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Coordinated down-regulation of NBC-1 and NHE-3 in sodium and bicarbonate loading

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Coordinated down-regulation of NBC-1 and NHE-3 in sodium and bicarbonate loading.

Background. Bicarbonate reabsorption in the kidney proximal tubule is predominantly mediated via the apical Na^+/H^+ exchanger (NHE-3) and basolateral Na^+:HCO_3^- cotransporter (NBC-1). The purpose of these studies was to examine the effects of Na^+ load and altered acid-base status on the expression of NHE-3 and NBC-1 in the kidney.

Methods. Rats were placed on 280 mmol/L of $NaHCO_3$, NaCl, or NH_4Cl added to their drinking water for 5 days and examined for the expression of NHE-3 and NBC-1 in the kidney.

Results. Serum [HCO_3^-] was unchanged in $NaHCO_3$- and NaCl-loaded animals versus control ($P > 0.05$). However, a significant hyperchloremic metabolic acidosis was developed in NH_4Cl-loaded animals. A specific polyclonal antibody against NBC-1 recognized a 130 kD band, which was exclusively expressed in the basolateral membrane of proximal tubules. Immunoblot studies indicated that the protein abundance of NBC-1 and NHE-3 in the cortex decreased by 74% ($P < 0.04$) and 66% ($P < 0.03$), respectively, in $NaHCO_3$ loading and by 72% ($P < 0.003$) and 55% ($P < 0.04$), respectively, in NaCl loading. Switching from $NaHCO_3$ to distilled water resulted in rapid recovery of NHE-3 and NBC-1 protein expression toward normal levels. Metabolic acidosis increased the abundance of NHE-3 ($P < 0.0001$) but not NBC-1 ($P > 0.05$).

Conclusions. $NaHCO_3^-$ or NaCl loading coordinately down-regulates the apical NHE-3 and basolateral NBC-1 in rat kidney proximal tubule, presumably due to increased Na^+ load. We propose that the down-regulation of these two Na^+- and HCO_3-absorbing transporters is, to a large degree, responsible for enhanced excretion of excess of Na^+ and alkaline load and prevention of metabolic alkalosis in rats subjected to $NaHCO_3^-$ loading.

The majority of the filtered load of HCO_3^- is reabsorbed in the proximal tubule via the luminal Na^+/H^+ exchanger NHE-3 [reviewed in 1]. The exit of HCO_3^- across the basolateral membrane of proximal tubule is via the Na^+:HCO_3^- cotransporter [2–7]. A number of acid-base or electrolyte disorders are associated with altered HCO_3^- reabsorption in the kidney proximal tubule [8–10]. These changes result from an adaptation in the activity of luminal NHE-3 and basolateral NBC-1. Coordinated regulation of apical Na^+/H^+ exchanger (NHE-3) and basolateral Na^+:HCO_3^- cotransporter (NBC-1) has been proposed in several acid-base or electrolyte disorders [11–15]; however, molecular studies in support of this hypothesis are few.

Mammalians (rat or human) with normal vascular volume and electrolyte balance do not develop metabolic alkalosis despite a huge amount of bicarbonate intake [16]. Indeed, serum bicarbonate either remains unchanged or increases mildly in bicarbonate loading [16]. This has been attributed to increased renal bicarbonate excretion in bicarbonate-loaded subjects [16]. The nephron segments and the transporters that mediate this adaptive alteration remain unknown. The proximal tubule plays an important regulatory role in acid-base and electrolytes disorders. This is due to the presence of high capacity bicarbonate- and sodium-absorbing transporters in this segment. In the case of sodium or bicarbonate loading, however, functional and/or molecular studies in support of adaptive regulation of proximal tubule acid-base transporters are lacking.

An Na^+:HCO_3^- cotransporter (NBC-1) was recently cloned from human [17], rat [18], and amphibian kidney [19]. Cloning experiments have identified additional NBC isoforms (NBC-2, NBC-3, and NBC-4) and variants (pancreas NBC1, muscle NBC3 and melanoma cell NBC2) [5, 20–22]. NBC-1 expression in the kidney is exclusively limited to the basolateral membrane of the proximal tubule [23, 24]. The apical Na^+/H^+ exchanger works in tandem with basolateral Na^+:HCO_3^- cotransporter to reabsorb the filtered bicarbonate [1–5].

The purpose of the current studies was to examine possible molecular regulation of the apical Na^+/H^+ exchanger (NHE-3) and basolateral Na^+:HCO_3^- cotrans-

Key words: acid-base transporters, metabolic alkalosis, renoprotection, transporters, basolateral membrane, kidney proximal tubule.

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porter (NBC-1) in kidney proximal tubule in response to increased sodium intake, and altered acid-base status.

METHODS

Animal model

Male Sprague-Dawley rats (125 to 150 g) were placed in metabolic cages and had free access to normal diet and distilled water. In each study, a group of experimental animals was compared with controls that were obtained from the same shipment and studied for the same period of time. After three days of adjustment to metabolic cages, rats were subjected to the following treatments.

For $NaHCO_3$ loading, animals were allowed free access to 280 mmol/L $NaHCO_3$ in the drinking solution for five days and compared with rats on distilled water. Both groups were fed regular rat chow for the duration of the experiment.

For NaCl loading, animals were placed on 280 mmol/L NaCl added to the drinking solution for five days. The control group had free access to distilled water. All groups were fed regular rat chow for the duration of the experiment.

For NH_4Cl loading, animals were placed on a 280 mmol/L NH_4Cl-containing solution for five days. The control rats were placed on distilled water. Both groups were fed regular rat chow for the duration of the experiment.

At the end of the experiments, rats were anesthetized by an intraperitoneal injection of 50 mg of sodium pentobarbital. Intracardiac blood was obtained for serum $[K^+]$ and $[HCO_3^-]$ measurements, and both kidneys were rapidly removed for RNA isolation and membrane protein preparation.

Proximal tubules and medullary thick ascending limb suspensions

Proximal tubules (PTs) and medullary thick ascending limb (mTAL) tubular suspensions were prepared from cortex and the inner stripe of the outer medulla harvested from normal Sprague-Dawley rats, respectively, as previously described [13, 25].

NBC-1–specific antibodies

Peptide-derived polyclonal antibody specific to NBC-1 was raised using commercial services (Alpha Diagnostics, San Antonio, TX, USA). Two peptides corresponding to rat NBC-1 amino acid residues 153 to 172 and 1016 to 1035 were synthesized and conjugated. Two rabbits were immunized with the conjugate. Both rabbits developed enzyme-linked immunosorbent assay (ELISA) titers greater than 1:100,000. The antisera were affinity purified by covalently immobilizing the immunizing peptide on commercially available columns (Sulfo-Link Immobilization kit 2; Pierce, Rockford, IL, USA). For NHE-3, a specific immune serum raised against the C-terminal of rat NHE-3 (Alpha Diagnostics, San Antonio, TX, USA) was utilized. The specificity of the NHE-3 antibody was verified by the pre-adsorption of the antibody with the excess of the immunizing peptide (data not shown).

Immunocytochemistry of NBC-1 in the kidney

Kidneys from experimental animals were cut into slices and mounted on holders to form tissue blocks. The tissues were fixed in a solution containing 0.1% glutaraldehyde plus 2% paraformaldehyde in 0.1 mol/L sodium cacodylate buffer, pH 7.20, and stored in 0.1 mol/L cacodylate buffer, pH 7.2, at 4°C. For immunohistochemistry, the tissue blocks were sectioned into 5 μm sections, placed on slides (Fisher brand-Superfrost/Plus), and incubated at 75°C in the oven for one hour. To deparaffinize the tissues, the slides were placed in xylene for five minutes, then acetone for one minute, and washed in phosphate-buffered saline (PBS) three times for three minutes each. To remove endogenous peroxidase activity, slides were incubated in a solution containing 190 mL of MeOH and 3 mL of 30% H_2O_2 for 15 minutes at room temperature. The slides were then washed twice in PBS for two minutes, followed by two washes in distilled H_2O for three minutes. The slides were placed in a coplin jar containing 1% $ZnSO_4$ in distilled H_2O and heated in the microwave twice for 5 minutes, then cooled at room temperature for 15 minutes, and washed in distilled H_2O twice for 3 minutes and in PBS twice for 3 minutes. To block the nonspecific binding, the slides were blotted, treated with a normal rabbit serum in a dilution of 1:10 in PBS + 1% bovine serum albumin (BSA), and incubated in a humidified chamber for 30 minutes at room temperature. The primary antibody was applied to the slides in the appropriate optimized dilution in PBS + 1% BSA and incubated in a humidified chamber for two hours at room temperature. The slides were washed three times in 200 mL PBS supplemented with horse serum at 6, 4, and 2 mL and in 5, 5, and 10 minutes at room temperature. The secondary antibody was applied to the slides in a dilution of 1:25. Each slide was treated in 4 μL secondary antibody + 5 μl normal horse serum + 91 μL PBS + 1% BSA, incubated in a humidified chamber for one hour at room temperature, and then washed in PBS three times for two minutes each. The peroxidase-anti-peroxidase (PAP) conjugate diluted in 1:100 in PBS + 1% BSA was applied to the slides. Thereafter, the slides were incubated in a humidified chamber for one hour at room temperature and then washed in PBS three times for two minutes each. To develop a colored reaction product, the diaminobenzidine (DAB) was used. Finally, the tissues were counterstained with Harris hematoxylin and mounted down onto a slide using fluoromount G and covered with cover glass.

Preparation of cortical or whole kidney membrane fractions

A total membrane fraction containing plasma membrane and intracellular membrane vesicles was prepared as previously described [26]. Briefly, the tissue samples were homogenized in ice-cold isolation solution (250 mmol/L sucrose and 10 mmol/L triethanolamine, pH 7.6) containing protease inhibitors (phenazine methyl sulfonyl fluoride, 0.1 mg/mL; leupeptine 1 μg/mL), using a Polytron homogenizer. The homogenate was centrifuged at low speed (1000 × *g*) for 10 minutes at 4°C to remove nuclei and cell debris. The supernatant was spun at 150,000 × *g* for 90 minutes at 4°C. The pellet containing plasma membrane and intracellular vesicles was suspended in an isolation solution with protease inhibitors. The total protein concentration was measured, and the membrane fractions were solubilized at 65°C for 20 minutes in Laemmli sample buffer.

Electrophoresis and immunoblotting

Semiquantitative immunoblotting experiments were carried out as previously described [26]. Briefly, the solubilized membrane proteins were size fractionated on polyacrylamide minigels (Novex, San Diego, CA, USA) under denaturing conditions. Using a BioRad transfer apparatus (BioRad Laboratories, Hercules, CA, USA), the separated proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 5% milk proteins and then probed with affinity purified anti-NBC-1 or NHE-3 immune serum at an IgG concentration of 0.8 μg/mL and 0.35 μg/mL, respectively. The secondary antibody was donkey anti-rabbit IgG conjugated to horseradish peroxidase (Pierce). The sites of the antigen-antibody complex formation on the nitrocellulose membranes were visualized using chemiluminescence method (SuperSignal Substrate; Pierce) and captured on light-sensitive imaging film (Kodak). Bands corresponding to NBC-1 and NHE-3 proteins were quantitated by densitometric analysis using UN-SCAN-IT gel software (Silk Scientific, Inc., Orem, UT, USA) and were expressed as a percentage of controls. The equity in protein loading in all blots was first verified by gel staining using the Coomassie brilliant blue R-250 (Bio-Rad, Hercules, CA, USA) [10].

RNA isolation and Northern hybridization

Total cellular RNA was extracted from whole kidney or cortex by the method of Chomczynski and Sacchi [27], quantitated spectrophotometrically, 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 (MSI) using ×10 sodium chloride-sodium phosphate-EDTA (SSPE) as the transfer buffer. Membranes were cross-linked by ultraviolet light and baked for one hour. Hybridization was performed according to Church and Gilbert [28]. Briefly, membranes were pre-prehybridized for one hour in 0.1 × SSPE-1% sodium dodecyl sulfate (SDS) solution at 65°C and then prehybridized for three hours at 65°C with 0.5 mol/L sodium phosphate buffer, pH 7.2, 7% SDS, 1% BSA, 1 mmol/L EDTA, and 100 μg/mL sonicated carrier DNA. Thereafter, the membranes were hybridized overnight in the above solution with ^{32}P-labeled DNA probe for NBC-1 and NHE-3. The cDNA probes (25 ng) were labeled with ^{32}P deoxynucleotides using the RadPrime DNA labeling kit (GIBCO-BRL). The membranes were washed twice in 40 mmol/L sodium phosphate buffer, pH 7.2., 5% SDS, 0.5% BSA, and 1 mmol/L EDTA for 10 minutes at 65°C, washed four times in 40 mmol/L sodium phosphate buffer, pH 7.2, 1% SDS, and 1 mmol/L EDTA for 10 minutes at 65°C, exposed to PhosphorImager cassette at room temperature for 24 to 72 hours, and read by PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). For NBC-1, a PCR product corresponding to nucleotides 509 to 3237 was used as a specific probe. For NHE-3, the polymerase chain reaction (PCR) fragment encoding the nucleotides 1883 to 2217 was used as a specific probe.

Materials

^{32}P-dCTP was purchased from New England Nuclear (Boston, MA, USA). Nitrocellulose filters and other chemicals were purchased from Sigma Chemical Co. The RadPrime DNA labeling kit was purchased from GIBCO BRL. BCECF was from Molecular Probes, Inc. (Eugene, OR, USA).

Statistics

Results are expressed as means ± SE. Statistical significance between experimental groups was determined by the paired or unpaired Student *t* test. $P < 0.05$ was considered significant.

RESULTS

Serum [K^+] and [HCO_3^-] in sodium bicarbonate, sodium chloride, and NH_4Cl loading

Similar to the previously published studies from this laboratory [18], rats did not develop metabolic alkalosis or hypokalemia after five days of increased $NaHCO_3$ intake. Serum [HCO_3^-] was 27 ± 1.4, 24 ± 1.7 and 23.3 ± 1.3 mEq/L in $NaHCO_3$, distilled water and NaCl-loaded rats ($N = 4$, $P > 0.05$). Serum [K^+] was 4.7 ± 0.2 mEq/L in the bicarbonate-loaded group versus 4.9 ± 0.3 in rats on distilled water ($N = 4$, $P > 0.05$). Similarly, no difference was observed between sodium chloride-loaded rats and their control (water) in term of serum [K^+] or [HCO_3^-] (data not shown). However, a significant hyperchloremic metabolic acidosis in NH_4Cl-loaded animals

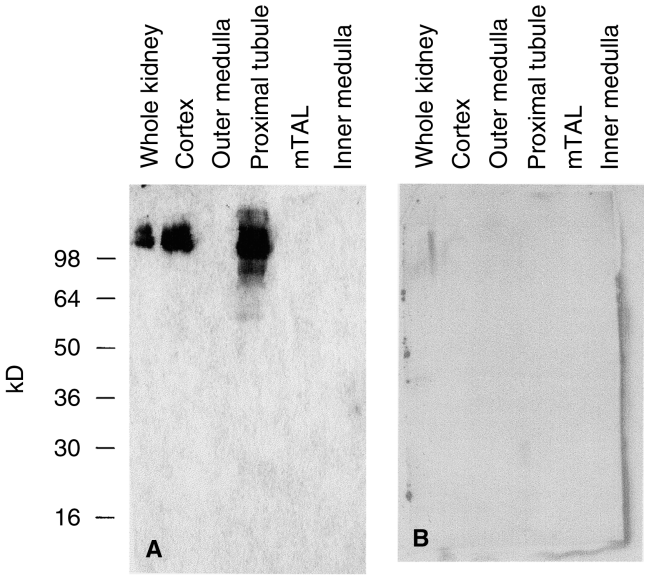

Fig. 1. Specificity of NBC-1 immune serum. Immunoblot of total membrane fractions of rat kidney tissues probed with either affinity-purified anti-NBC-1 (IgG concentration, 0.8μg/mL; *A*) or the NBC-1 antibody pre-adsorbed overnight with 0.8 mg of the immunizing peptides (*B*). Each lane was loaded with 10 μg of total protein harvested from whole kidney, cortex, outer medulla, proximal tubules, medullary thick ascending limb tubules (mTAL), and inner medulla.

developed as indicated by the fall in serum [HCO_3^-] [15 ± 0.5 vs. 25 ± 1.3 mEq/L in control rats (water), $P < 0.01$, $N = 5$ for each], and an increase in serum [Cl^-] (111 ± 1.5 vs. 101 ± 1.2 mEq/L in control rats, $P < 0.05$, $N = 5$ for each).

Specificity of NBC-1 immune serum

In the next series of experiments, the specificity of NBC-1 immune serum was tested. As shown in Figure 1A, NBC-1 immune serum detects a ~130 kD band in the cortex and proximal tubules. No labeling was detected in the medulla. Pre-adsorption of the immune serum with the synthetic peptide prevented the labeling (Fig. 1B), indicating the specificity of the immune serum.

Immunohistochemical staining of the rat kidney with NBC-1 immune serum

To examine the distribution of NBC-1 in the kidney, immunocytochemical staining of rat kidney sections was performed as described in the **Methods** section. As shown in Figure 2A, NBC-1 immune serum labels the basolateral membranes of proximal tubule cells. No labeling was detected with the pre-immune serum (Fig. 2B).

Effect of $NaHCO_3$ loading on NBC-1 protein abundance in the cortex

To determine the effect of sodium bicarbonate loading on NBC-1 protein abundance, an immunoblot analysis of NBC-1 on membrane proteins isolated from rat kidney cortex was performed. As shown in Figure 3, NBC-1 protein abundance decreased by 74% after five days of sodium bicarbonate loading [26±4 vs. 100±15, in bicarbonate-loaded ($N = 4$) vs. control ($N = 3$) rats, $P < 0.04$].

Immunohistochemical staining of NBC-1 in the kidneys of $NaHCO_3$ loaded rats

Next, the effect of $NaHCO_3$ loading on NBC-1 was examined by immunohistochemical staining. NBC-1 staining of basolateral membranes of proximal tubule was decreased in $NaHCO_3$-loaded rats (Fig. 4B) as compared with controls (Fig. 4A).

Effect of $NaHCO_3$ loading on NHE-3 protein abundance and mRNA expression in the cortex

In this series of experiments, the effect of sodium bicarbonate loading on NHE-3 abundance was examined in membrane proteins isolated from rat kidney cortex. As shown in Figure 5A, NHE-3 protein abundance decreased by 66% after five days of sodium bicarbonate loading (34 ± 4 vs. 100 ± 10, in bicarbonate-loaded vs. control rats, $N = 5$ for each, $P < 0.03$; Fig. 5A). Northern hybridization experiments using total RNA isolated from cortex show that the expression levels of NHE-3 mRNA was not altered in sodium bicarbonate-loaded animals ($P > 0.05$, $N = 3$ for each; Fig. 5B).

Effect of recovery from $NaHCO_3$ loading on NBC-1 expression in the kidney

In these experiments, two groups of rats were studied. One group (4 rats) had free access to water (control) and the other group (12 rats) was supplemented with 280 mmol/L $NaHCO_3$ in their drinking water for five days. Four rats from this latter group (sodium bicarbonate loading) were sacrificed after five days, and the other eight rats were switched to distilled water and sacrificed after three ($N = 4$) or 14 ($N = 4$) hours (recovery). Total RNA and membrane fractions isolated from kidneys of each group were used for Northern hybridization and immunoblotting, respectively. The first series of experiments compared the expression of NBC-1 at 14 hours of recovery versus Na-bicarbonate loading. As shown in Figure 6A (left upper panel), the abundance of NBC-1 was decreased in sodium bicarbonate loading but recovered to almost normal levels 14 hours after switching to normal drinking water. The second set of experiments examined the abundance of NBC-1 at 3 and 14 hours of recovery from sodium bicarbonate loading. As shown in Figure 6A (left lower panel), the abundance of NBC-1 decreased by 50% in sodium bicarbonate loading [50 ± 2% vs. 100 ± 7%, in sodium bicarbonate-loaded ($N = 4$) vs. control ($N = 4$) rats, $P < 0.03$]. Three hours after switching to water, NBC-1 protein remained low [43 ± 1% vs. 100 ± 7 in control (water), $P < 0.02$, $N = 4$ for

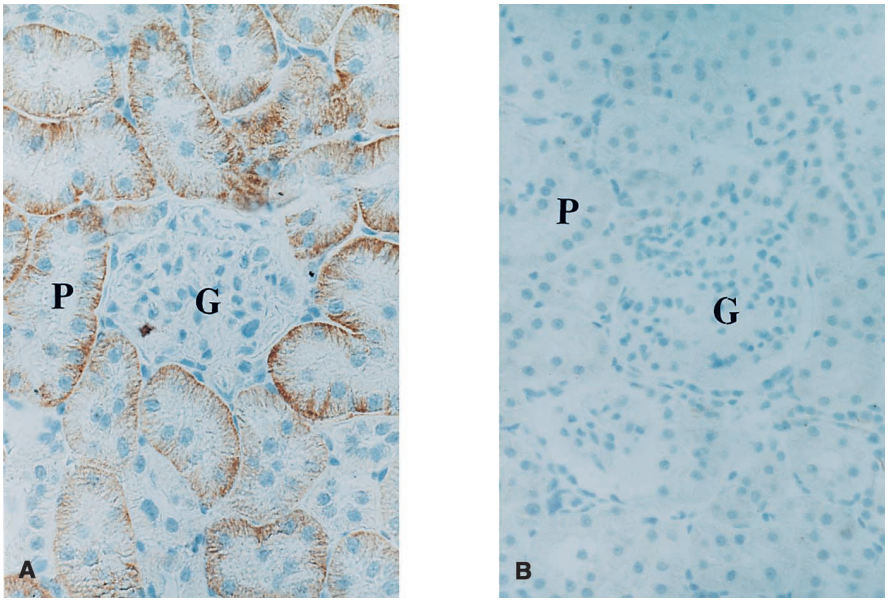

Fig. 2. Immunohistochemical localization of NBC-1 in the rat cortex. Immunohistochemical staining of rat kidney with NBC-1 immune serum (*A*) or pre-immune serum (*B*). NBC-1 protein is detected exclusively in the basolateral membrane of proximal tubule cells as indicted by the arrows. Abbreviations are: G, glomerulus; P, proximal tubule.

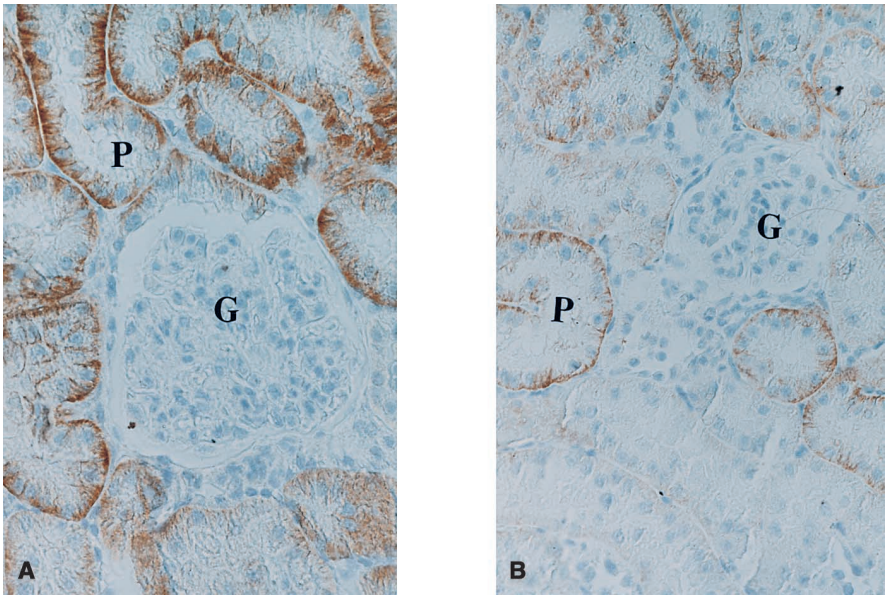

Fig. 4. Immunohistochemical staining of NBC-1 in the kidneys of $NaHCO_3$ loaded rats. Representative immunohistochemical staining of NBC-1 protein in the cortical slices harvested form control (*A*) or $NaHCO_3$ loaded animals (*B*). As shown, $NaHCO_3$ loading elicited a significant reduction in the basolateral staining of the proximal tubules. Abbreviations are: G, glomerulus; P, proximal tubule.

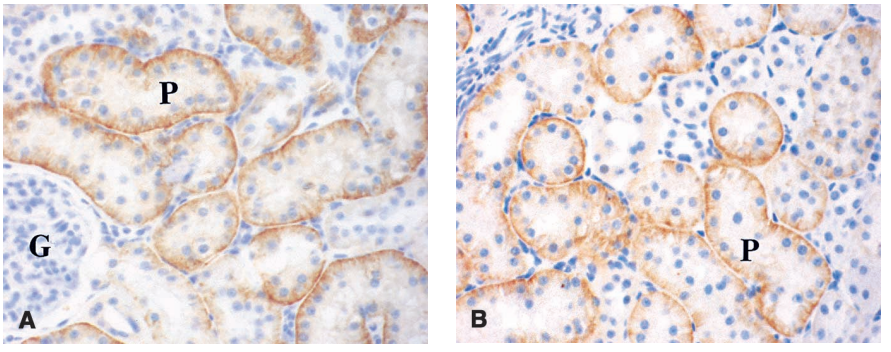

Fig. 11. Immunohistochemical staining of NBC-1 in kidneys of acidotic rats. Representative immunohistochemical staining of NBC-1 protein in cortical slices harvested form control (*A*) or NH_4Cl loaded animals (*B*). As shown, NH_4Cl loading did not alter the intensity of the basolateral staining in the proximal tubules. Abbreviations are: G, glomerulus; P, proximal tubule.

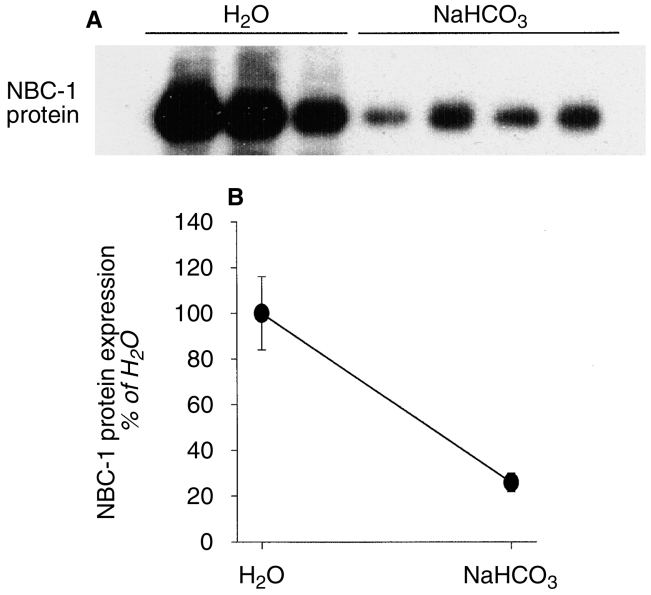

Fig. 3. Effect of $NaHCO_3$ loading on NBC-1 protein expression in the cortex. (*A*) Immunoblot of NBC-1 protein abundance in the cortex of rats on water or $NaHCO_3$ for five days. (*B*) Corresponding densitometry analysis of NBC-1 band showing a 74% decrease in NBC-1 abundance in $NaHCO_3$ loaded animals ($N = 4$, $P < 0.04$ vs. water, $N = 3$). Each lane was loaded with 10 μg total protein from a different rat.

each; Figure 6A, left lower panel] and recovered to almost baseline levels 14 hours after switching to normal drinking water [87 ± 9 vs. 100 ± 7, in 14 hours of recovery ($N = 4$) vs. control ($N = 4$), $P > 0.05$; Figure 6A]. Figure 6A (right panel) depicts the densitometric analysis of the blots. NBC-1 mRNA expression decreased by 46% after five days of sodium bicarbonate loading [54 ± 8 vs. 100 ± 9, in sodium bicarbonate-loaded vs. control, $P < 0.04$, $N = 3$ for each; Fig. 6B), but recovered to almost normal levels after switching to drinking water (89 ± 5 vs. 100 ± 9 in recovery vs. control, $P > 0.05$, $N = 3$ for each; Fig. 6B).

Effect of recovery from $NaHCO_3$ loading on NHE-3 protein abundance in the kidney

Microsomes harvested from experimental groups (discussed previously in this article) were examined for NHE-3 abundance. As shown in Figure 7 (left upper panel), the abundance of NHE-3 protein decreased in sodium bicarbonate loading but recovered to almost normal levels 14 hours after switching to normal drinking water. The second set of experiments examined the abundance of NHE-3 at 3 and 14 hours of recovery from Na-bicarbonate loading. The results depicted in Figure 7 (left lower panel) indicate that NHE-3 protein abundance decreased by 47% after five days of sodium bicarbonate loading [53 ± 3 vs. 100 ± 9, in sodium bicarbonate loading ($N = 4$) vs. control ($N = 4$), $P < 0.004$]. Interestingly, NHE-3 protein abundance decreased further to 27 ± 9% three hours after switching to normal drinking water ($P < 0.002$ vs. water, $N = 4$ for each), but returned to near baseline levels after 14 hours of recovery (91 ± 5 vs. 100 ± 9 in recovery vs. control, $N = 4$ for each, $P > 0.05$; Fig. 7). Figure 7 (right panel) depicts the densitometric analysis of the blots.

Effect of NaCl loading on NBC-1 mRNA and protein expression in the kidney

In studies shown in Figures 2 to 7, the effect of $NaHCO_3$ loading was compared to water alone. In the next series of experiments we attempted to examine the effect of NaCl load, as a control for the Na load. As shown in Figure 8A, NBC-1 protein abundance in the cortex decreased by 72% after five days of NaCl loading (28 ± 12 vs. 100 ± 13, in NaCl loading vs. control, $N = 5$ for each, $P < 0.003$). Furthermore, Northern hybridization experiment indicated that the NBC-1 mRNA correlates with the protein abundance as it was decreased by 55% in NaCl-loaded rats compared to control (45 ± 9 vs. 100 ± 10, in NaCl vs. control, $N = 4$ for each, $P < 0.04$; Fig. 8B).

Effect of NaCl loading on NHE-3 mRNA and protein expression in the kidney

This series of experiments examined the expression of NHE-3 in NaCl loading. Accordingly, cortical total RNA and membrane fractions were harvested and used for assessment of NHE-3 mRNA expression and protein abundance. The results are summarized in Figure 9 and show that NHE-3 protein abundance was decreased by 55% in the cortex of NaCl-loaded animals as compared with control (45 ± 9 vs. 100 ± 12, in NaCl loading vs. control, $N = 5$ for each, $P < 0.04$; Fig. 9A). However, unlike NBC-1, the expression of NHE-3 mRNA was not altered in response to NaCl loading ($P > 0.05$, $N = 4$ for each; Fig. 9B).

Effect of NH_4Cl loading on NBC-1 and NHE-3 expression in the kidney

Rats subjected to NH_4Cl loading for five days developed metabolic acidosis as shown by a decrease in plasma bicarbonate concentration (discussed previously in this article). Several studies have reported that metabolic acidosis increases NHE-3 activity/expression in the cortex [11, 15, 29–31]. However, the molecular nature of the adaptation of basolateral NBC-1 to metabolic acidosis remains unknown. We therefore examined the expression of NBC-1 protein abundance in the kidney cortex of acidotic rats. Figure 10 shows that the expression of NBC-1 protein was not affected in response to metabolic acidosis as compared to water alone (106 ± 3 vs. 100 ± 4 in acidosis vs. control, $N = 5$ for each, $P > 0.05$). Immunohistochemical studies showed no changes in the intensity of staining by NBC-1 immune serum in basolat-

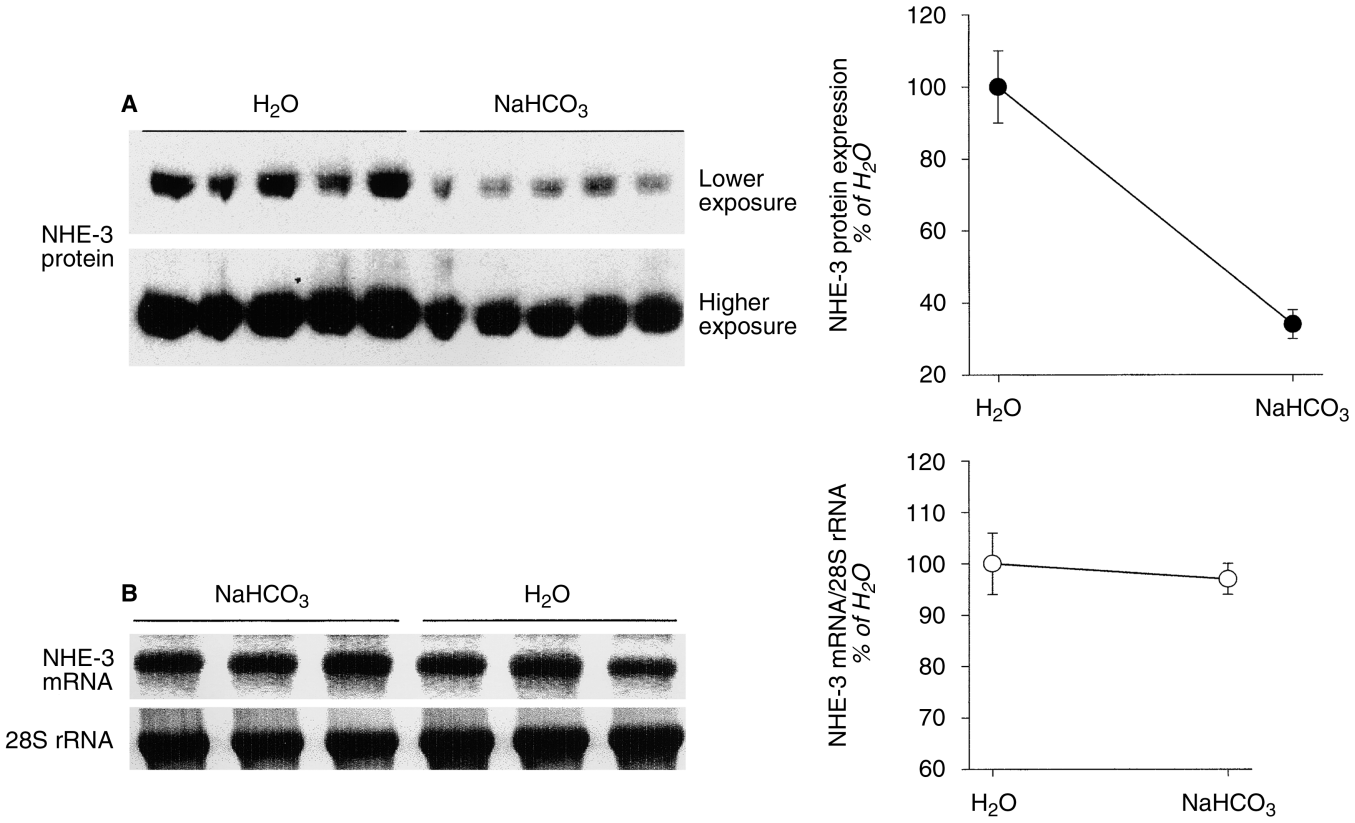

Fig. 5. Effect of $NaHCO_3$ loading on NHE-3 protein abundance in the cortex. (*A*, left panel) Immunoblot of NHE-3 protein abundance in the cortex harvested from rats on water or $NaHCO_3$ for five days. (Right panel) Corresponding densitometric analysis of the NHE-3 band showing a 66% decrease in NHE-3 protein abundance ($P < 0.03$ vs. water, $N = 5$ for each). Each lane was loaded with 12 μg total protein from a different rat. (*B*, left panel): Representative Northern hybridization of NHE-3 mRNA and 28S rRNA levels in the cortex. (Right panel) Mean NHE-3 mRNA-to-28S rRNA ratio in the cortex of rats on water or $NaHCO_3$. The mean expression levels were not altered in $NaHCO_3$-loaded animals ($P > 0.05$ compared with control, $N = 3$ for each). Each lane was loaded with 30 μg total RNA from a different rat.

eral membranes of kidney proximal tubule of acidotic animals (Fig. 11). These results indicate that increased NBC activity in metabolic acidosis is likely due to a post-translational mechanism. The other plausible explanation in this regard was the possibility that rats on NH_4Cl did not adapt to acidosis. To investigate this latter possibility, immunoblot analysis of NHE-3 was performed. As indicated in Figure 12, NHE-3 abundance in the cortex was increased significantly in acidotic rats (100 ± 5 in control vs. 181 ± 2.6, $P < 0.0001$, $N = 5$ for each group).

DISCUSSION

The present studies examined the effect of sodium loading and altered acid-base status on the proximal tubule apical NHE-3 and basolateral NBC-1. Immunohistochemical staining and immunoblotting experiments demonstrated that NBC-1 is exclusively expressed on the basolateral membrane of the proximal tubule (Figs. 1 and 2). The mRNA and protein abundance of NBC-1 in the proximal tubule cells decreased significantly in bicarbonate loading (Figs. 3, 4, and 6). Bicarbonate loading was associated with a significant decrease in NHE-3 protein abundance in the cortex (Fig. 5A) without altering its mRNA expression (Fig. 5B). Switching to normal drinking water resulted in rapid recovery of both NBC-1 and NHE-3 protein abundance to near normal levels (Figs. 6 and 7). Rat subjected to the same level of Na^+ loading with NaCl showed a significant reduction in the expression of NBC-1 and NHE-3 (Figs. 8A and 9A). The magnitude of the reduction of NHE-3 and NBC-1 abundance in NaCl loading was comparable to $NaHCO_3$ loading, indicating that the down-regulation of NBC-1 and NHE-3 in both conditions resulted from increased Na^+ load. Finally, our results demonstrate that metabolic acidosis increased NHE-3 protein abundance (Fig. 12) but did not alter the abundance of NBC-1 protein (Figs. 10 and 11).

Mammalians (rat or human) are generally resistant to the generation of metabolic alkalosis in response to increased bicarbonate intake [16]. Serum bicarbonate concentration in the current experiments was not significantly altered in response to increased bicarbonate intake (**Results** section). This is similar to other studies,

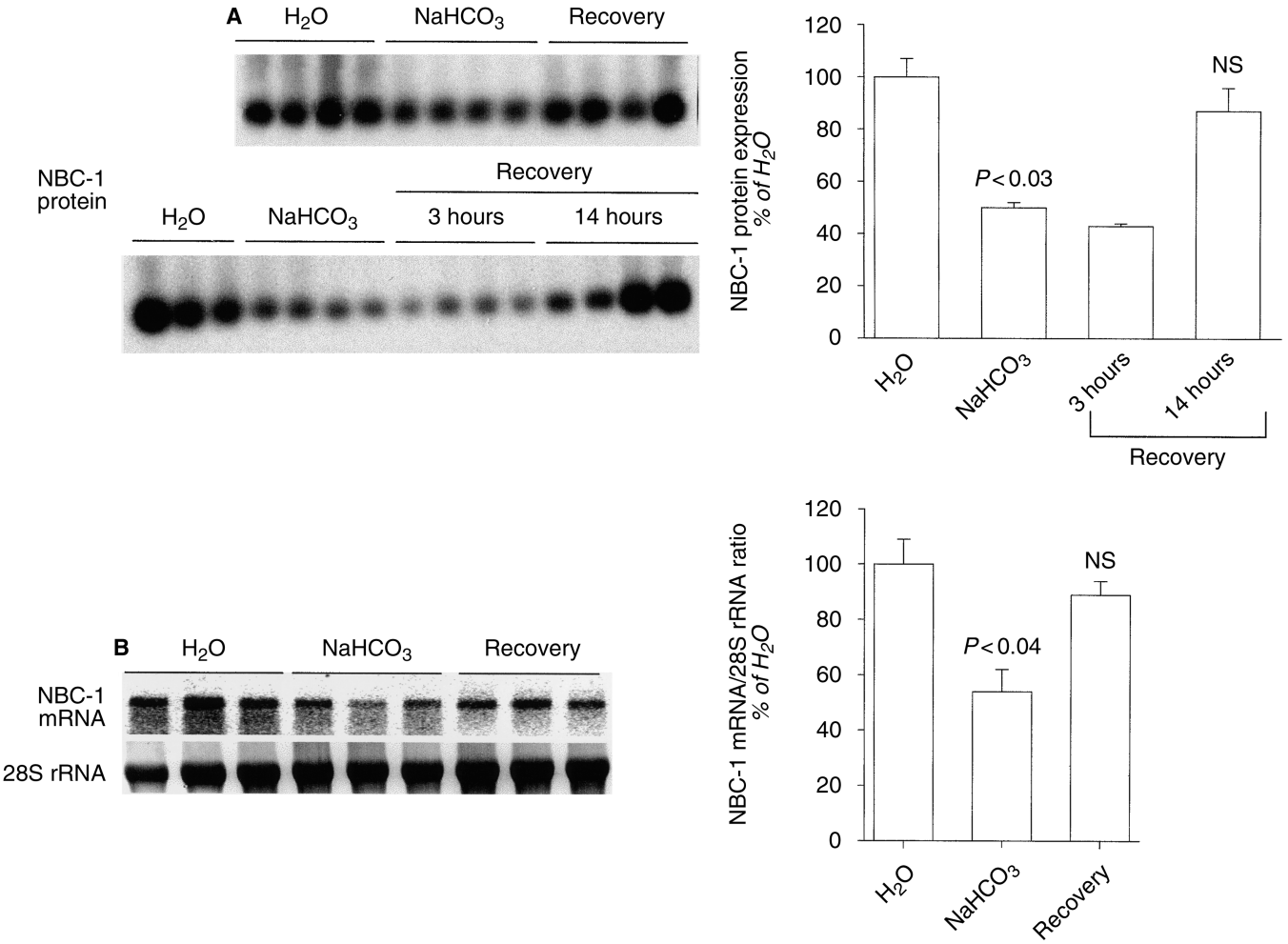

Fig. 6. Effect of $NaHCO_3$ loading and recovery from $NaHCO_3$ loading on NBC-1 mRNA and protein expression in the kidney. (*A*, left upper panel) Immunoblot of NBC-1 protein abundance in kidneys harvested from rats either on water or on $NaHCO_3$, or after 14 hours of recovery from $NaHCO_3$ loading. (Left lower panel) NBC-1 protein abundance in kidneys of animals either on water or on $NaHCO_3$ or after 3 and 14 hours of recovery from $NaHCO_3$ loading. (Right panel) Corresponding densitometric analysis of NBC-1 blots showing a 50% decrease in NBC-1 protein abundance in $NaHCO_3$ loaded animals ($N = 4$, $P < 0.03$ vs. water, $N = 4$). NBC-1 protein abundance remained low at three hours (−57%, $P < 0.02$), but recovered significantly at 14 hours after switching to water ($N = 4$, $P > 0.05$ vs. water, $N = 4$). Each lane was loaded with 10 μg total protein from a different rat. (*B*, left panel) Northern hybridization of NBC-1 mRNA and 28S rRNA levels in the left kidneys of the same animals. (Right panel) Mean NBC-1 mRNA-to-28S rRNA ratio showing a 46% decrease in NBC-1 mRNA expression in $NaHCO_3$ loaded animals ($P < 0.04$ vs. water, $N = 3$ for each), and the recovery of NBC-1 mRNA after switching to water ($P > 0.05$ vs. water, $N = 3$ for each). Each lane was loaded with 30 μg total RNA from a different rat.

which have demonstrated that increasing bicarbonate intake has a minimal effect on serum bicarbonate concentration [reviewed in 16]. These results indicate that adaptive mechanisms in the kidney enhance bicarbonate secretion in bicarbonate-loaded subjects. The nephron segments that are responsible for enhanced bicarbonate excretion in subjects on excess bicarbonate intake remain speculative. Both proximal tubule and cortical collecting duct are reasonable candidates; however, data in support of this conclusion are scant. Proximal tubule is predominantly responsible for the bulk of bicarbonate reabsorption under normal conditions. This process is predominantly accomplished via the Na^+/H^+ exchanger NHE-3 and Na^+:HCO_3^- cotransporter NBC-1 acting in series [1–3, 5, 11, 15]. The localization of these two transporters in the proximal tubule has been well documented. NHE-3 is located on the apical domain of proximal tubule membrane. NBC-1 localization, based on immunoelectron microscopic studies, is confined to convoluted segments (S1 and S2) but not the S3 segment of the proximal tubule [32].

The down-regulation of cortical apical NHE-3 in rats subjected to bicarbonate loading is consistent with decreased bicarbonate reabsorption in proximal tubule. This, in turn, can lead to increased delivery of bicarbonate to the distal nephron and eventually the urine. The

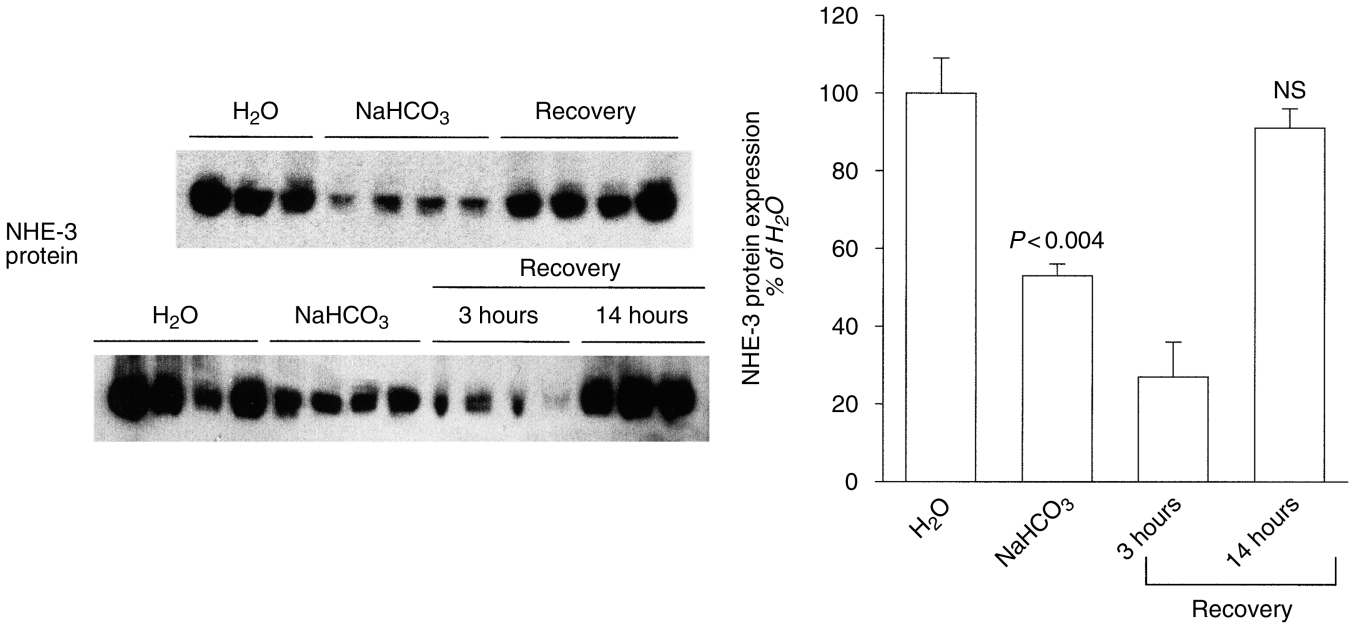

Fig. 7. Effect of $NaHCO_3$ loading and recovery from $NaHCO_3$ loading on NHE-3 protein abundance in the kidney. (Left upper panel) Immunoblot of NHE-3 protein abundance in kidneys harvested from rats either on water or on $NaHCO_3$, or after 14 hours of recovery from $NaHCO_3$ loading (left lower panel), NHE-3 protein abundance in animals treated with water or $NaHCO_3$, or after 3 and 14 hours of recovery from $NaHCO_3$ loading. (Right panel) Corresponding densitometric analysis of blots showing a 47% decrease in NHE-3 protein abundance in $NaHCO_3$-loaded animals ($N = 4$, $P < 0.03$ vs. water, $N = 4$ for each). NHE-3 protein abundance further decreased at 3 hours (−73%, $P<0.002$) but recovered significantly at 14 hours after switching to water ($N = 4$, $P > 0.05$ vs. water, $N = 4$ for each). Each lane was loaded with 10 μg total protein from a different rat.

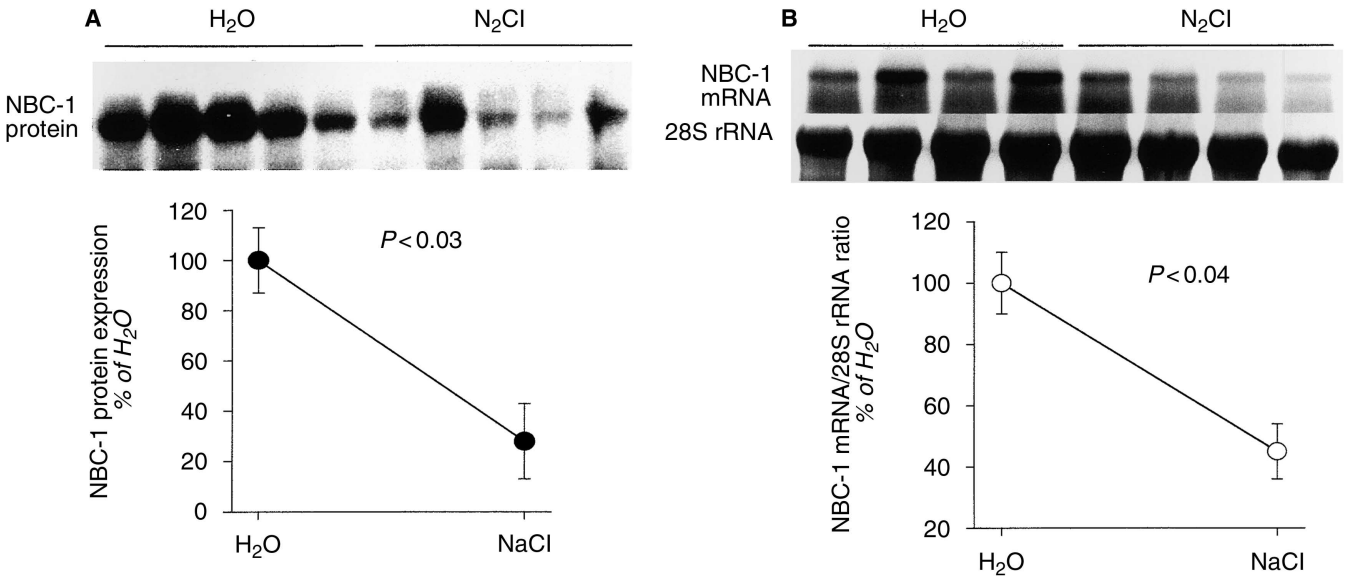

Fig. 8. Effect of NaCl loading on NBC-1 mRNA and protein expression in the kidney. (*A*, upper panel) Immunoblot of NBC-1 protein abundance in the cortex harvested from rats on water or NaCl loading. (Lower panel) Corresponding densitometric analysis of NBC-1 band showing a 72% decrease in NBC-1 protein abundance in NaCl loaded animals ($P < 0.003$ vs. water, $N = 5$ for each). Each lane was loaded with 10 μg total protein from a different rat. (*B*, upper panel) Northern hybridization of NBC-1 mRNA and 28S rRNA levels in the cortex of the same animals. (Lower panel) Mean NBC-1 mRNA-to-28S rRNA ratio showing a 55% decrease in NBC-1 mRNA expression in NaCl loaded animals ($P < 0.04$ vs. water, $N = 4$ for each). Each lane was loaded with 30 μg total RNA from a different rat.

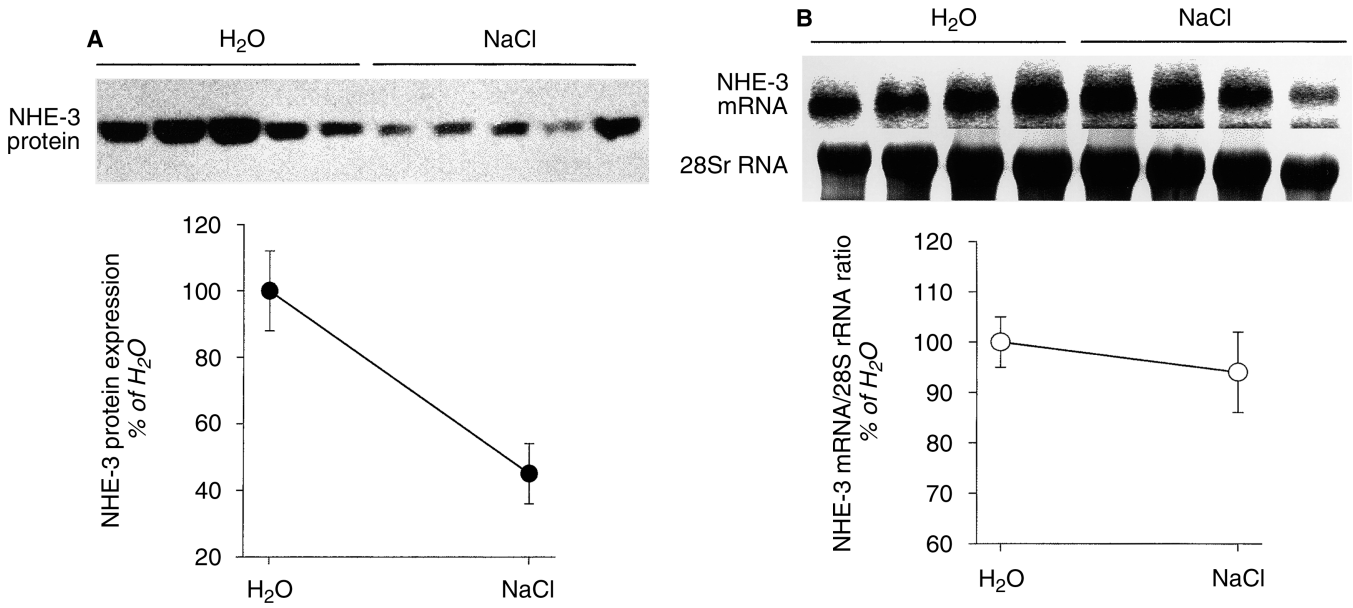

Fig. 9. Effect of NaCl loading on NHE-3 mRNA and protein expression in the kidney. (*A*, upper panel) Immunoblot of NHE-3 protein abundance in the cortex harvested from rats on water or (lower panel) NaCl loading. Corresponding densitometric analysis of NHE-3 band showing a 55% decrease in NHE-3 protein abundance in NaCl loaded animals ($P < 0.04$ vs. water, $N = 5$ for each). Each lane was loaded with 12μg total protein from a different rat. (*B*, upper panel) Northern hybridization of NHE-3 mRNA and 28S rRNA levels in the cortex of the same animals. (Lower panel) Mean NBC-1 mRNA-to-28S rRNA ratio showing no change in NHE-3 mRNA expression in NaCl loaded animals ($P > 0.05$ vs. water, $N = 4$ for each). Each lane was loaded with 30 μg total RNA from a different rat.

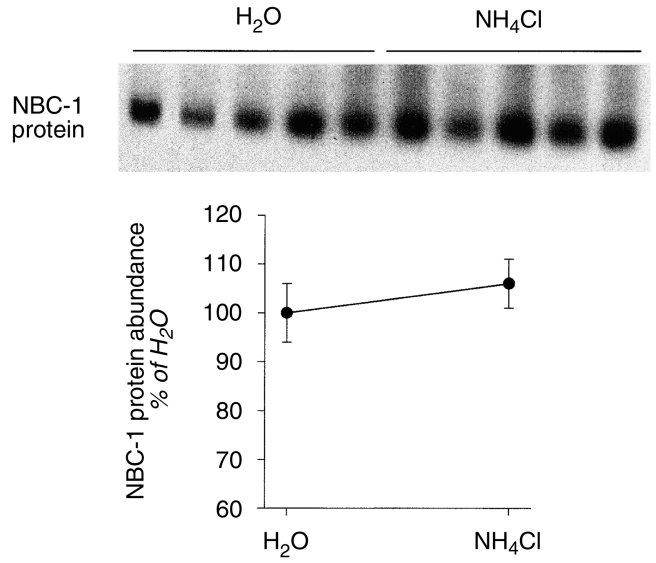

Fig. 10. Effect of NH_4Cl loading on NBC-1 protein abundance in the kidney. (Upper panel): Immunoblot of NBC-1 protein abundance in the cortex harvested from rats on water or NH_4Cl (metabolic acidosis). (Lower panel) Corresponding densitometric analysis of NBC-1 band showing no effect of metabolic acidosis on NBC-1 protein abundance ($P > 0.05$ vs. water, $N = 5$ for each). Each lane was loaded with 10 μg total protein from a different rat.

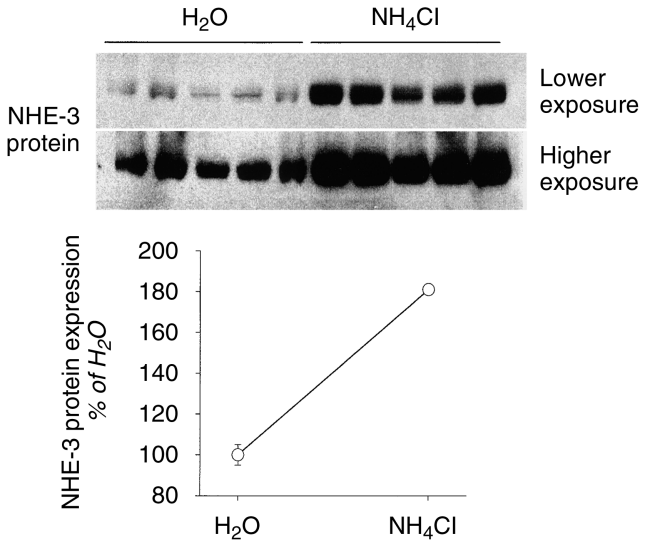

Fig. 12. Effect of NH_4Cl loading on NHE-3 protein abundance in the cortex. (Upper panel) Immunoblot of NHE-3 protein abundance in the kidneys harvested from rats on water or NH_4Cl (metabolic acidosis). (Lower panel) Corresponding densitometric analysis demonstrating increased NHE-3 protein abundance in acidosis ($P < 0.0001$ vs. water, $N = 5$ for each). Each lane was loaded with 10 μg total protein from a different rat.

down-regulation of basolateral NBC-1 in bicarbonate-loaded rats further confirms the decreased bicarbonate reabsorption in proximal tubule and indicates a coordinated down-regulation of apical NHE-3 and basolateral NBC-1. This in turn should increase renal bicarbonate excretion and prevent generation of metabolic alkalosis.

The mechanism of the down-regulation of cortical NHE-3 and NBC-1 in bicarbonate loading remains speculative. Nevertheless, the present studies clearly indicate that this adaptive process is triggered by an increased Na^+ load but not the HCO_3^- load. It is well known that increased Na^+ intake is associated with extracellular fluid volume (ECFV) expansion and subsequent increase in glomerular filtration rate (GFR) [33, 34]. However, whether the down-regulation of NHE-3 and NBC-1 resulted from an increased filtered load of Na^+ or was mediated via peritubular physical and/or neurohumoral factors related to volume expansion remains unclear at this time. Chronic studies in rats subjected to uninephrectomy or protein loading showed a significant up-regulation in the activity of proximal tubule NHE-3, along with increased GFR [35]. However, acute increases in renal perfusion pressure with subsequent increase in GFR were shown to be associated with a rapid redistribution of NHE-3 from the apical membrane to intracellular vesicles in the proximal tubules [36]. In our studies, the abundance of NHE-3 in the total membrane proteins was significantly decreased in response to both NaCl and $NaHCO_3$ loading (Figs. 5 and 7). Whether an initial redistribution of NHE-3 protein to intracellular vesicles occurred during the early time points of Na loading remains speculative at this time.

Clearance studies demonstrated that extracellular fluid volume expansion decreased whole kidney sodium and bicarbonate reabsorption in both human and experimental animals [37–39]. Subsequent micropuncture/microperfusion studies indicated that extracellular fluid volume expansion was associated with a significant reduction in proximal bicarbonate reabsorption, which was assumed to account for the observed enhanced sodium bicarbonate excretion in whole kidney experiments [33, 34, 40]. An increase in the renal interstitial hydrostatic pressure was thought to play an important role in the inhibition of Na^+ reabsorption by the proximal tubule in response to acute ECFV expansion [41]. In separate studies, a chronic increase in Na^+ intake was reported to decrease the activity of the proximal tubule apical Na^+/H^+ exchanger (NHE-3) [15, 40]. These latter studies are in agreement with our results demonstrating a significant down-regulation of both NHE-3 and NBC-1 protein abundance in response to increased Na^+ intake.

The signals mediating the effect of increased Na^+ intake could be complex and multifactorial. While no attempt was made to measure the status of the ECFV in the present study, it is likely that changes in the ECFV may provide a key to the dysregulation of NHE-3 and NBC-1 expression in response to Na^+ loading. Previous studies have demonstrated that α2-catecholamine, angiotensin II, dopamine, parathyroid hormone, and adenosine all directly regulate the activity of both NHE-3 [42–45] and NBC-1 [46–50] in the proximal tubule. Furthermore, the synthesis and the circulating levels of most of these hormones are highly sensitive to alterations in Na^+ intake or vascular volume. Hence, it is likely that one or more of these hormones may be involved in the coordinated down-regulation of cortical NHE-3 and NBC-1 in response to increased Na^+ intake.

With regard to NH_4Cl-induced acidosis, studies have demonstrated that chronic metabolic acidosis stimulates bicarbonate reabsorption in the proximal tubule [10, 15]. The increased ability to reabsorb bicarbonate results at least in part from increased NHE-3 activity [13, 15, 30, 31], which is a consequence of enhanced NHE-3 protein abundance as shown in Figure 11 and previously reported [29, 51]. Further, several studies have demonstrated a parallel increase in the activity of apical Na^+/H^+ exchanger and basolateral Na^+:HCO_3^- cotransporter [11, 13, 15] in metabolic acidosis. Our previous studies [18] and the present work indicate that NBC-1 mRNA expression and protein remain unchanged, strongly suggesting that increased NBC activity in metabolic acidosis involves a post-translational regulatory process.

The sequential localization of NHE-3 and NBC-1 (apical vs. basolateral) in kidney proximal tubule links the activity of each transporter to the other, in that altered activity of one transporter can change the activity of the other transporter by altering the availability of the substrate for that transporter. For example, decreased NHE-3 activity increases intracellular H^+ concentration and, as a result, decreases intracellular bicarbonate concentration. This in turn can decrease the activity of basolateral NBC-1. Alternatively, decreased basolateral NBC decreases the exit of bicarbonate across the basolateral membrane and results in increased intracellular bicarbonate concentration. This will decrease the availability of intracellular H^+ for transport via apical NHE-3. Whether the down-regulation of NHE-3 in bicarbonate loading is secondary to the down-regulation of basolateral NBC or vice versa remains speculative at present.

In conclusion, sodium bicarbonate or sodium chloride loading coordinately down-regulates the expression of the apical Na^+/H^+ exchanger NHE-3 and basolateral $Na:HCO_3$ cotransporter NBC-1 in the rat proximal tubule. This effect likely results from increased sodium rather than bicarbonate or chloride intake. The down-regulation of these transporters in conditions associated with increased $NaHCO_3$ intake results in increased bicarbonate and sodium excretion, which should prevent the development of metabolic alkalosis and limit the expansion of the extracellular fluid volume, respectively.

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APPENDIX

Abbreviations used in this article are: BSA, bovine serum albumin; ECFV, extracellular fluid volume; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; GFR, glomerular filtration rate; mTAL, medullary thick ascending limb; NBC, Na^+:HCO_3^- cotransporter; NHE, Na^+/H^+ exchanger; NHE-3, Na^+/H^+ exchanger isoform 3; PAP, peroxidase–anti-peroxidase; PBS, phosphate-buffered saline; PT, proximal tubules; SDS, sodium dodecyl sulfate.

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