Metabolic acidosis in rats increases intestinal NHE2 and NHE3 expression and function

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ALVARO LUCIONI, CHRISTOPHER WOMACK, MARK W. MUSCH, FLAVIO L. ROCHA, CRES BOOKSTEIN, AND EUGENE B. CHANG. Metabolic acidosis in rats increases intestinal NHE2 and NHE3 expression and function. Am J Physiol Gastrointest Liver Physiol 283: G51–G56, 2002. First published March 6, 2002; 10.1152/ajpgi.00529.2001.—Chronic metabolic acidosis increases intestinal Na absorption, although through undefined mechanisms. Whether this occurs through enhanced expression and/or function of the brush-border Na+/H+ exchangers (NHE2 and NHE3 is unknown. Metabolic acidosis was induced in rats by feeding ammonium chloride through their drinking water. Intestinal NHE activities were measured using brush-border 22Na uptake. Western and Northern blots measured changes in protein and mRNA expression, respectively. Acidosis occurred within 2 days of ammonium chloride feedings but increased after 6 days. NHE2 and NHE3 activities, protein expression, and mRNA levels increased in acidic rats compared with controls. In contrast, basolateral NHE1 expression was not affected. Brush-border alkaline phosphatase showed no effect of metabolic acidosis on cellular differentiation. This study demonstrated a direct effect of metabolic acidosis on NHE2 and NHE3 activity, expression, and gene transcription. Metabolic acidosis is one of the few circumstances shown to affect NHE2 function and expression, thus providing insights into the role of NHE2 on intestinal physiology.

Although it is well known that chronic metabolic perturbations in systemic acid-base balance affect Na absorptive functions of the gut, the cellular mechanisms mediating these changes are incompletely characterized (3–5). In vivo studies (6, 13), for instance, have shown that alterations in pH, Pco2, and Hco3− may affect colonic Na+ and Cl− absorption. The fact that one of the major pathways for NaCl absorption in the gastrointestinal tract is mediated by Na+/H+ exchanger (NHE) and the Cl-/HCO3− exchanger suggests a possible link between acid-base balance and NaCl absorption. Studies using specific inhibitors of these exchangers corroborate the presence of this link. The effect of increased CO2 on NaCl absorption is significantly reduced when amiloride is used to inhibit NHE2 (16). To better understand the link between chronic metabolic acidosis and NaCl absorption, Charney and colleagues (10, 11) performed studies showing that changes in Pco2 cause an increase in NaCl absorption regardless of the HCO3− or pH.

Changes in NHE activity may also occur with acute changes in acid-base parameters. This is immediately apparent, because the H+ gradient leaving the cell is a major determinant of exchanger activity. Additionally, cellular mechanisms exist that may alter pH microdomains and may thus regulate NHE activity. Changes in pH in this microdomain (not total changes in cellular pH) is what causes apical NHE to respond (2). This pH microdomain may be regulated by Pco2 and HCO3−. Carbonic anhydrase II (CA II) is found predominantly in the subapical domain of colonic cells (18). Studies (15) performed on erythrocytes show the presence of an interaction between CA II and the erythrocyte membrane, suggesting a possible role of CA II in directing the generated H+ toward the NHE so that it can be excreted from the cell. The effect of CA II on NaCl absorption varies in different tissues. For example, inhibition of CA II in renal cortical cells has no effect on NHE activity, whereas inhibition of this enzyme in colonic tissue does affect the function of the exchanger (11).

Six different NHE isoforms have been cloned and described in the literature (23, 24). Of these six isoforms, three have been shown to be present in the small and large bowel: NHE1, NHE2, and NHE3. The former isoform is believed to perform housekeeping duties, such as maintenance of intracellular pH, cell volume, and cell proliferation (12, 22). In the intestine, NHE2 and NHE3 have only been identified on the brush-border membrane and are believed to be the major nonnutrient-dependant sodium-absorptive pathway in the intestine (9).

Although NHEs have been suggested to be responsible for the increase in intestinal NaCl absorption during chronic acidic conditions, there are no studies showing a direct effect of acidosis on intestinal NHE activity and protein expression. On the other hand, in

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the kidney, chronic metabolic acidosis has been shown to enhance NHE3 activity and protein expression in the thick ascending limb (26). This study, therefore, examined the effect of chronic metabolic acidosis on intestinal NHE2 and NHE3 expression and function. We show that chronic metabolic acidosis leads to an upregulation of intestinal NHE2 and NHE3 activity and expression, effects paralleled by increased NHE2 and NHE3 mRNA production. In contrast, no changes in NHE1 expression were noted.

MATERIALS AND METHODS

Experiments used in this study were approved by the University of Chicago Institutional Animal Care and Use Committee.

Induction of metabolic acidosis in rats. Rats were fed ammonium chloride (5% wt/vol) in their drinking water for 2 or 6 days. To confirm acidosis, blood gases were measured immediately before death from blood taken from the left ventricle. Blood gases were always immediately analyzed.

Apical membrane unidirectional \( { }^{22}\text{Na} \) influx as a measure of NHE activity. Activity of NHE2 and NHE3 in the brush-border membranes was measured as previously described (7, 8). Briefly, ileal and colonic mucosal scrapings were weighed and added to 30 ml hypotonic lysis buffer (10 mM Tris, pH 7.4, and 3 mM EDTA with protease inhibitors as described previously) and homogenized for 30 s at a speed of 15,000 rpm in an Ultra-Turrax homogenizer. Samples were taken for enzyme enrichment studies and were spun at 2,000 g for 5 min at 4°C to remove nuclei and unbroken cells. Supernatants were removed and spun at 10,000 g for 10 min at 4°C to remove mitochondria. Supernatants were removed, and 15 mM CaCl2 was added. Samples were gently stirred at 4°C for 15 min and then spun at 8,000 g for 8 min to remove the endoplasmic reticulum, Golgi, and basolateral membranes. Supernatants were spun at 45,000 g for 45 min at 4°C to obtain brush-border membranes. Membranes were resuspended in a small volume of intravesicular transport buffer (in mM): 10 HEPES, pH 7.4, 80 mannitol and then resuspended using a Teflon pestle homogenizer. A sample was removed for protein determination and enzyme enrichment studies. Five microliters of the vesicles were added to 45 \( \mu l \) of extravesicular transport buffer (in mM): 10 HEPES, pH 7.4, 80 mannitol, and 1 Na with \( { }^{22}\text{Na} \) (1 \( \mu Ci/ml \) giving a specific activity of 2,200 disintegrations per minute/nmol Na). All brush-border uptakes were of a 10-s duration. Uptake of Na into the vesicles was stopped by the addition of 2-ml ice-cold 90 mM mannitol and immediate placement onto a 0.45-\( \mu \)m cellulose filter (HAWP; Millipore, Milford, MA). The filter was washed once with 4-ml ice-cold 90 mM mannitol, and the filter was removed and solubilized in liquid scintillation fluid. \( { }^{22}\text{Na} \) was determined by liquid scintillation spectroscopy. For the present experiments, \( { }^{22}\text{Na} \) uptakes were always performed under three conditions: 1) no amiloride addition, 2) HOE-694 (30 \( \mu M \)), and 3) dimethylamiloride (DMA; 500 \( \mu M \)). HOE-694 and DMA were used to distinguish NHE2 and NHE3 activities. With the use of 1 mM Na, NHE2 is completely inhibited by the amiloride analog HOE-694 at 30 \( \mu M \), whereas NHE3 is inhibited <5% (20). Both exchangers are sensitive to DMA. Therefore, the total flux minus the flux after HOE-694 inhibition provided NHE2 activity, and the flux after DMA minus the flux after HOE-694 yielded the NHE3 activity. Fluxes in experimental rats were expressed as a percentage of the flux in untreated control rats.

Western blotting. Brush-border membrane vesicles (BBMV) isolated as above were resuspended in homogenization buffer (10 mM Tris, 5 mM MgSO4, 5 U/ml RNase, 50 U/ml DNAse, 10 \( \mu g/ml \) leupeptin, 10 \( \mu g/ml \) aprotonin, and 1 mM phenylmethylsulfonyl fluoride). An aliquot was removed for protein determination, and the remaining cell protein was then solubilized in Laemml stop buffer. Proteins were separated by 7.5% SDS-PAGE and transferred to the polyvinylidene difluoroide membranes. After blocking with Tris-buffered saline (TBS)/0.1% Nonidet P-40 (NP-40)/5% milk, blots were incubated with primary antibody diluted in TBS/1% BSA overnight at 4°C. Blots were incubated with specific polyclonal antisera to NHE2 and NHE3, developed and characterized by our lab (1, 20). After three washes with TBS/0.1% NP-40/5% milk and once with TBS/0.1% NP-40/1% milk, blots were incubated with horseradish peroxidase-conjugated secondary antibody diluted in TBS/0.1% NP-40/1% milk for 1 h at 25°C. After three washes with TBS/0.1% NP-40/1% milk and once with TBS/0.1% NP-40, the membrane was developed using an enhanced chemiluminescence system. For rat intestinal scrapings, NHE2 and NHE3 protein levels were analyzed by taking an aliquot of the brush-border membranes used for flux studies. The brush-border proteins were solubilized in Laemml stop solution and resolved on a 7.5% SDS-PAGE, and Western blots were performed as above.

Northern blotting. For RNA isolation, intestinal scrapings were homogenized in Trizol. RNA was then extracted once with acid phenol-chloroform, reprecipitated, and quantified by absorbance at 260 nm. Twenty micrograms of intestinal total RNA were size-separated on a denaturing formaldehyde agarose gel, transferred to a positively charged nylon membrane by capillary action, and RNA linked to the membrane by ultraviolet light. Blots were prehybridized and hybridized in XOTCH (7% wt/vol SDS, 1% wt/vol albumin, 200 mM NaH2PO4, and 10 mM EDTA with 15% vol/vol deionized formamide) solution as previously described (1) by the use of cDNA probes for rat NHE2 and NHE3. Glyceraldehyde phosphate dehydrogenase was used as a constitutive control. Probes were labeled with \([^{32}P]dCTP\) by random prime labeling. Blots were hybridized at 55°C overnight and then washed to a stringency of 0.5× SSC/0.5% SDS at 55°C.

Effect of acidosis on differentiation. To determine whether acidosis had effects on the differentiation of the C2 cells or the rat intestine, activities of the brush-border enzymes sucrase and alkaline phosphatase were measured. Additionally, levels of the microvillus structural protein villin were determined by Western blotting.

RESULTS

Metabolic acidosis in vivo induces NHE2 and NHE3. Rats were made acidic by addition of \( \text{NH}_4\text{Cl} \) (5% wt/vol) in their drinking water, confirmed by measurements of blood gases and \( \text{HCO}_3^- \) (see blood pH, \( \text{PCO}_2 \), and \( \text{HCO}_3^- \) in Table 1). Acidosis was clearly present within 2 days (blood pH 7.36 ± 0.04 vs. 7.30 ± 0.03, \( \text{PCO}_2 \) 35.6 ± 1.1 vs. 34.2 ± 1.4) but was greater by 6 days (Table 1).

To determine whether there were functional changes in absorptive NHE transport pathways, intestinal tissues were harvested from these rats, and brush-border membrane activities of NHE2 and NHE3 were determined. By 2 days, increased activities of both NHE2 and NHE3 were observed in acidic rats compared with the control group in all sections of the intestinal tract analyzed (Fig. 1). These changes were greater at
6 days compared with 2 days (Fig. 2). Because the expression of NHE2 and NHE3 is maturation (villus cell) dependent (1), the differentiation parameter alkaline phosphatase was measured at the maximal time (6 days) of NHE activity increase. No changes in alkaline phosphatase activity were observed (Fig. 3). This suggests that alterations in maturation of the intestinal epithelial cells were not responsible for the increased NHE activities, but the latter are a specific effect of the metabolic acidosis.

Increased NHE2 and NHE3 protein levels. Because activity increases could be due to either increased

### Table 1. Blood values of control and acidotic rat

<table>
<thead>
<tr>
<th>Group</th>
<th>pH</th>
<th>Pco2, Torr</th>
<th>[HCO₃]⁻, mM</th>
<th>[Na], mM</th>
<th>[K], mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.37 ± 0.05</td>
<td>35.7 ± 1.2</td>
<td>24.6 ± 0.7</td>
<td>144.7 ± 0.8</td>
<td>4.5 ± 0.3</td>
</tr>
<tr>
<td>Acidotic</td>
<td>7.31 ± 0.04</td>
<td>33.8 ± 1.1</td>
<td>20.7 ± 0.9*</td>
<td>146.2 ± 0.6</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>Acidotic</td>
<td>7.24 ± 0.07*</td>
<td>32.4 ± 1.8*</td>
<td>17.8 ± 1.2†</td>
<td>145.2 ± 0.6</td>
<td>4.0 ± 0.2*</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4 rats in each group. *P < 0.05, †P < 0.01 compared with control mean by paired Student's t-test. § Rats fed ammonium chloride for 2 days; ‡ rats fed ammonium chloride for 6 days. Brackets denote concentration.

Fig. 1. Effect of 2-day metabolic acidosis on brush-border membrane Na⁺/H⁺ exchanger (NHE2 (top) and NHE3 (bottom) activities. Rats were fed 5% (wt/vol) NH₄Cl in their drinking water (acidotic) or regular drinking water (control). Brush-border membrane vesicles (BBMV) were isolated and ²²Na fluxes performed as described in MATERIALS AND METHODS. Values shown are means ± SE for 6 rats. *P < 0.05, †P < 0.01 compared with control by paired Student's t-test.

Fig. 2. Effect of 6-day metabolic acidosis on brush-border membrane NHE2 (top) and NHE3 (bottom) activities. Rats were fed 5% (wt/vol) NH₄Cl in their drinking water (acidotic) or regular drinking water (control). BBMV were isolated and ²²Na fluxes performed as described in MATERIALS AND METHODS. Values shown are means ± SE for 6 rats. *P < 0.05, †P < 0.01 compared with control by paired Student's t-test.
activity of the transporters or increased protein levels, levels of NHE2 and NHE3 were measured in ileal and colonic brush-border membranes at the maximal change in NHE activities (6 days). As shown in Fig. 4, NHE2 and NHE3 protein levels were increased. The images from all rats were analyzed by scanning densitometry by NIH Image 1.45 software. At 6 days, ileal NHE2 increased 65\% and NHE3 increased 89\% (both \(P < 0.01\) by paired Student's t-test). Colonic NHE2 increased 103.4\% and NHE3 increased 95.5\% (both \(P < 0.01\) by paired Student's t-test).

Increases in protein correlated well with the functional changes in NHE2 and NHE3 activity in both tissues. In contrast, no changes were noted in expression of NHE1 in basolateral membranes from acidotic rats (Fig. 4).

NHE2 and NHE3 are increased by transcriptional activation. Increased levels of NHE2 and NHE3 protein could be due to stabilization of protein, levels of the mRNA, or increased transcription of NHE2 and NHE3 genes. To examine these possibilities, mRNA was isolated from ileal and colonic mucosa 6 days after induction of acidosis. Northern blots were probed first with NHE2 probe (a Pst1 cut of bases 260-3598 of the coding region), next with NHE3 probe (a full-length 3.6 kb coding region cDNA), and last with a murine glyceraldehyde-3-phosphate dehydrogenase probe (GAPDH; full-length coding region). A representative image is shown in Fig. 5. Levels of both NHE2 and NHE3 mRNA increased in both ileal and colonic regions. Increases in NHE2 were 97\% in ileum and 116.5\% in colon (both \(P < 0.05\) by paired Student's t-test). NHE3 increases were 82.9\% in ileum and 84.2\% in colon (both \(P < 0.05\) by paired Student's t-test). Percent increases in mRNA were similar to those of the protein levels and activity, suggesting that transcriptional control was a major determinant of the increased NHE in the acidotic rats. No changes in GAPDH were appreciated, suggesting no difference in loading.
DISCUSSION

Past studies (1, 16) have shown that NHEs are responsible for the increase in NaCl absorption during chronic metabolic acidosis; however, this study is the first to report a direct effect of chronic metabolic acidosis on increasing intestinal NHE2 and NHE3 activity and protein expression. Furthermore, this commensurate increase in NHE mRNA expression suggests that this effect is due to increased gene transcription, although posttranslational modifications of the protein that may affect its stability and activity cannot be ruled out. Additionally, changes in mRNA could also be due to changes in mRNA stability rather than increased transcription. In this case, the effect would have to be specific, because no changes in the housekeeping gene GAPDH were observed. At the protein level, the effect of chronic metabolic acidosis on NHE2 and NHE3 activity and protein expression occurs both in the small and large intestine, but upregulation seems to be more pronounced in the latter. This is consistent with past studies that suggest a stronger effect of acid-base balance on colonic transport function.

Little is known about the chronic regulation of NHE2 and NHE3, but NHE3 has been found to respond to a variety of physiological stimuli, such as glucocorticoids, aldosterone, and other neurohormones released after meals (28). Significantly more is known about the acute regulation of NHE2 and NHE3, which may be regulated by various signal transduction pathways that involve protein kinase C, cAMP, cGMP, intracellular calcium (20), or accessory proteins, such as NHE regulatory factor, and E3KARP (17, 29). This short-term regulation may involve processes such as phosphorylation/dephosphorylation of the transporter and possibly the presence of a subapical pool and an active pool of NHE3 in the apical membrane of the cell and exocytosis of exchangers (21).

Our studies, however, focus on changes in NHEs that occur over days and are therefore chronic, rather than acute regulation. Increases of intestinal Na transport have been measured after chronic metabolic acidosis, and our studies demonstrate that this is due to upregulation of function and expression of the apical NHE2 and NHE3. Certain studies (19) in rat intestine have suggested cellular hypertrophy as part of the adaptation process in metabolic acidosis. In vitro studies (14), on the other hand, show no hypertrophic changes after exposure to acidic media. We did not observe increased brush-border protein expression in our studies (as determined by milligrams of brush-border protein isolated per length, data not shown) nor was the gut grossly hypertrophied after 6-day induction of metabolic acidosis. However, it is possible that subtle adaptive changes in the morphology or number of cells could be occurring. Our studies are relevant here, because in isolated brush borders, we observed increased function and expression of NHE2 and NHE3 but no change in alkaline phosphatase, suggesting a specific upregulation of function in response to chronic metabolic acidosis. Intestinal cells could adapt to chronic metabolic acidosis by increasing their individual capacity to buffer acid. In vitro studies with OKP cells, cells derived from proximal tubules of the opossum kidney, have shown that inhibition of tyrosine kinase prevented the activation of NHE3 activity after acid incubation. Furthermore, this study also stipulates that inactivation of c-src nonreceptor protein tyrosine kinase by overexpression of COOH-terminal src kinase also inhibits the upregulation in NHE3 activity. The role of tyrosine kinase in rat intestine is yet to be determined (27). It should be noted that even after 2 days of ammonium chloride feeding, both NHE2 and NHE3 increased. Changes in blood gases and electrolytes were not as large as at 2 days as at 6 days; however, a clear trend toward metabolic acidosis was observed (Table 1). After 2 days of ammonium chloride feedings, blood pH did not change significantly but HCO₃⁻ levels did change significantly. During acidic conditions excess H⁺ is rapidly buffered by HCO₃⁻, ameliorating any changes in blood pH. Thus although one does not observe changes in blood pH by 2 days of ammonium chloride feeding, renal and respiratory compensatory mechanism may already be activated.

The regulation of NHE2 is understood to a lesser degree than the regulation of NHE3. Only limited studies have demonstrated changes in NHE2 function and expression, while NHE3 has been found to respond to glucocorticoids and mineralocorticoids (6, 7) and, as previously discussed, acidosis, although in a renal cell system. Thus having found that metabolic acidosis leads to an upregulation in intestinal NHE2 function and expression may provide important clues into NHE2 regulation. Further studies would provide understanding of the NHE2 physiological role and the intracellular processes that regulate its function.

In summary, this study demonstrates a direct effect of chronic metabolic acidosis on NHE2 and NHE3 activity, expression, and gene transcription. Further studies are necessary to determine both the short- and long-term regulation of intestinal NHEs after metabolic acidosis and the signaling pathways that may be responsible for the activation of NHE and increase in gene transcription.

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Acidosis induces intestinal Na\(^+\)/H\(^+\) exchange


