable change in NHE3 protein abundance in cortex in response to NH₄Cl loading (normalized band densities: NH₄Cl-loaded, 91 ± 12%; control, 100 ± 6%). In contrast to the response in the cortex, the abundance of NHE3 in the outer medulla (Fig. 4, bottom) was significantly elevated in NH₄Cl-loaded rats relative to control animals (normalized band densities: NH₄Cl-loaded, 191 ± 25%; control, 100 ± 9%, P < 0.05) as previously demonstrated (29).

Effects of acute NH₄Cl loading on Na transporters. To test whether short-term NH₄Cl loading causes changes in the expression of Na transporter and channel proteins in the rat kidney similar to those seen with long-term acid loading, immunoblots were run by using cortical homogenates from rats 16 h after receiving a single dose of NH₄Cl (7.2 mmol/220 g BW). As summarized in Table 2, these immunoblots demonstrated the same pattern as seen for 7-day NH₄Cl loading. Specifically, the relative abundances of NHE3 and α-ENaC were unaltered by the short-term NH₄Cl load, whereas the abundances of NaPi-2, TSC/NCC, β-ENaC, and γ-ENaC were significantly decreased.

Effects of chronic NaHCO₃ loading on Na transporters and channels. To test the effect of chronic base loading on the abundances of TSC/NKCC and ENaC subunits in the kidney, immunoblots were run by using whole kidney homogenates from six rats receiving 7.2 mmol·220 g BW⁻¹·day⁻¹ of NaHCO₃ for 7 days and from six control rats treated identically except for the NaHCO₃ load (Fig. 5). In general, the pattern of effects appears to be opposite of that seen in response to long-term NH₄Cl loading. There were marked increases in the abundances of β-ENaC (normalized band densities: NaHCO₃-loaded, 249 ± 33%; control, 100 ± 12%, P < 0.01) and the γ-ENaC (normalized band densities: NaHCO₃-loaded, 221 ± 20%; control, 100 ± 12%, P < 0.0005) in response to chronic NaHCO₃ loading. The abundance of α-ENaC protein was not significantly changed by chronic NaHCO₃ loading (normalized band densities: NaHCO₃-loaded, 77 ± 14%; control, 100 ± 16%). In addition, there was no significant difference in the abundance of TSC/NCC protein.

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**Table 2. Response to short-term (16-h) NH₄Cl loading: normalized band densities**

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 6)</th>
<th>NH₄Cl-Loaded (n = 6)</th>
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<tr>
<td>NHE3</td>
<td>100 ± 9%</td>
<td>124 ± 12%</td>
</tr>
<tr>
<td>NaPi-2</td>
<td>100 ± 9%</td>
<td>48 ± 3%*</td>
</tr>
<tr>
<td>TSC/NCC</td>
<td>100 ± 12%</td>
<td>61 ± 6%*</td>
</tr>
<tr>
<td>α-ENaC</td>
<td>100 ± 19%</td>
<td>101 ± 9%</td>
</tr>
<tr>
<td>β-ENaC</td>
<td>100 ± 13%</td>
<td>65 ± 6%*</td>
</tr>
<tr>
<td>γ-ENaC</td>
<td>100 ± 12%</td>
<td>57 ± 8%*</td>
</tr>
</tbody>
</table>

Band density data are normalized to the mean of the control value and presented as means ± SE. n, No. of rats; NH₄, type 3 NaH exchanger; NaPi-2, type 2 sodium-phosphate cotransporter; α-, β-, and γ-ENaC, epithelial sodium channel subunits. *P < 0.05.
between the two groups although the data suggest a tendency toward an increase (normalized band densities: NaHCO₃-loaded, 173 ± 45%; control, 100 ± 13%).

DISCUSSION

In this paper, we illustrate a new approach to the investigation of integrative questions in renal physiology, i.e., the use of an ensemble of antibodies to transporters expressed along the entire nephron for comprehensive assessment of the renal adaptation. For this, we have developed rabbit polyclonal antibodies to each of the major apical Na transporters and channels expressed along the renal tubule: NHE3 of the proximal tubule (24, 29), BSC-1/NKCC2 of the thick ascending limb (20, 30), TSC/NCC of the distal convoluted tubule (31), and ENaC of the connecting tubule and collecting duct (35). The use of potent diuretics as transporter-specific inhibitors in isolated perfused tubule and micropuncture studies supports the view that the Na absorption in the thick ascending limb is mediated by BSC-1/NKCC2 (10, 28, 43), the Na reabsorption in the distal convoluted tubule is mediated by TSC/NCC (21, 47), and the Na reabsorption in the collecting duct is mediated by ENaC (40, 42). Therefore, these three Na transport proteins are believed to account for the apical component of nearly all of the Na absorption in the distal nephron and collecting duct.¹ Thus regulation of distal Na reabsorption is likely to involve one or more of these three apical transporters. In addition, antibodies to two apical Na transporters in the proximal tubule, NHE3 and NaPi-2, have allowed us to assess the abundances of transporters that account for the apical component of most of the Na reabsorption in the proximal tubule.

In this paper, we have used our ensemble of Na transporter antibodies to profile the nephron with regard to its adaptive responses to altered acid-base balance. We hypothesized that an increase in Na/H exchange activity in the proximal tubule in response to acid loading may be compensated for by decreases in the abundances of one or more Na transporter or Na channel proteins downstream from the proximal tubule. Indeed, in response to either long- or short-term acid loading, there were marked decreases in the renal abundances of TSC/NCC and β- and γ-ENaC. In addition, we confirmed the previously demonstrated fall in NaPi-2 abundance in the proximal tubule (2). In the following discussion we analyze these findings in the context of the foregoing literature.

TSC/NCC. TSC/NCC of the distal convoluted tubule is an important target for regulation of renal Na excretion. In the present study, NH₄Cl administration was associated with a marked fall in the renal cortical abundance of this cotransporter, presumably providing part of the compensation for increased Na absorption via NHE3 in the proximal tubule as hypothesized in the introduction to this study. We have recently demonstrated that a long-term increase in circulating aldosterone levels due to either aldosterone administration or dietary NaCl restriction is associated with a marked increase in TSC/NCC abundance in the distal convoluted tubule (31), raising the possibility that aldosterone could play some role in the response to NH₄Cl administration. However, acid loading in the present study was not associated with a decrease in plasma aldosterone concentration. Therefore, the effect of acid loading to decrease TSC/NCC abundance must be attributable to a different, unknown mediator.

The fall in TSC/NCC in response to long-term acid loading was profound (<50% of control) and would be expected to ameliorate the effect of the acid loading on systemic pH, judging from the known effect of thiazide diuretics to cause an indirect stimulation of acidification in the collecting duct system, often resulting in metabolic alkalosis in the clinical setting (49). This effect of thiazide diuretics is believed to be due in part to increased Na delivery to the collecting duct, thereby increasing collecting duct Na absorption, which would indirectly increase apical plasma membrane voltage in α-intercalated cells and stimulate proton secretion (27).

Before the availability of antibodies or cDNA probes for TSC/NCC, Fanestil and colleagues (23) developed a method for investigating the abundance of TSC/NCC by measuring the density of metolazone binding sites in renal cortical membrane fractions. In one such study, Fanestil and colleagues demonstrated that long-term NH₄Cl administration in rats significantly reduces cortical metolazone binding, a finding that foreshadowed the present results. In addition, in the same study, Fanestil and colleagues demonstrated a significant increase in cortical metolazone binding after long-term NaHCO₃ administration. In the present study, the mean TSC/NCC band density was 173% of controls after 7 days of NaHCO₃ administration, although this difference was not statistically significant. The previous results of Fanestil et al. support the view that TSC/NCC abundance is indeed upregulated in response to long-term alkali loading.

ENaC. In the kidney, ENaC is expressed in both connecting tubule cells and in principal cells of the collecting duct (17). It is a heteromultimer composed of three different subunits (12). It is a target for long-term regulation by both aldosterone (35) and vasopressin (19). Long-term increases in circulating aldosterone concentrations result in increases in the abundances of the α-subunit protein (35) and mRNA (5, 22, 37, 41) with no effect on β- and γ-subunit abundance. In contrast, long-term exposure to high circulating vasopressin levels stimulates a marked increase in the abundances of the β- and γ-subunit proteins, with little or no effect on the abundance of the α-subunit (18). Thus differential regulation of the ENaC subunits in the kidney is well established. The present study provides

¹ In the thick ascending limb, approximately one-half of the Na absorption is via the transcellular pathway and approximately one-half is via the paracellular pathway. However, the paracellular transport is driven by the lumen positive transepithelial voltage that is dependent on the apical Na-K-2Cl cotransporter BSC-1/NKCC2. Thus furosemide and other loop diuretics inhibit virtually 100% of Na absorption in the thick ascending limb.
another example of differential regulation of ENaC subunits, demonstrating that NH₄Cl loading decreases the abundances of β- and γ-ENaC proteins while not affecting the abundance of α-subunit protein. This pattern of response resembles what would be expected with a decrease in circulating vasopressin. However, direct measurements of plasma vasopressin level showed no decrease. Thus the effect of acid loading must be attributable to a different, unknown mediator.

Acid loading produced a stable decrease in β- and γ-ENaC protein abundances. In contrast to the effect of acid loading, alkali loading caused an increase in β- and γ-ENaC protein abundances. Thus the physiological range of the long-term effect of acid-base intake on β- and γ-ENaC expression is relatively wide. In addition to the long-term effects of acid and base loading, short-term acid-base perturbations may also regulate ENaC by mechanisms involving direct effects of pH changes. Patch-clamp studies in microdissected cortical collecting duct principal cells have demonstrated an increase in open probability of ENaC with alkalinization of the solution bathing the cytoplasmic side of the patch (38), a finding consistent with previous observations in amphibian urinary bladders and skin (26). Recent studies of ENaC expressed in Xenopus laevis oocytes have demonstrated that, through effects on the α-subunit, decreased pH inhibits Na ion conductance while increased pH increases ENaC conductance (13). These effects parallel the long-term effects of acid loading and base loading on ENaC abundance that we have demonstrated in the present study.

The demonstrated decreases in β- and γ-ENaC protein abundances in response to acid loading presumably combine with the observed decrease in TSC/NCC abundance to compensate for increased Na/H exchange in the proximal tubule, maintaining Na balance as proposed in the introduction to this study. This conclusion, however, is contingent on the assumption that changes in β- and γ-ENaC protein abundances may result in parallel changes in the amount of functional ENaC at the cell surface. Because the validity of this assumption was not addressed in this paper, the physiological significance of the observed decreases in β- and γ-ENaC abundances with regard to the effect on Na transport must be regarded as tentative. Indeed, there is evidence that the synthesis of the α-subunit may be rate limiting for the assembly of the mature ENaC complex and that β- and γ-ENaC may be in excess under normal circumstances (36).

**NHE3 abundance in cortex and outer medulla.** Previous studies have demonstrated that metabolic acidosis in animals is associated with an increase in the abundance of NHE3 protein in brush-border membrane fractions from the renal cortex (1, 50). The increase in brush-border NHE3 occurred despite the lack of an increase in NHE3 mRNA (1), indicating that the adaptation was due to an effect on a process other than regulation of gene transcription or mRNA stability. The findings in the present study showed a lack of an increase in NHE3 protein abundance in response to acid loading when cortical homogenates were analyzed. These homogenates contain both plasma membrane and cytoplasmic membranes, whereas the previous measurements showing increased NHE3 protein abundance were accomplished in membrane fractions enriched in plasma membranes (1, 50). Studies by Biemesderfer et al. (7) have established that a substantial amount of proximal tubule NHE3 resides in intracellular vesicles. The combination of results suggests that the activation of NHE3 by acid loading in the mammalian proximal tubule does not involve increases in cellular abundance of NHE3 but rather may involve a redistribution of NHE3 from the cytoplasmic compartment.

An assumption of this study is that activation of NHE3 increases apical Na/H exchange in the proximal tubule of the intact rat and would increase proximal tubule Na reabsorption. Generally, Na absorption in the proximal tubule is assessed indirectly through measurements of fluid absorption. Micropuncture measurements by Kunau et al. (32) in rats and by Sutton et al. (44) in dogs demonstrated marked increases in proximal fluid absorption in response to chronic acid loading in rats. Thus the observations have been variable, suggesting that factors other than the acid-base state of the animals may have affected proximal tubule function. For example, circulating angiotensin II levels, renal nerve activity, the extracellular fluid volume, and the mode, depth, and duration of anesthesia are all factors that are likely to affect proximal tubule function and could have been variable in the studies performed. In contrast to measurements of fluid absorption, the ability of systemic acid loading to stimulate proximal tubule NHE3-mediated Na-H exchange activity has been a consistent finding, as reviewed above.

In contrast to the findings in renal cortex, we found an increase in NHE3 protein in the renal outer medullary homogenates after NH₄Cl loading, confirming previous observations (29). The outer medulla contains two segments that express NHE3, i.e., the thin descending limb and the thick ascending limb of Henle’s loop (3, 7). Thus the kidney displays different regulatory mechanisms for NHE3 in the proximal tube and the loop of Henle with an acidosis-induced increase in cellular NHE3 protein abundance only in the loop of Henle.

**Decreased cortical NaPi-2 protein with acid loading.** Our results, showing a decrease in NaPi-2 protein in homogenates from rat renal cortex in response to acid loading, are consistent with the observations of Ambuhl et al. (2) showing that metabolic acidosis is associated with a decrease in Na-phosphate cotransport activity and immunoreactive NaPi-2 protein in brush-border membranes from the renal cortex. In addition, our results combine with the findings of Ambuhl et al. to indicate that the decrease in brush-border NaPi-2 is
not due solely to redistribution of NaPi-2 to the cytoplasmic compartment but represents a true decrease in cellular NaPi-2. The decrease in NaPi-2 abundance was associated with an increase in urinary phosphate excretion (Table 1), an effect which can be viewed as homeostatic with regard to acid-base balance because the increase in urinary buffer can be expected to facilitate increase net acid excretion at any given urinary pH. Of course, as pointed out in the introduction to this study, a decrease in apical Na uptake due to a decrease in proximal Na-phosphate cotransport may compensate in part for the stimulation of Na uptake that would result from activation of NHE3.

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