Role of NHE3 in Mediating Renal Brush Border Na\(^{+}\)-H\(^{+}\) Exchange

ADAPTATION TO METABOLIC ACIDOSIS

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The aims of the present study were to estimate the fraction of renal brush border membrane Na\(^{+}\)-H\(^{+}\) exchange activity mediated by the isoform NHE3 and to evaluate whether the increased brush border Na\(^{+}\)-H\(^{+}\) exchange observed in metabolic acidosis is due to increased expression of NHE3 protein. Compared with other isoforms, NHE3 is known to have a unique profile of sensitivity to pharmacologic inhibitors, including relative resistance to amiloride analogs and HOE694. We therefore assessed the inhibitor sensitivity of pH gradient-stimulated \(^{22}\)Na uptake in renal brush border vesicles isolated from normal rats. The \(I_{50}\) values for amiloride (30 \(\mu\)M), dimethylamiloride (10 \(\mu\)M), ethylisopropylamiloride (6 \(\mu\)M), and HOE694 (>100 \(\mu\)M) were markedly dissimilar from those reported for NHE1 and NHE2 but were nearly identical to reported values for NHE3. Na\(^{+}\)-H\(^{+}\) exchange activity in renal brush border vesicles isolated from rats with 5 days of NH\(_{4}\)Cl-induced metabolic acidosis was increased 1.5-fold compared with control rats, with no change in inhibitor sensitivity. Western blot analysis indicated that NHE3 protein expression was greater in brush border membranes from acidotic compared with control rats. We conclude that virtually all measured Na\(^{+}\)-H\(^{+}\) exchange activity in brush border membranes from control and acidotic rats is mediated by NHE3 and that metabolic acidosis causes increased expression of renal brush border NHE3 protein.

A major fraction of HCO\(_{3}\) reabsorption in the mammalian proximal tubule is mediated by apical membrane Na\(^{+}\)-H\(^{+}\) exchange (1). Molecular cloning studies have led to the identification of five Na\(^{+}\)-H\(^{+}\) exchanger isoforms, NHE1–5 (2–7). Expression of NHE1–4 but not NHE5 has been detected in the kidney (3–7). Immunocytochemical studies using isoform-specific antibodies have localized NHE3 expression to the apical membrane of cells in the proximal tubule and thick ascending limb of Henle (8, 9) and NHE1 to the basolateral membrane of multiple nephron segments (10). Membrane fractionation studies indicate that NHE4 is also a basolateral isoform in the kidney (11). The localization of NHE2 is presently uncertain.

The aims of the present study were 2-fold. First, we estimated the fraction of brush border Na\(^{+}\)-H\(^{+}\) exchange activity mediated by NHE3 based on the pattern of sensitivity to inhibitors. Second, we evaluated whether the increased brush border Na\(^{+}\)-H\(^{+}\) exchange observed in metabolic acidosis is due to increased expression of NHE3 protein. We found that virtually all measured Na\(^{+}\)-H\(^{+}\) exchange activity in brush border membranes from control and acidotic rats is mediated by NHE3 and that metabolic acidosis causes increased expression of renal brush border NHE3 protein. A preliminary account of this study has been previously published as an abstract (12).

EXPERIMENTAL PROCEDURES

Induction of Metabolic Acidosis—Male Sprague-Dawley rats (240–260 g) were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Metabolic acidosis was induced by administration of 1.5% NH\(_{4}\)Cl in the drinking water and by feeding a 1:1 (mL/g) mixture of 3% NH\(_{4}\)Cl and powdered rat chow (Agway ProLab RML 3200, Syracuse, NY). Animals received this diet for 5 days prior to sacrifice. Pair-fed rats were used as the control group. After anesthesia was induced with intraperitoneal phenobarbital, arterial blood was withdrawn from the bifurcation of the lower abdominal aorta for blood gas measurement, and the kidneys were then harvested for vesicle preparation. As shown in Fig. 1, significant metabolic acidosis was achieved as evidenced by a decline in pH from 7.37 ± 0.01 (n = 35) to 7.17 ± 0.01 (n = 35) and a fall in bicarbonate from 27.0 ± 0.5 to 14.8 ± 0.4.

Isolation of Brush Border Membrane Vesicles and Measurement of \(^{22}\)Na Uptake—On a given day, preparations of brush border vesicles were isolated from the pooled kidney cortices of paired groups of eight acidotic and eight control rats. Renal brush border membrane vesicles were isolated from 1- to 2-mm thickness of outer renal cortex by magnesium aggregation and differential centrifugation as described previously (13), except for the following modifications. To increase yield, the second of the low speed pellets that had been previously discarded was resuspended and centrifuged at 3100 \(\times\) g. The two 3100 \(\times\) g supernatants were then combined for the subsequent high speed centrifugation. Similarly, the third low speed pellet was resuspended and centrifuged at 5700 \(\times\) g and then the two 5700 \(\times\) g supernatants combined for the last high speed centrifugation. The high speed centrifugation steps were performed at 30,000 \(\times\) g. Vesicles were stored at −70°C until subsequent transport or enzyme assays.

Specific activities of the apical membrane marker γ-glutamyl-transpeptidase and the basolateral enzyme Na,K-ATPase were assayed as described (14, 15). As indicated in Fig. 2, brush border vesicles from control and acidotic animals were equivalently enriched in γ-glutamyl-transpeptidase and had a similar degree of contamination with Na,K-ATPase. Measurements of \(^{22}\)Na uptake into microvillus membrane vesicles were performed in triplicate at room temperature (20–22°C) using the rapid filtration technique (16). Membrane vesicles were pre-equilibrated for 120 min at room temperature in 105 mM mannitol, 22 mM KOH, 52 mM Hepes, 32 mM Mes, pH 6.1, or with equimolar replacement of Mes by mannnitol, pH 7.5. Subsequently, in a reaction volume of 50 \(\mu\)L containing 100–250 \(\mu\)g of membrane protein and 0.1 mC of \(^{22}\)Na, the uptake of 1 mM Na\(^{+}\) was assayed either in the presence of 134 mM mannitol, 32 mM KOH, 51 mM KCl, 1 mM NaCl, 42 mM Hepes, 10 mM Mes, pH 7.5, for vesicles that had been preincubated at pH 6.1 or in the presence of 157 mM mannitol, 22 mM KOH, 51 mM KCl, 1 mM NaCl, 42 mM Hepes, pH 7.5, for vesicles that had been preincubated at pH 7.5. Experiments were performed by placing 40 \(\mu\)L of solution containing \(^{22}\)Na in the bottom of a 10-ml test tube and by placing 10 \(\mu\)L of preincubated membrane suspension on the side of the tube just above the solution.
the radioactive solution. The uptake reaction was started by vortex mixing the two droplets together. For inhibitor studies, an additional 25-μl droplet containing inhibitors in 200 mM mannitol, 42 mM KOH, 80 mM Hepes, pH 7.5, was added to the radioactive solution in the bottom of the tube. Inhibitors were initially dissolved in 100% Me₂SO, resulting in a final Me₂SO concentration of 0.2% after dilution into the reaction media. The uptake reaction was stopped by dilution with 3 ml of an ice-cold medium containing 100 mM KCl, 42 mM KOH, 80 mM Hepes, pH 7.5. Vesicles were collected on 0.65-μm Millipore filters (Dawan), and washed with an additional 9 ml of the same ice-cold solution. Intravesicular 23Na was determined by liquid scintillation spectroscopy. Values of solute uptake were corrected for retention of isotope by the Millipore filters when vesicles were diluted with stopping solution prior to the addition of 23Na. A metronome was used for timing of the 4-s uptake period.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting—Aliquots (25 μg) of brush border membranes from control and acidotic rats were solubilized in sample buffer, and the proteins were separated by SDS-polyacrylamide gel electrophoresis using 7.5% polyacrylamide gels according to Laemmli (17). For immunoblotting, proteins were transferred to polyvinylidene difluoride (Immobilon-P, Millipore Corp.) from polyacrylamide gels at 400 mA for 6 h at 4 °C with a Transphor transfer electrophoresis unit ( Hoefer Scientific Instruments, San Francisco, CA) and stained with Ponceau S in 0.5% trichloroacetic acid. Sheets of polyvinylidene difluoride containing transferred protein were incubated first in Blotto (5% nonfat dry milk in phosphate-buffered saline, 1% Tween, pH 7.4) for 2–3 h to block nonspecific binding of antibody, followed by overnight incubation with monoclonal antibody 2B9, an isoform-specific anti-NHE3 antibody (18), diluted 1:500 in Blotto. The sheets were then washed repeatedly in Blotto and incubated for 1 h with secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgG). After additional washing for 1 h, antibody was detected with the ECL chemiluminescence system (Amersham Corp.) according to the manufacturer’s protocols.

After stripping of the first antibody by incubation in 2% SDS, 100 mM β-mercaptoethanol, 50 mM Tris, pH 6.9, for 1 h at 70 °C, polyvinylidene difluoride blots were reprobed with a rabbit polyclonal antiserum against γ-glutamyltranspeptidase (1:5000), kindly provided by Dr. David Castle at the University of Virginia (19). The sheets were then washed in Blotto and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG. Films were scanned and digitized and then band intensities were quantified using a Bio-Image system with Visage applications software.

Materials—25Na (carrier-free) was obtained from Amersham. Amiloride, dimethylamiloride, and ethylisopropylamiloride were purchased from Molecular Probes (Eugene, OR). HOE694 was a gift from Dr. H. Lang, Hoechst Pharmaceuticals (Frankfurt, Germany). Horseradish peroxidase-conjugated goat anti-rabbit and goat anti-mouse IgG were purchased from Zymed Laboratories (San Francisco, CA).

RESULTS

We first evaluated the inhibitor sensitivity of Na⁺-H⁺ exchange in brush border membrane vesicles isolated from normal rats. Na⁺-H⁺ exchange was assayed as the rate of 22Na uptake in the presence of an outward H⁺ gradient (pHᵢ, 6.1; pHₒ, 7.5). As indicated in Fig. 3, Na⁺-H⁺ exchange was markedly inhibited by 100 μM amiloride, dimethylamiloride, and ethylisopropylamiloride but was almost completely resistant to HOE694. As reported by Counillon et al. (20), NHE3 is markedly less sensitive to HOE694 (Kᵢ, 650 μM) than are the isoforms NHE1 (Kᵢ, 0.2 μM) and NHE2 (Kᵢ, 5 μM). Thus, these inhibitor data, along with previous immunochemical studies, suggest that NHE3 is the principal isoform accounting for Na⁺-H⁺ exchange activity in rat renal brush border membrane vesicles.

To define inhibitor sensitivity more precisely, we evaluated the concentration dependence of inhibition of Na⁺-H⁺ exchange by HOE694, amiloride, dimethylamiloride, and ethylisopropylamiloride. Shown in Fig. 4, the rank order and approximate I₅₀ values for inhibition were: ethylisopropylamiloride (6 μM) > dimethylamiloride (10 μM) > amiloride (30 μM) > HOE694. These values are similar to those reported by Counillon et al. for NHE3 (20), further indicating that NHE3 is the isoform accounting for Na⁺-H⁺ exchange activity in renal brush border membrane vesicles.

To evaluate the possible role of NHE3 in the renal adaptation to acidosis, we induced metabolic acidosis in groups of male Sprague-Dawley rats by NH₄Cl administration for 5 days. Additional groups of rats were pair-fed daily as controls. Brush border membrane vesicles were isolated in a paired fashion from groups of acidic and control rats sacrificed on the same day. As shown in Fig. 5, the initial rate (4-s value) of H⁺ gradient-stimulated 22Na uptake was approximately 50% greater in membranes isolated from acidic compared with control animals. Equilibrium values of 22Na uptake were similar, indicating comparable values for intravesicular volume in...
We then evaluated whether the enhanced rate of Na\(^{+}\)-H\(^{+}\) exchange in membranes from acidotic animals was due to expression of an NHE isoform with a different inhibitor sensitivity. Indicated in Fig. 6, we found that the dose-response curves for inhibition by HOE694, amiloride, and ethylisopropylamiloride were identical when membrane preparations from acidotic and control animals were compared. These data indicate that up-regulation of brush border Na\(^{+}\)-H\(^{+}\) exchange in acidosis is due to increased expression of NHE3, the same NHE isoform accounting for base-line transport activity.

To directly measure the expression of NHE3 protein in brush border membranes, we performed immunoblotting using an anti-NHE3 monoclonal antibody. Expression of NHE3 was compared with that of the brush border enzyme \(\gamma\)-glutamyltranspeptidase. In all eight pairs of membrane preparations that were examined, there was a marked increase in NHE3 abundance in brush border membranes isolated from acidotic compared with normal rats, as illustrated in Fig. 7. Abundance of \(\gamma\)-glutamyltranspeptidase was either unchanged or decreased in membranes from acidotic animals, arguing against a nonspecific effect on brush border protein expression.

Shown in Fig. 8, densitometry confirmed the increased abundance of NHE3 relative to \(\gamma\)-glutamyltranspeptidase in brush border membranes of acidotic animals. NHE3 protein expression was increased approximately 3-fold, whereas abundance of \(\gamma\)-glutamyltranspeptidase was decreased. Interestingly, the acidosis-induced increase in NHE3 protein expression was disproportionate to the increase in measured brush border Na\(^{+}\)-H\(^{+}\) exchange activity, suggesting the presence of an inactive or less active subpopulation of transporters under the conditions of these experiments.

**DISCUSSION**

The predominant route for H\(^{+}\) secretion in the proximal tubule is apical membrane Na\(^{+}\)-H\(^{+}\) exchange (1). Among the renal adaptive responses to metabolic acidosis is an increase in renal brush border membrane Na\(^{+}\)-H\(^{+}\) exchange activity (21–25). Although the brush border Na\(^{+}\)-H\(^{+}\) exchanger is allosterically activated by internal H\(^{+}\) (26), such activation cannot account for the enhanced Na\(^{+}\)-H\(^{+}\) exchange activity in brush border vesicles isolated from acidic compared with control animals because these studies were performed in vitro using constant intravesicular pH. Possible explanations for the intrinsic adaptation leading to increased Na\(^{+}\)-H\(^{+}\) exchange ac-

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**Fig. 4.** Dose response relations for inhibition of Na\(^{+}\)-H\(^{+}\) exchange. Results represent mean ± S.E. for 8 membrane preparations. EIPA, ethylisopropylamiloride; DMA, dimethylamiloride.

**Fig. 5.** Rates of Na\(^{+}\)-H\(^{+}\) exchange in vesicles isolated from control and acidotic rats. \(^{22}\)Na uptake was assayed in the absence (pH\(_{i}\), 7.5; pH\(_{o}\), 7.5) or presence (pH\(_{i}\), 6.1; pH\(_{o}\), 7.5) of an outward H\(^{+}\) gradient. Values are normalized to the 2 h uptake value measured in the absence of a pH gradient for each control membrane preparation. Results represent mean ± S.E. for 8 pairs of membrane preparations.

**Fig. 6.** Inhibitor sensitivity of Na\(^{+}\)-H\(^{+}\) exchange in vesicles isolated from control and acidotic rats. Results represent mean ± S.E. for 8 pairs of membrane preparations. EIPA, ethylisopropylamiloride.

**Fig. 7.** Abundance of NHE3 and \(\gamma\)-glutamyltranspeptidase (\(\gamma\)-GT) in vesicles isolated from control and acidotic rats. Results shown represent eight pairs of membrane preparations from normal (N) and acidic (A) rats.
tivity are an increased turnover rate with no change in transporter protein expression and/or increased expression of transporter protein. Increased expression of transporter protein could, in turn, be due to an increment in the abundance of the same isoform mediating Na\(^+\)-H\(^+\) exchange under control conditions or to enhanced expression of a different isoform.

Indeed, cloning studies have identified five Na\(^+\)-H\(^+\) exchanger isoforms, NHE1–5 (2–7). Expression of NHE1–4 but not NHE5 has been detected in the kidney (3–7). Immunocytochemical studies have localized NHE3 to the brush border membrane of proximal tubule cells (8, 9) and to the apical membrane of the thick ascending limb of Henle (9). In contrast, NHE1 is expressed along the basolateral membrane of multiple nephron segments (10), and membrane fractionation studies indicate that NHE4 is also a basolateral isoform in the kidney (11). Thus, NHE3 is the leading candidate to mediate brush border membrane Na\(^+\)-H\(^+\) exchange activity, although NHE2 may also be expressed at this site (27).

An important distinguishing feature among the NHE isoforms is their unique patterns of sensitivity to inhibition by amiloride analogs and HOE694 (20). Our finding that brush border membrane Na\(^+\)-H\(^+\) exchange activity is relatively resistant to amiloride analogs and is completely insensitive to inhibition by 100 μM HOE694 strongly supports the conclusion that NHE3 is the principal isoform accounting for transport activity in these membranes. Moreover, because the pattern of inhibitor sensitivity was unchanged in acidosis, the acidosis-induced increment in Na\(^+\)-H\(^+\) exchange activity also must have been due to NHE3.

To evaluate the mechanism underlying the observed increase in functional activity of NHE3, we estimated the expression of NHE3 protein by immunoblotting. Similar to the results of Ambühl et al. (25), we found that NHE3 protein expression was significantly increased in metabolic acidosis. It is interesting to note that metabolic acidosis does not cause a change in NHE3 mRNA abundance (25). Accordingly, the acidosis-induced increase in NHE3 protein expression most likely arises at the post-transcriptional level, involving an alteration in translation, processing, or degradation.

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