Mechanism of proximal tubule bicarbonate absorption in NHE3 null mice

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Wang, Tong, Chao-Ling Yang, Thecla Abbati, Patrick J. Schultheis, Gary E. Shull, Gerhard Giebisch, and Peter S. Aronson. Mechanism of proximal tubule bicarbonate absorption in NHE3 null mice. Am. J. Physiol. 277 (Renal Physiol. 46): F298–F302, 1999.—NHE3 is the predominant isoform responsible for apical membrane Na+/H+ exchange in the proximal tubule. Deletion of NHE3 by gene targeting results in an NHE3−/− mouse with greatly reduced proximal tubule HCO3− absorption compared with NHE3+/+ animals (P. J. Schultheis, L. L. Clarke, P. Meneton, M. L. Miller, M. Soleimani, L. R. Gawenis, T. M. Riddle, J. J. Duffy, T. Doetschman, T. Wang, G. Giebisch, P. S. Aronson, J. N. Lorenz, and G. E. Shull. Nature Genet. 19: 282–285, 1998). The purpose of the present study was to evaluate the role of other acidification mechanisms in mediating the remaining component of proximal tubule HCO3− reabsorption in NHE3−/− mice. Proximal tubule transport was studied by in situ microperfusion. Net rates of HCO3− (JHCO3) and fluid absorption (JF) were reduced by 54 and 63%, respectively, in NHE3 null mice compared with controls. Addition of 100 µM ethylisopropylamiloride (EIPA) to the luminal perfusate caused significant inhibition of JHCO3 and JF in NHE3+/+ mice but failed to inhibit JHCO3 or JF in NHE3−/− mice, indicating lack of activity of NHE2 or other EIPA-sensitive NHE isoforms in the null mice. Addition of 1 µM bafilomycin caused a similar absolute decrement in JHCO3 in wild-type and NHE3 null mice, indicating equivalent rates of HCO3− absorption mediated by H+-ATPase. Addition of 10 µM Sch-28000 did not reduce JHCO3 in either wild-type or NHE3 null mice, indicating lack of detectable H+-K+-ATPase activity in the proximal tubule. We conclude that, in the absence of NHE3, neither NHE2 nor any other EIPA-sensitive NHE isoform contributes to mediating HCO3− reabsorption in the proximal tubule. A significant component of HCO3− reabsorption in the proximal tubule is mediated by bafilomycin-sensitive H+-ATPase, but its activity is not significantly upregulated in NHE3 null mice.

A major fraction of proximal tubule HCO3− reabsorption is mediated by apical membrane Na+/H+ exchange (1, 4). Molecular cloning studies have shown that at least four Na+/H+ exchanger (NHE) isoforms are expressed in the mammalian kidney (23, 26, 30, 31). Immunocytochemical studies using isoform-specific antibodies have indicated that, whereas NHE1 and NHE4 have a basolateral distribution (6, 9, 24), NHE2 and NHE3 are located along the apical membranes of various nephron segments, including the proximal tubule (3, 5, 7, 10, 28, 29, 39, 40). Analysis of inhibitor sensitivity has suggested that virtually all of the measured Na+/H+ exchange activity in isolated renal cortical brush-border membrane vesicles is mediated by NHE3 (38).

Direct evidence for the functional importance of NHE3 is that proximal tubule HCO3− reabsorption is reduced by ∼60% in NHE3 null mice (27). However, the mechanisms accounting for the remaining component of HCO3− reabsorption in the proximal tubules of NHE3 null mice are not known. Possible mechanisms include participation of another apical NHE isoform, such as NHE2, or of primary active H+ transport pathways, such as H+-ATPase and H+-K+-ATPase. The aims of the present study are to evaluate the relative contributions of these possible mechanisms to mediating HCO3− reabsorption in the proximal tubules of NHE3 null mice.

METHODS

Animals and surgical preparation. Knockout mice deficient in NHE3 were generated by targeted gene disruption (27). Genotype analysis of tail DNA was performed by PCR. Heterozygous wild-type (NHE3+/−) and null (NHE3−/−) mice resulting from breeding of heterozygotes were maintained on a regular diet and tap water until the day of the experiment. Ages of mutant animals were matched with their wild-type controls. The mice were anesthetized by intraperitoneal injection of 100 mg/kg body wt Inactin [5-ethyl-5-(L-methylpropyl)-2-thiobarbituric acid; BYK-Gulden, Konstanz, Germany] and were placed on a thermostatically controlled surgical table to maintain body temperature at 37°C. After tracheotomy, the left jugular vein was exposed and cannulated with a PE-10 catheter for intravenous infusion. A carotid artery was also catheterized with PE-10 tubing for arterial blood collection for blood gas analysis and for measurement of mean arterial pressure. Blood gas analysis was performed on freshly drawn blood by use of a Corning Blood Gas Analyzer.

Microperfusion of proximal tubules in situ. After surgical preparation, saline solution (0.9%) was infused at a rate of 0.15 ml/h (1/10 of the infusion rate used in rat). The left kidney was exposed by lateral abdominal incision, carefully isolated, and immobilized in a special kidney cup filled with light mineral oil (37°C). The kidney surface was illuminated by a fiber optical light. The details of the method for microperfusion of proximal tubules in vivo were described previously for the rat (35). Briefly, a proximal convoluted tubule with three to five loops on the kidney surface was selected and perfused at a rate of 15 nl/min with a proximal oil block. The perfusion
solution contained 20 µCi/ml of low-sodium [methoxy-\(^{3}H\)]inulin for measuring volume absorption and 0.1% FD&C green dye for identification of the perfused loops. Tubule fluid collections were made downstream with another micropipette with distal oil block. One collection was made in each perfused tubule, and two to four collections were taken in each kidney. The perfused tubules were marked after collection with sudan black heavy mineral oil. To determine the length of the perfused segment, tubules were filled with high-viscosity microfil (Canton Bio-Medical Products, Boulder, CO), the kidney was partially digested in 20% NaOH, and silicone rubber casts of the tubule segments were dissected.

Measurement of net HCO\(_3\)- and fluid absorption. The rates of net HCO\(_3\)- and fluid (Jv) absorption were calculated based on changes in the concentrations of [\(^{3}H\)]inulin and total CO\(_2\) as described previously (35). The total CO\(_2\) concentrations in both initial and collected fluids were measured by a microcalorimetric (Picapnotherm) method (35). Jh CO\(_3\) and Jv were expressed per millimeter tubule length. The composition of the perfusion solution was the same as used previously in the rat (36) (in mM): 115 NaCl, 25 NaHCO\(_3\), 4 KCl, 1 CaCl\(_2\), 5 sodium acetate, 2.5 NaHPO\(_4\), 0.5 NaH\(_2\)PO\(_4\), 5 \(L\)-alanine, and 5 glucose. Solutions were bubbled at room temperature with \(5\%\) CO\(_2\)-95% O\(_2\) gas mixture before use. The pH was titrated to 7.4 with NaOH or HCl as required.

Statistics. Data are presented as means ± SE. Student’s t-test was used when a single experimental group was compared with a control group (Table 1). Several experimental groups were compared with a control group (Table 2) by use of Dunnett’s test. Differences were considered significant at P < 0.05.

**RESULTS**

Blood gas analysis, as shown in Table 1, indicated that NHE3 null mice have a mild to moderate metabolic acidosis with reduction in arterial blood HCO\(_3\)- concentration from 25.7 to 20.4 mM and reduction of pH from 7.34 to 7.18. A moderate respiratory acidosis was also noted in both sets of animals (Pco\(_2\), 50–55), most likely secondary to anesthesia. The mild metabolic acidosis in NHE3 null mice confirms previous results (27).

As indicated in Table 2 and Fig. 1, Jh CO\(_3\) and Jv were reduced by 54 and 63%, respectively, in NHE3 null mice compared with controls. These findings confirm previous results indicating a major role of NHE3 in mediating proximal tubule HCO\(_3\)- and fluid absorption (27). Clearly, a significant fraction of Jh CO\(_3\) persists in the NHE3 null mice, indicating contributions from alternative acidification processes. We therefore used inhibitors to investigate the mechanisms mediating the remaining Jh CO\(_3\) and Jv.

To investigate whether any other NHE isoform such as NHE2 contributes to the remaining component of Jh CO\(_3\) in NHE3 knockout mice, we examined the effects of the Na\(^{+}\)/H\(^{+}\) exchange inhibitor ethylisopropylamiloride (EIPA). This inhibitor was added to the lumen perfusate at a concentration of 100 µM. As shown in Table 2 and Fig. 1, EIPA significantly decreased Jh CO\(_3\) and Jv by 40 and 47%, respectively, in wild-type mice. These results are similar to previous studies in rat proximal tubule (25, 35). In contrast, EIPA did not reduce either Jh CO\(_3\) or Jv in NHE3 knockout mice. These results indicate that other NHE isoforms such as NHE2 do not play a role in mediating HCO\(_3\)- absorption in the absence of NHE3. It may be noted that EIPA did not reduce Jh CO\(_3\) and Jv in the wild-type mice to the levels observed in the knockout mice. This can be explained by the known resistance of NHE3 to amiloride analogs in the presence of physiological Na\(^{+}\) concentrations (12, 18, 25, 35), resulting in incomplete inhibition of NHE3 activity in the wild-type mice.

To evaluate the contribution of the H\(^{+}\)-ATPase to mediating HCO\(_3\)- absorption in the proximal tubules of wild-type and NHE3 null mice, we studied the effect of 1 µM bafilomycin A1 (17, 32, 34). The results in Table 2 and Fig. 1 show that bafilomycin decreased Jh CO\(_3\) in wild-type mice by 22%, indicating that, under physiological conditions, a significant fraction of proximal tubule HCO\(_3\)- absorption is mediated by the H\(^{+}\)-ATPase. This result is also consistent with previous findings that a similar fraction of HCO\(_3\)- absorption in the proximal tubule of the rat is mediated by a Na\(^{+}\)-independent and/or bafilomycin-sensitive mechanism (11, 32).

Fractional inhibition of Jh CO\(_3\) by bafilomycin was far greater (59%) in NHE3 null mice compared with wild-type controls, indicating that the major fraction of the Jh CO\(_3\) that persists in the absence of NHE3 activity is mediated by the H\(^{+}\)-ATPase. However, the absolute decrement in Jh CO\(_3\) (25 vs. 30 pmol·min\(^{-1}\)·mm\(^{-2}\)) was virtually the same in NHE3 knockout and wild-type animals, indicating a lack of significant compensatory upregulation of H\(^{+}\)-ATPase in the NHE3 null mice. Interestingly, although there was a trend for bafilomycin to reduce Jv, this inhibition did not reach statistical significance in either wild-type or NHE3 null mice.

Finally, we studied the effect of the inhibitor SCH-28080 to assess the role of H\(^{+}\)-K\(^{+}\)-ATPase (14) in mediating proximal tubule HCO\(_3\)- and fluid absorption in wild-type and NHE3 null mice. The data in Table 2 and Fig. 1 demonstrate that 10 µM SCH-28080 failed to inhibit Jh CO\(_3\) and Jv in either wild-type or NHE3 knockout mice. These findings indicate that H\(^{+}\)-K\(^{+}\)-ATPase activity does not contribute significantly to proximal tubule HCO\(_3\)- absorption in wild-type or NHE3 null mice.

**DISCUSSION**

We have evaluated the relative contributions of apical \(H^{+}\) extrusion mechanisms to mediating HCO\(_3\)- reabsorption in the proximal tubules of NHE3 null mice. First, we confirmed the previous findings (27)
that a large fraction (50–60%) of HCO₃⁻ and fluid absorption in the microperfused mouse proximal tubule is dependent on the operation of the apical NHE isoform NHE3. These findings are also in accord with free-flow micropuncture data indicating marked impairment of fluid reabsorption in the proximal tubule of NHE3 null mice (20).

Second, we could detect no EIPA-sensitive component of HCO₃⁻ absorption in the proximal tubules of NHE3 null mice. Because NHE2 is more sensitive to amiloride analogs than is NHE3 (12), these findings indicate that NHE2 does not appreciably contribute to HCO₃⁻ absorption, although its expression has been detected in the proximal tubule (39, 40).

Third, we found a significant component of HCO₃⁻ absorption mediated by bafilomycin-sensitive H⁺-ATPase in the proximal tubules of both wild-type and NHE3 null mice, consistent with previous demonstrations of Na⁺-independent and/or bafilomycin-sensitive acidification in this nephron segment (11, 13, 32, 33). The contribution of apical membrane H⁺-ATPase to proximal tubule acidification is also supported by studies of H⁺ transport in isolated brush-border vesicles (17), the observation of Na⁺-independent acid extrusion from intact cells (19, 34), and the expression of H⁺-ATPase on the apical membrane as detected by immunostaining (8). However, we detected no upregulation of the bafilomycin-sensitive component of HCO₃⁻ absorption in the proximal tubule of the NHE3 null mice.

Fourth, we were unable to detect a component of Sch-28080-sensitive HCO₃⁻ absorption, arguing against a significant role for Sch-28080-sensitive K⁺-ATPase in mediating proximal tubule acidification in either wild-type or NHE3 null mice, although Sch-28080-sensitive K⁺-ATPase activity has been detected in this nephron segment (41). It should be noted that the concentration of Sch-28080 used in our experiments, 10 µM, should have been sufficient to inhibit at least 80% of the K⁺-ATPase activity that had been identified in the proximal tubule (41).

A component of proximal tubule HCO₃⁻ reabsorption (21 pmol·min⁻¹·mm⁻¹) persisted in the NHE3 null mice in the presence of bafilomycin (see Table 2 and Fig. 1).

Table 2. Fluid and bicarbonate absorption in proximal tubules of wild-type and NHE3 null mice

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Vᵣ, nl/min</th>
<th>L, mm</th>
<th>[HCO₃]ₒ, mM</th>
<th>[HCO₃]ᵢ, mM</th>
<th>Jᵥ, nl·min⁻¹·mm⁻¹</th>
<th>Jₑ[HCO₃], pmol·min⁻¹·mm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>9</td>
<td>15.41 ± 0.11</td>
<td>1.60 ± 0.08</td>
<td>25.34 ± 0.06</td>
<td>16.84 ± 0.83</td>
<td>1.57 ± 0.16</td>
<td>110.6 ± 9.6</td>
</tr>
<tr>
<td>EIPA</td>
<td>8</td>
<td>15.92 ± 0.11</td>
<td>1.34 ± 0.19</td>
<td>25.44 ± 0.24</td>
<td>21.20 ± 0.98*</td>
<td>0.81 ± 0.23*</td>
<td>66.7 ± 7.1*</td>
</tr>
<tr>
<td>BAF</td>
<td>12</td>
<td>15.48 ± 0.28</td>
<td>1.85 ± 0.15</td>
<td>24.96 ± 0.11</td>
<td>16.97 ± 1.29</td>
<td>1.24 ± 0.19</td>
<td>86.1 ± 6.3*</td>
</tr>
<tr>
<td>SCH</td>
<td>12</td>
<td>15.11 ± 0.03</td>
<td>1.45 ± 0.13</td>
<td>25.21 ± 0.16</td>
<td>18.11 ± 0.49</td>
<td>1.69 ± 0.18</td>
<td>113.1 ± 8.3</td>
</tr>
</tbody>
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NHE3⁻/⁻

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Vᵣ, nl/min</th>
<th>L, mm</th>
<th>[HCO₃]ₒ, mM</th>
<th>[HCO₃]ᵢ, mM</th>
<th>Jᵥ, nl·min⁻¹·mm⁻¹</th>
<th>Jₑ[HCO₃], pmol·min⁻¹·mm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>13</td>
<td>15.14 ± 0.04</td>
<td>1.50 ± 0.17</td>
<td>24.87 ± 0.49</td>
<td>21.35 ± 0.65</td>
<td>0.58 ± 0.07</td>
<td>51.2 ± 3.4</td>
</tr>
<tr>
<td>EIPA</td>
<td>9</td>
<td>15.72 ± 0.10</td>
<td>1.71 ± 0.16</td>
<td>24.18 ± 0.14</td>
<td>20.12 ± 0.90</td>
<td>0.49 ± 0.12</td>
<td>47.8 ± 7.3</td>
</tr>
<tr>
<td>BAF</td>
<td>10</td>
<td>15.17 ± 0.23</td>
<td>1.97 ± 0.22</td>
<td>24.95 ± 0.03</td>
<td>23.54 ± 0.41</td>
<td>0.45 ± 0.09</td>
<td>21.1 ± 2.8*</td>
</tr>
<tr>
<td>SCH</td>
<td>11</td>
<td>15.24 ± 0.10</td>
<td>1.30 ± 0.16</td>
<td>25.00 ± 0.10</td>
<td>21.52 ± 0.62</td>
<td>0.58 ± 0.08</td>
<td>55.4 ± 6.0</td>
</tr>
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</table>

Values are means ± SE; n, no. of perfused tubules. Vᵣ, perfusion rate; L, tubular length; [HCO₃]ₒ, HCO₃⁻ concentration in the original perfusate; [HCO₃]ᵢ, HCO₃⁻ concentration in collected fluid; Jᵥ, fluid absorption; Jₑ[HCO₃], HCO₃⁻ absorption; EIPA, ethylisopropylamiloride (100 µM) added to the luminal perfusate; BAF, bafilomycin (1 µM) added to the luminal perfusate; SCH, Sch-28080 (10 µM) added to the luminal perfusate. *Significant difference from control (P < 0.05).
corresponding to 19% of the control rate of \( \text{HCO}_3^- \) absorption in wild-type mice. There are several possible explanations for this small remaining component of inhibitor-insensitive \( \text{HCO}_3^- \) absorption in the NHE3 null mice. First, a passive driving force for \( J_{\text{HCO}_3} \) was present in these experiments because plasma \( \text{HCO}_3^- \) concentration was reduced to \( \sim 20 \text{ mM} \) in NHE3 null mice, whereas the concentration of \( \text{HCO}_3^- \) in the tubule microperfusion solution was 25 mM. Assuming a mean transtubular \( \text{HCO}_3^- \) gradient of 3 mM (Table 2), a negligible transtubular potential difference (15), and a \( \text{HCO}_3^- \) permeability of 1.6 \( \text{nl} \cdot \text{mm}^{-1} \cdot \text{min}^{-1} \) as found in the rat proximal tubule (2), we calculate that only a minor fraction (5 \( \text{pmol} \cdot \text{min}^{-1} \cdot \text{mm}^{-1} \)) of the remaining \( J_{\text{HCO}_3} \) can be attributed to passive transport.

A second possible explanation for the remaining component of inhibitor-insensitive \( \text{HCO}_3^- \) absorption in NHE3 null mice is the use of insufficient inhibitor concentrations to abolish \( \text{H}^+/-\text{K}^+/-\text{ATPase} \) activity completely. However, it is possible that the inhibitor is absorbed in the perfused segment so that its concentration decreases, thereby resulting in incomplete inhibition of \( \text{H}^+/-\text{ATPase} \) activity. As mentioned above, 10 \( \mu \text{M} \) Sch-28080 should have been sufficient to reduce \( \text{H}^+/-\text{K}^+/-\text{ATPase} \) by 80% in the proximal tubule (41), yet no inhibition by this agent was observed. NHE2, the only apical NHE isoform other than NHE3 so far identified, should have been significantly inhibited by 100 \( \mu \text{M} \) EIPA (12), but no EIPA inhibition of \( J_{\text{HCO}_3} \) was observed in NHE3 null mice. Taken together, these considerations indicate that \( \text{H}^+/-\text{K}^+/-\text{ATPase} \) and non-NHE3-mediated \( \text{Na}^+/-\text{H}^+ \) exchange are not likely to have contributed substantially to the inhibitor-insensitive \( J_{\text{HCO}_3} \) in NHE3 null mice.

Third, it should be noted that acetate was present in the microperfusion solution used in these studies. Absorption of acetate via \( \text{Na}^+/-\text{acetate} \) cotransport in parallel with recycling of acetate back to the lumen by nonionic diffusion has been shown to effect net acid extrusion across the apical membrane of proximal tubule cells (22). However, acetate was found to inhibit rather than stimulate transtubular \( \text{HCO}_3^- \) absorption (16), so that this mechanism is unlikely to account for significant \( \text{HCO}_3^- \) absorption in NHE3 null mice. Alternatively, it is possible that secretion from blood to lumen of some other organic anion that crosses the apical membrane by nonionic diffusion or anion/\( \text{OH}^- \) exchange may contribute to proximal acidification in these experiments (4).

Finally, despite a profound impairment of proximal tubule \( \text{HCO}_3^- \) absorption capacity in NHE3 null mice, we confirmed previous findings (27) that only a mild metabolic acidosis is present in these animals. Although, as described above, we detected no upregulation of acidification mechanisms in the proximal tubule itself, at least two other compensatory responses to limit acidosis have so far been identified. First, there is a marked reduction in glomerular filtration rate in NHE3 null mice due to tubuloglomerular feedback (20). This in turn would be expected to reduce the filtered load of \( \text{HCO}_3^- \) and thereby limit the delivery of \( \text{HCO}_3^- \) out of the proximal tubule even in the presence of a reduced capacity for \( \text{HCO}_3^- \) absorption. Second, \( \text{HCO}_3^- \) absorption capacity is increased in cortical and outer medullary collecting ducts of NHE3 null mice due to upregulation of \( \text{H}^+/-\text{K}^+/-\text{ATPase} \) isoforms (21).

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