Regulation of Thick Ascending Limb Ion Transporter Abundance in Response to Altered Acid/Base Intake

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Abstract. Changes in ammonium excretion with acid/base perturbations are dependent on changes in medullary ammonium accumulation mediated by active NH₄⁺ absorption by the medullary thick ascending limb. To investigate whether alterations in the abundance of medullary thick ascending limb ion transporters, namely the apical Na⁺/K⁺/(NH₄⁺)/2Cl⁻-cotransporter (BSC-1), the apical Na⁺/H⁺-exchanger (NHE3), and the Na⁺/K⁺-ATPase α₁-subunit, may be responsible in part for altered medullary ammonium accumulation, semiquantitative immunoblotting studies were performed using homogenates from the inner stripe of the rat renal outer medulla. After 7 d of NH₄Cl (7.2 mmol/220 g body wt per d) loading (associated with increased medullary ammonium accumulation), neither BSC-1 nor Na⁺/K⁺-ATPase protein expression was altered, but NHE3 protein abundance was significantly increased. On the other hand, both BSC-1 and Na⁺/K⁺-ATPase protein abundance was increased significantly in rats fed NaHCO₃ (7.2 mmol/220 g body wt per d) for 7 d. Rats fed a high-NaCl diet (7.7 mEq Na⁺/220 g body wt per d) for 5 d also showed marked increases in both BSC-1 and Na⁺/K⁺-ATPase expression. The expression level of NHE3 protein did not change with either NaHCO₃ or high NaCl intake. None of these three transporters showed a significant difference in abundance between the groups fed equimolar (7.2 mmol/220 g body wt per d for 7 d) NaHCO₃ or NaCl. It is concluded that outer medullary BSC-1 and Na⁺/K⁺-ATPase α₁-subunit protein abundance is increased by chronic Na⁺ loading but not by acid/base perturbations and that outer medullary NHE3 protein abundance is increased by chronic NH₄Cl loading.

The medullary thick ascending limb (MTAL) of the loop of Henle participates in acid/base transport processes as well as in active absorption of NaCl (1,2). In vivo micropuncture studies have revealed a substantial amount of HCO₃⁻ absorption in the rat loop of Henle (3,4). Most of this absorption is thought to occur in the thick ascending limb (5) as a result of apical membrane Na⁺/H⁺ exchange (6). Ammonium absorption also occurs along the loop of Henle (7), predominantly by a secondary active transport process in which NH₄⁺ substitutes for K⁺ in the apical membrane Na⁺/K⁺/2Cl⁻-cotransporter system of the thick ascending limb (8,9). This active absorption enhances urinary NH₄⁺ excretion by driving countercurrent multiplication of ammonium in the renal medulla, resulting in accumulation of ammonium in the medullary interstitium. This ammonium accumulation facilitates secretion of ammonium into the medullary collecting ducts (10,11). Energy for both NH₄⁺ absorption via the Na⁺/K⁺/(NH₄⁺)/2Cl⁻-cotransporter and H⁺ secretion via the Na⁺/H⁺-exchanger is derived from the Na⁺ concentration difference that is generated across the apical membrane secondary to active Na⁺ transport from the cell mediated by the basolateral Na⁺/K⁺-ATPase (1,2).

Changes in renal net acid excretion that maintain the systemic acid/base balance occur primarily through controlled changes in the rate of urinary NH₄⁺ excretion. Packer et al. (12) showed that in rats the extent of medullary interstitial ammonium accumulation varied with perturbations in the acid/base balance, such that greater accumulation was seen with acid loading and less accumulation was seen with base loading. Because active NH₄⁺ absorption by the thick ascending limb provides a single effect for medullary countercurrent multiplication of ammonium and because countercurrent multiplication yields a gradient for ammonium along the corticomedullary axis (13), we hypothesized that the variation in ammonium accumulation could be attributable in part to regulation of the MTAL ion transporters in response to changes in acid/base intake. To address the possibility that such regulation occurs via changes in the abundance of these transporters, we performed semiquantitative immunoblotting. To do this, we used isoform-specific antibodies raised to the thick ascending limb type 1 Na⁺/K⁺/(NH₄⁺)/2Cl⁻-cotransporter (BSC-1), to the apically expressed type 3 Na⁺/H⁺-exchanger (NHE3), and to the α₁-subunit of Na⁺/K⁺-ATPase to probe immunoblots prepared from homogenates of the inner stripes of the outer medullas of rats undergoing acid or base loading.

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Materials and Methods

Animals and Study Design

Male Sprague Dawley rats (Taconic Farms, Inc., Germantown, NY), weighing between 205 and 255 g, were kept in metabolism cages throughout the study period. Control and treated rats were chosen randomly and all were provided with a gelled agar (1%) diet, modified from an approach originally designed by others (14), so that a daily fixed amount of water (37 ml/220 g body wt per d) and regular rat chow (15 g/220 g body wt per d, NIH-07; Zeigler, Gardners, PA) was given to each rat. The rats were fed once daily, at 10 a.m., and ate all of the offered food during the course of the day. There were no significant changes in body weight during the 5-7-d period of observation in any of the groups of rats. Urine was collected, under mineral oil (with added thymol), from each rat during the final 24 h of each protocol, for analysis of urine pH and urinary ammonium excretion.

Four different loading protocols were used, as follows. The first was NH₄Cl loading. NH₄Cl was included in the gelled diet at 7.2 mmol/220 g body wt per d and was administered for 7 d. Control rats received the same gelled diet but without NH₄Cl. The second protocol was NaHCO₃ loading. Rats were fed the gelled diet containing NaHCO₃, at 7.2 mmol/220 g body wt per d, for 7 d. The control diet was identical except for the absence of added NaHCO₃. The third protocol was NaCl loading. For high sodium intake, a daily fixed amount of NaCl was included in the gelled diet for 5 d. The control diet contained no added NaCl. The amount of sodium in the diet corresponded to 2.2 mEq/220 g body wt per d for controls and 7.7 mEq/220 g body wt per d for high-NaCl-treated rats. The fourth protocol was HCO₃⁻ loading. To determine whether the changes in transporter expression observed with NaHCO₃ loading could be attributed specifically to the effect of HCO₃⁻ administration, equimolar (7.2 mmol/220 g body wt per d) NaHCO₃ or NaCl was given to experimental and control rats, respectively, for 7 d. Detailed characterization of the physiologic responses of the rats to the NH₄Cl loading and HCO₃⁻ loading protocols has been reported previously (15).

Antibodies

Peptide-derived polyclonal antibodies to BSC-1 and NHE3 and a monoclonal antibody to Na⁺/K⁺-ATPase α₁-subunit were used for immunoblotting. To obtain a polyclonal antibody against BSC-1, a 24-amino acid synthetic peptide corresponding to amino acids 33 to 55 of the amino-terminal tail of rat BSC-1 was synthesized (16), on the basis of the cloned sequence reported by Gamba et al. (17) (with an added carboxy-terminal cysteine; sequence, NH₂-SDSTDPPHY-EETSFGDEAQNRLKC-COOH). To obtain a polyclonal antibody against NHE3, a 24-amino acid synthetic peptide corresponding to amino acids 809 to 831 of the carboxyl-terminal tail of rat NHE3 was synthesized, on the basis of the cloned sequence reported by Orłowski et al. (18) (with an added amino-terminal cysteine; sequence, NH₂-CDSFLQADGPEEQLOPASPESTHM-COOH). Each synthetic peptide was purified by HPLC and was conjugated to maleimide-activated keyhole limpet hemocyanin via covalent linkage to the amino-terminal cysteine. Two rabbits each were immunized with these conjugates, using a combination of Freund’s complete and incomplete adjuvants. Rats with enzyme-linked immunosorbent assay titers greater than 1:32,000 before exsanguination were chosen, and all subsequent studies were performed with their immune sera (L320 for BSC-1 and L546 for NHE3). Each antisemum was affinity-purified using a column on which 2 mg of the relevant synthetic peptide was immobilized via covalent linkage to maleimide-activated agarose beads (immobilization kit 2; Pierce, Rockford, IL). The mouse monoclonal Na⁺/K⁺-ATPase α₁-subunit-specific antibody was obtained from a commercial source (Upstate Biotechnology, Lake Placid, NY).

Electrophoresis and Immunoblotting of Proteins

The rats were euthanized by decapitation, and the right 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 (United States Biochemicals, Toledo, OH), titrated to pH 7.6. The kidneys were then dissected to obtain outer medullary tissue. Only the inner stripe of the outer medulla was collected, using the inner/outer medullary junction and the inner-stripe/outer-stripe junction as the boundaries for dissection (19). Tissue samples were homogenized in ice-cold isolation solution using a tissue homogenizer (Omni 1000, fitted with a micro-sawtooth generator; Omni International, Inc., Warrenton, VA). After homogenization, protein concentrations were measured using the Pierce bicinchoninic acid protein assay reagent kit. Samples were then solubilized at 60°C for 15 min in Laemmli sample buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed using 7.5% polyacrylamide minigels (to assess BSC-1 or Na⁺/K⁺-ATPase α₁-subunit protein expression) or 10% polyacrylamide minigels (to assess NHE3 protein expression). In all cases, to confirm equality of loading among the lanes, electrophoresis was initially performed for the entire set of samples in a given experiment on a single 12% polyacrylamide/sodium dodecyl sulfate gel, which was then stained with Coomassie blue. Selected bands from these gels were analyzed using 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 electrophoretically transferred from gels to nitrocellulose membranes. After blocking with 5 g/dl nonfat dry milk, proteins were probed overnight at 4°C with the desired antibody at the following IgG concentrations: 0.12 μg/ml for BSC-1, 0.397 μg/ml for NHE3, and 0.05 μg/ml for Na⁺/K⁺-ATPase α₁-subunit. The antibodies were prepared in an antibody diluent containing 150 mM NaCl, 50 mM sodium phosphate, 10 mg/dl sodium 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. 31468) and was used at a concentration of 0.16 μg/ml. Sites of antibody-antigen reaction were detected using luminol-based enhanced chemiluminescence (LumiGLO; Kirkegaard and Perry Laboratories, Gaithersburg, MD) before exposure to x-ray film (Kodak 165-1579 scientific imaging film). Exposures were made with a broad range of exposure times to allow us to choose films for analysis in which the band densities for controls were approximately 30 to 50% of the density at saturation. On the basis of previous observations, this criterion produces films in which the relationship between the band density and the protein amount is in the linear range (19,20). All blots presented in this report were repeated at least once.

Statistical Analyses

Relative quantification of the resulting immunoblot band densities was performed densitometrically, using a laser scanner (Molecular Dynamics, San Jose, CA) and ImageQuaNT software (Molecular Dynamics). Results were presented as mean ± SEM. The statistical significance of the effects of various treatments on transporter expression was assessed using unpaired t tests when SD were the same or using Welch t tests when SD were significantly different (INSTAT; Graphpad Software, San Diego, CA). To facilitate comparisons, we
normalized the densitometric values such that the mean for the control group in each study was defined as 100%. P values of <0.05 were accepted as indicating significant differences between means.

Results
Characterization of NHE3 Antibody
Figure 1 shows the relative regional distribution of NHE3 protein in the rat kidney, as determined by immunoblotting of crude homogenates from the cortex, outer medulla, and inner medulla. When 10 μg protein/lane was loaded for each region, a narrow band of approximately 84 kD was noted, which was most abundant in the cortex and outer medulla. A stronger band density was noted in the cortex than in the outer medulla, consistent with greater abundance of the protein in the proximal tubule, compared with the MTAL. When the anti-NHE3 antibody was preadsorbed with an excess of the immunizing peptide, the band was fully ablated.

Effects of Chronic NH4Cl Loading
To test whether chronic NH4Cl loading would change the abundance of BSC-1, NHE3, or Na+/K+-ATPase protein in the rat renal outer medulla, immunoblots were obtained using outer medullary (inner stripe) homogenates from five rats that had received 7.2 mmol/220 g body wt per d NH4Cl for 7 d and from five control rats. NH4Cl-loaded rats exhibited lower urine pH (5.47 ± 0.02 versus 6.4 ± 0.07, P < 0.005) and higher urinary ammonium excretion (6.43 ± 0.24 versus 0.55 ± 0.05 mmol/d, P < 0.005), compared with controls.

Figure 2 shows the results of immunoblots probed with anti-BSC-1, anti-NHE3, and anti-Na+/K+-ATPase α1-subunit. Each lane was loaded with an outer medullary protein sample from a different rat. Statistical analysis of densitometric data revealed no significant difference in BSC-1 protein expression between NH4Cl-loaded rats and controls (normalized band densities: NH4Cl-loaded, 126 ± 18%; control, 100 ± 21%). There was also no significant difference in the expression of the Na+/K+-ATPase α1-subunit between the two groups (normalized band densities: NH4Cl-loaded, 164 ± 42%; control, 100 ± 30%). On the other hand, the expression level of NHE3 protein in the outer medulla was significantly elevated in NH4Cl-loaded rats, compared with controls (normalized band densities: NH4Cl-loaded, 167 ± 10%; control, 100 ± 13%; P < 0.005).

Effects of Chronic NaHCO3 Loading
To test whether chronic NaHCO3 loading would change the abundance of BSC-1, NHE3, or Na+/K+-ATPase protein in

* Our immunoblots of the α1-subunit of Na+/K+-ATPase consistently show a doublet in 7.5% polyacrylamide gels. The doublet is seen not only with the monoclonal antibody used in this study, but also with a polyclonal antibody obtained from the same commercial supplier. The doublet is not apparent when higher percentages of polyacrylamide (10% and 12%) are used, because of lower resolution. The explanation for the existence of two bands is currently unclear, but the possibilities include differential splicing of the mRNA, post-translational modification (which would alter the mobility of the protein), and physiologic processing by proteases. We think that artifactual proteolysis during preparation of the samples is unlikely, because none of the other proteins studied showed any evidence of proteolysis.
the rat renal outer medulla, immunoblots were obtained using outer medullary (inner stripe) homogenates from six rats that had received 7.2 mmol/220 g body wt per d NaHCO$_3$ for 7 d and from six control rats. NaHCO$_3$-loaded rats exhibited higher urine pH than controls (8.19 ± 0.03 versus 6.59 ± 0.02, $P < 0.005$).

Figure 3 shows the results of immunoblots probed with anti-BSC-1, anti-NHE3, or anti-Na$^+$/K$^+$/ATPase $\alpha_1$-subunit. Each lane was loaded with an outer medullary protein sample from a different rat. In contrast to the effects of NH$_4$Cl loading, the expression level of BSC-1 protein was significantly elevated in NaHCO$_3$-loaded rats, compared with controls (normalized band densities: NaHCO$_3$-loaded, 164 ± 6%; control, 100 ± 12%; $P < 0.001$). The expression level of the Na$^+$/K$^+$/ATPase $\alpha_1$-subunit was also significantly increased in NaHCO$_3$-loaded rats, compared with controls (normalized band densities: NaHCO$_3$-loaded, 209 ± 30%; control, 100 ± 10%; $P < 0.01$). However, there was no significant difference in the expression of NHE3 protein between the two groups (normalized band densities: NaHCO$_3$-loaded, 120 ± 16%; control, 100 ± 16%).

Effects of High-NaCl Diet

Dietary sodium intake has been reported to be an important determinant of the MTAL bicarbonate and ammonium transport capacity (21). In addition, Ecelbarger et al. (22) showed that chronic oral saline loading increased BSC-1 protein expression. We tested the effects of high NaCl intake (7.7 mEq Na$^+$/220 g body wt per d) on transporter abundance, in comparison with control (2.2 mEq Na$^+$/220 g body wt per d). Figure 4 shows the results of immunoblots for BSC-1, NHE3, and Na$^+$/K$^+$/ATPase $\alpha_1$-subunit obtained using outer medullary (inner stripe) homogenates from four rats that had received high-NaCl diet for 5 d and from four controls. The expression of BSC-1 protein was significantly enhanced in the rats fed the high-NaCl diet, compared with controls (normalized band densities: NaCl-loaded, 150 ± 4%; control, 100 ± 4%; $P < 0.001$). The expression level of Na$^+$/K$^+$/ATPase $\alpha_1$-subunit was also significantly increased in the rats fed the high-NaCl diet, compared with controls (normalized band densities: NaCl-loaded, 189 ± 42%; control, 100 ± 9%; $P < 0.05$). However, no significant difference was seen in the expression of NHE3 protein (normalized band densities: NaCl-loaded, 140 ± 15%; control, 100 ± 21%).

Effects of Bicarbonate Loading

To test the effect of bicarbonate administration without changing Na$^+$ intake, we compared rats loaded with 7.2 mmol...
of NaHCO$_3$ and those loaded with 7.2 mmol of NaCl, each for 7 d. Figure 5 shows the results of immunoblots probed with anti-BSC-1, anti-NHE3, or anti-Na$^+$/K$^+$-ATPase α1-subunit. Each lane was loaded with an outer medullary protein sample from a different rat. None of the three transporters showed a significant difference in protein abundance between the two treatments.

**Discussion**

The tools are now available to study the regulation of ion transport at the molecular level. Most of the ion transporters of the thick ascending limb of the loop of Henle have been cloned, e.g., the cDNA for rat renal BSC-1 (17). This transporter is the primary apical Na$^+$/Cl$^-$-entry pathway of the thick ascending limb (23), and its expression level seems to be regulated by in vivo factors associated with altered NaCl transport across the thick ascending limb epithelium (22). Five Na$^+$/H$^+$-exchanger isoforms (NHE1 to NHE5) have been identified (24), and immunocytochemical studies using isoform-specific antibodies have localized NHE3 expression to the apical membrane of cells of the thick ascending limb of the loop of Henle (25,26). The α1-subunit of Na$^+$/K$^+$-ATPase has been detected by immunoblotting all along the rat nephron (27,28). The highest Na$^+$/K$^+$-ATPase activities are found in the thick ascending limb and the distal convoluted tubule (29).

**Figure 5.** Effects of HCO$_3^-$ loading. Immunoblots of outer medullary homogenates from Sprague Dawley rats that were fed gelled food with NaCl (7.2 mmol/220 g body wt per d; n = 6) or NaHCO$_3$ (7.2 mmol/220 g body wt per d; n = 6) are shown. Each lane was loaded with a sample from a different rat. The BSC-1 blot was loaded with 2 μg total protein/lane and was probed with anti-BSC-1. The NHE3 blot was loaded with 10 μg total protein/lane and was probed with anti-NHE3. The Na$^+$/K$^+$-ATPase blot was loaded with 2 μg total protein/lane and was probed with anti-Na$^+$/K$^+$-ATPase α1-subunit. Preliminary 12% sodium dodecyl sulfate-polyacrylamide gels were run and were stained with Coomassie blue to confirm equality of loading in all lanes (not shown). No statistically significant differences were found for BSC-1 protein, NHE3 protein, or Na$^+$/K$^+$-ATPase α1-subunit protein.

**Regulation of Apical Transporters**

Previous in vivo microperfusion studies showed adaptive increases in the capacity of the MTAL to absorb NH$_4^+$ and HCO$_3^-$ in response to metabolic acidosis (21). Similar conclusions were reached in in vivo micropuncture studies (30). These increases are likely to be mediated by increases in the activity of the apical membrane BSC-1 and NHE3, respectively. Our results suggest that the increase in ammonium absorption via BSC-1 is not a consequence of increased co-transporter expression. However, the increase in NHE3 abundance demonstrated here is likely to have contributed to the regulation of countercurrent multiplication of ammonium. This result is consistent with a recent report (31).

Increased acidification of the thick ascending limb lumen (associated with increased expression of NHE3) in response to ammonium chloride loading would have a detrimental effect on net ammonia absorption, on the basis of the effect it would have to accelerate NH$_3$ secretion in the thick ascending limb because of nonionic diffusion. However, this effect is thought

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b We cannot rule out a change in cotransporter abundance that was smaller than the detection limit of our method and yet could have contributed to the 36% increase in ammonium absorption measured by Good (21). This caveat applies to all of the negative results presented in this study.
to be very small, because of the fact that the epithelium of the MTAL has a relatively low permeability to NH$_3$ ($3 \times 10^{-3}$ cm/s) (8). Nevertheless, luminal acidification would be expected to augment net ammonium excretion by enhancing NH$_3$ secretion by nonionic diffusion in the distal nephron and collecting ducts.

Isolated perfused tubule studies (21) also demonstrated that NaHCO$_3$-induced chronic metabolic alkalosis caused paradoxical increases in the NH$_4^+$ and HCO$_3^-$ absorptive capacity of the MTAL. Those results were interpreted as being attributable to an adaptation of the MTAL to high sodium intake, rather than to the alkalosis itself (21). Our results are consistent with this view, at least with respect to the observed increase in NH$_4^+$ absorption. Although NaHCO$_3$ loading caused a significant increase in BSC-1 expression, a similar increase was seen in response to administration of equivalent amounts of NaCl (Table 1). The increase in BSC-1 expression with increased dietary NaCl intake is consistent with the finding of Ecelbarger et al. (22) that chronic oral saline loading resulted in a marked increase in BSC-1 protein abundance in the renal outer medulla. In contrast to BSC-1, NHE3 expression was altered by neither NaHCO$_3$ loading nor NaCl loading, indicating that the observed increase in HCO$_3^-$ absorption in isolated perfused rat MTAL (21) must have been attributable to some other mode of regulation. It seems likely that the increase in HCO$_3^-$ absorption seen with NaHCO$_3$ loading and NaCl loading was attributable instead to increases in Na$^+$/K$^+$-ATPase abundance (see below).

**Physiologic Role of Adaptation of MTAL NaCl Absorption**

An adaptive increase in MTAL sodium absorption in response to NaCl loading seems contrary to requirements for the regulation of sodium balance. However, this response is appropriate as a means to maintain water balance. NaCl loading markedly increases NaCl excretion, presumably by inhibition of NaCl absorption in renal tubule segments other than the MTAL. This large increase in NaCl excretion would tend to

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*Table 1. Summary of changes in relative expression levels of rat renal outer medullary ion transporter proteins*
increase water excretion via an osmotic effect. However, increased medullary interstitial sodium accumulation, secondary to an increase in NaCl absorption by the MTAL, would tend to balance the osmotic effect of high NaCl levels in the collecting duct lumen and allow NaCl to be excreted without obligating large amounts of water. Therefore, because the MTAL is able to adapt by increasing BSC-1 and Na\textsuperscript{+/K\textsuperscript{+}}-ATPase expression in response to Na\textsuperscript{+} loading, water conservation can be maintained.

### Conclusion

In conclusion, the results of the studies presented here do not support a role for adaptation of BSC-1 and Na\textsuperscript{+/K\textsuperscript{+}}-ATPase expression in the regulation of ammonium accumulation in the renal medulla. However, the findings support the conclusion that increased dietary sodium intake provides a strong stimulus to adapt by increasing BSC-1 and Na\textsuperscript{+} loading, water conservation can be maintained.

### Acknowledgments

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