Human duodenal mucosal brush border Na\(^+/\)H\(^+\) exchangers NHE2 and NHE3 alter net bicarbonate movement

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The present study was based on the hypothesis that one or more of the epithelial, amiloride-sensitive NHE isoforms is located and functional on the human duodenal enterocyte apical surface. Thus immunofluorescence studies for NHE2 and NHE3 were performed on proximal duodenal mucosal biopsies. Furthermore, the effect of graded doses of amiloride that selectively decreases surface epithelial HCO\(_3^-\) secretion is involved in prevention of mucosal acid peptic damage (8). Moreover, duodenal ulcer patients infected with Helicobacter pylori have diminished basal and stimulated HCO\(_3^-\) secretion that normalizes after H. pylori eradication (11). Duodenal mucosa in vitro obtained from patients with cystic fibrosis has decreased resting and cAMP-stimulated HCO\(_3^-\) secretion (18). Thus duodenal mucosal bicarbonate secretion (DMBS) is important in both health and disease.

Similar to other cells that are involved in ion transport, duodenal enterocytes contain apical and basolateral membrane transporters that serve to maintain the intracellular pH near 7.1 (1). To date, the acid/base transporters that have been identified in animal and human duodenal enterocytes include 1) an amiloride-sensitive Na\(^+/\)H\(^+\) exchanger (NHE) that functions largely as an acid extruder; 2) a stilbene-sensitive Na/HCO\(_3^-\) cotransporter that functions as a base loader; and 3) a stilbene-sensitive Cl\(^-\)/HCO\(_3^-\) antiporter that is a base extruder (1, 2). Additionally, the cystic fibrosis transmembrane regulator (CFTR) has also been implicated in HCO\(_3^-\) secretion, in addition to Cl\(^-\) conductance (17, 20). There are, however, limited in vivo human studies that integrate mucosal structure with function of acid/base transporters. Nyberg et al. (16) demonstrated in humans that PGE\(_2\)-stimulated DMBS was decreased significantly by putative blockade of the Cl\(^-\)/HCO\(_3^-\) exchanger with luminal perfusion of the stilbene derivative DIDS, whereas theophylline-stimulated DMBS (presumably acting by inhibition of cyclic nucleotide phosphodiesterases and thereby increasing cAMP content) was unaltered. These findings suggest that separate luminal transport mechanisms are involved in human PGE\(_2\) and cAMP-stimulated duodenal HCO\(_3^-\) transport.

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inhibit NHE2 and NHE3 were determined. We observed that 1) both NHE2 and NHE3 are located principally on the apical surface of human duodenal villus cells and 2) suppression of NHE2 activity (10^{-5}–10^{-4} M amiloride) significantly increased net duodenal HCO_3^- output; moreover, inhibition of NHE3 (by 10^{-3} M amiloride) resulted in an additional significant increase in HCO_3^- output.

METHODS

Subjects. Five male subjects aged 32–55 yr participated in these studies. Each was in excellent health, taking no medications, and free of any acute or chronic disease. The experimental protocol was approved by the University of California at San Diego Human Subjects Committee, and each subject gave signed informed consent. Duodenal biopsies and measurement of DMBS were performed on days separated by at least 2 wk.

Duodenal mucosal biopsies and immunofluorescence of NHE2 and 3. Two to three endoscopic biopsies (Radial Jaw, Microvasive; Boston Scientific, Watertown, MA) were obtained in the midportion of the duodenal bulb. Tissues were fixed in 3% neutral buffered formaldehyde, processed on a Technicon, and embedded in paraffin. Immunohistochemistry was performed as described previously (12). After being embedded in paraffin, sections were cut at 4 nm and mounted on gelatin-coated slides. The slides were de waxed in xylene, rehydrated in ethanol, and rinsed in PBS buffer; endogenous peroxide was blocked by incubation in 0.3% H_2O_2 in methanol. The slides were blocked in PBS buffer (1% NFDM) with goat serum and incubated overnight at 4°C with primary rabbit polyclonal antibodies Ab 597 (rabbit polyclonal anti-GST-C terminal 87 AA of NHE2) or Ab 1381 (rabbit polyclonal anti-GST-C terminal 85 AA of NHE3) or, as control, secondary antibody alone (goat anti-rabbit IgG; Vector Laboratories, Burlingame, CA). Each of these antibodies had been characterized previously (12, 21). Labeling was visualized by light microscopy and horseradish peroxidase by experts in this technique using the Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

Duodenal mucosal HCO_3^- secretion. Proximal duodenal mucosal net HCO_3^- output (i.e., by the duodenal bulb; DMBS) was measured with well-documented and validated methods described previously (11, 13, 14). In brief, 3 cm of proximal duodenum was isolated by two balloons that straddled the pylorus and a third that was 3 cm beyond. The isolated segment was perfused with 154 mM NaCl at 2 ml/min containing 1^4C-labeled polyethylene glycol as a nonabsorbable marker, and the effluent was collected by gravity. HCO_3^- concentration ([HCO_3^-]) was determined in duplicate on samples obtained anaerobically. Measurements of pH and P_CO_2 were obtained (IL 1420, BG3 PCO_2 electrode, blood gas analyzer; Instrument Laboratories, Lexington, MA), and [HCO_3^-] was calculated by the Henderson-Hasselbalch equation (11, 13, 14). HCO_3^- outputs were calculated as the product of [HCO_3^-] times the [1^4C]-polyethylene glycol-corrected volume (≥85% of the infusates were recovered). Gastric and distal duodenal markers (phenol red and trypsin, respectively) were also infused continuously to assess for potential contamination of the isolated segment (11, 13, 14).

After measurement of basal HCO_3^- secretion for three 15-min periods, graded doses of amiloride (10^{-5}–10^{-4}, and 10^{-3} M; Sigma, St. Louis, MO) were infused into the test segment in increasing concentrations, each for 30 min, and the effects on net HCO_3^- output were determined. Moreover, additional control experiments were performed on a separate day in three subjects to determine whether there were time-related alterations in HCO_3^- secretion; thus isosmolar NaCl was infused alone for the 155-min test period.

Statistics. Data are presented as means ± SE and 95% confidence intervals (CI). Results were analyzed by repeated-measures analysis of variance and the Tukey-Kramer multiple-comparisons test. P values <0.05 were considered significant.

RESULTS

Immunofluorescence of NHE2 and NHE3. Light microscopic sections of the duodenal biopsies were examined using previously characterized polyclonal antibodies raised in rabbits. These antibodies had been shown to demonstrate the presence of NHE2 and NHE3 in multiple species, for example, in intestine (human [jejunum, ileum, and colon], rat [ileum and colon], chicken [small intestine and colon], and mouse [ileum and colon]) (22–24). As shown in Fig. 1, NHE2 and NHE3 were present principally in the brush border of villus cells from human proximal duodenum; a modest amount of staining extended into the upper crypt region. Neither NHE2 nor NHE3 was present on the basolateral surface of any epithelial cells. In each of the five subjects studied the immunofluorescence patterns were identical. In addition, no staining for either NHE2 or NHE3 was observed in the absence of primary antibody.

Duodenal HCO_3^- secretion. Basal DMBS was 355 ± 19 µmol·cm^{-1}·h^{-1} (95% CI: 300–410 µmol·cm^{-1}·h^{-1}). Amiloride resulted in significant (P < 0.003) concentration-dependent increases in duodenal HCO_3^- output compared with basal output (Fig. 2). The net mean (95% CI) incremental increases above baseline in response to 10^{-5}, 10^{-4}, and 10^{-3} M amiloride were 47 (11–82), 67 (32–103), and 111 (75–146) µmol·cm^{-1}·h^{-1}, respectively. In addition, although DMBS in response to each dose of amiloride was significantly greater than basal secretion, the DMBS response to 10^{-3} M amiloride was significantly greater than the response to either the 10^{-5} M or the 10^{-4} M infusion, which were not significantly different from one another. Furthermore, DMBS in the subjects in whom an additional NaCl infusion control test was performed revealed that basal HCO_3^- secretion was 336 (CI 289–383) µmol·cm^{-1}·h^{-1} and decreased only modestly to 306 (242–370) µmol·cm^{-1}·h^{-1} (P = not significant) during the final 30 min.

In addition, HCO_3^- concentration in the duodenal effluent (the infusate was nominally HCO_3^- free) increased significantly from basal concentration in response to 10^{-4} M and 10^{-3} M amiloride, and the effluent volume also increased modestly (Table 1). The increase in HCO_3^- concentration was caused primarily by an increase in P_CO_2 (basal: 13.70 ± 0.59 mmHg, 10^{-3} M amiloride: 15.75 ± 0.67 mmHg; P < 0.02). Thus, during amiloride perfusion, P_CO_2, [HCO_3^-], and net volume increased. An increase in effluent pH also occurred but did not attain statistical significance (basal: 7.22 ± 0.01, 10^{-3} M amiloride: 7.30 ± 0.02; P = 0.08).

DISCUSSION

The results of this study indicate that NHE2 and NHE3 are localized principally to the apical membrane of human proximal duodenal villus cells and that the
sequential inhibition of each NHE by amiloride results in stepwise and significant increases in net duodenal HCO$_3^-$ output. These findings indicate that NHE2 and NHE3 affect luminal duodenal pH by altering HCO$_3^-$ secretion and that human duodenal villus cell function contributes to net duodenal HCO$_3^-$ transport. Thus, similar to the mediation of renal proximal tubule HCO$_3^-$ absorption by apical membrane Na$^+$/H$^+$ exchange, duodenal HCO$_3^-$ transport is also influenced by apical NHEs (19). This process is likely caused by proton transport into the lumen via Na$^+$/H$^+$ exchange, neutralizing HCO$_3^-$ and resulting in H$_2$O and CO$_2$ production, the latter being partially permeable across the cell membrane. The intracellular CO$_2$ is then catalyzed by carbonic anhydrases to produce intracellular HCO$_3^-$ and H$^+$, which in turn are available for transport by their respective membrane acid/base antiporters. A potential alternative process would be inhibition of apically located NHEs resulting in intracellular acidification (due to decreased Na$^+$/H$^+$ exchange), thereby activating basolateral NaHCO$_3$ cotransport resulting in increased HCO$_3^-$ entry and transport into the lumen. However, it is also possible that the basolateral “housekeeping” NHE1 would transport H$^+$ out of the cell before activation of NaHCO$_3$ cotransport.

In health there is a sharp pH gradient from the non-acid-secreting antral portion of the stomach, where the luminal pH may be as low as 0.5, and the most proximal duodenum, where the luminal pH is near neutral, except for brief (~30 s) transient periods when the pH decreases to ~2–3 (5, 9). The mechanisms that contribute to duodenal neutrality are HCO$_3^-$ secretion by the proximal duodenal mucosa and pancreaticobiliary HCO$_3$ (25, 26). This study indicates that intraduodenal HCO$_3$ concentration is affected by Na$^+$/H$^+$ exchange that can result in neutralization of
luminal HCO$_3^\text{-}$ It is possible that under physiological conditions the exchangers may change direction. That is, when the lumen is very acidic, as occurs in the bulb, NHEs may take up protons and transport HCO$_3^\text{-}$ and Na$^+$ into the lumen. However, it has been demonstrated that a pH gradient is present in the in vivo duodenum and that the juxtamucosal pH is ~7 (3).

The cellular events that result in net HCO$_3^\text{-}$ secretion by the intestine, as well as other organs, have been the subject of intense study (15). In the intestine, the key transporters involved in HCO$_3^\text{-}$ transport across the apical membrane are 1) the Cl$^-$/HCO$_3^\text{-}$ exchanger, 2) CFTR, and, as this study demonstrates, 3) NHE2 and NHE3. CFTR conducts Cl$^-$ that can be exchanged after secretion into the lumen across the apical membrane for intracellular HCO$_3^\text{-}$ by the brush border Cl$^-$/HCO$_3^\text{-}$ exchanger. In addition, there is accumulating evidence that CFTR also has a HCO$_3^\text{-}$ conductance. There are fewer HCO$_3^\text{-}$ transporters across the basolateral membrane; HCO$_3^\text{-}$ enters the cell principally via the basolateral NaHCO$_3$ cotransporter (12, 22). However, the location of an anion exchanger in the duodenum is in question. A recent report by Alper et al. (4) describes in mice that AE2 immunostaining of enterocytes was restricted to a basolateral distribution. Further work is needed to delineate membrane transporters in human duodenum.

The relative amounts of NHE-induced CO$_2$ absorption and HCO$_3^\text{-}$ secretion can be estimated from these studies in which basal net HCO$_3^\text{-}$ secretion (which is made up of both basal CO$_2$ absorption and HCO$_3^\text{-}$ secretion) was 355 μmol·cm$^{-1}$·h$^{-1}$. Assuming that 10$^{-3}$ M amiloride blocks both apical NHEs and CO$_2$ absorption, it can be estimated that basal CO$_2$ absorption accounts for ~111 μmol·cm$^{-1}$·h$^{-1}$ of HCO$_3^\text{-}$, because this represents the net increase in HCO$_3^\text{-}$ secretion above basal levels caused by the 10$^{-3}$ M amiloride. Furthermore, it can be estimated that basal HCO$_3^\text{-}$ secretion is ~466 μmol·cm$^{-1}$·h$^{-1}$, calculated from the net basal HCO$_3^\text{-}$ transport rate of 355 μmol·cm$^{-1}$·h$^{-1}$ plus that estimated to be due to CO$_2$ absorption, 111 μmol·cm$^{-2}$·h$^{-1}$. Thus the contribution of CO$_2$ absorption by NHEs is estimated to be ~24% of the basal HCO$_3^\text{-}$ secretory rate. Moreover, these suggest that NHE2 and NHE3 contribute approximately

Table 1. Effect of amiloride on duodenal effluent \(\text{[HCO}_3^\text{-}\text{]}\) and secretion

<table>
<thead>
<tr>
<th>Amiloride</th>
<th>[HCO$_3^\text{-}$]</th>
<th>Volume</th>
<th>PCO$_2$</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>6.7±0.34</td>
<td>35.9±2.1</td>
<td>13.7±0.59</td>
<td>7.22±0.02</td>
</tr>
<tr>
<td>Amiloride (10$^{-6}$ M)</td>
<td>6.7±0.27</td>
<td>38.8±2.1ª</td>
<td>15.03±0.86</td>
<td>7.27±0.01</td>
</tr>
<tr>
<td>Amiloride (10$^{-3}$ M)</td>
<td>7.7±0.29ª</td>
<td>37.4±2.9</td>
<td>15.78±0.45ª</td>
<td>7.28±0.03</td>
</tr>
<tr>
<td>Amiloride (10$^{-2}$ M)</td>
<td>8.1±0.37ª</td>
<td>38.1±2.4</td>
<td>15.75±0.67ª</td>
<td>7.30±0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 segments. An isolated proximal duodenal segment was perfused with 154 mM NaCl, and effluent volume and HCO$_3^\text{-}$ concentration ([HCO$_3^\text{-}$]) were measured. After a 45-min basal period, amiloride was perfused luminally in increasing sequential concentration. [HCO$_3^\text{-}$] was calculated by pH/PCO$_2$ and the Henderson-Hasselbalch equation; volumes are corrected for [³¹C]polyethylene glycol recovery. Figure 2 depicts the net increases in HCO$_3^\text{-}$ transport. *P < 0.05 vs. basal measurement.
equally to basal HCO₃⁻ secretion. Additional studies are required to determine whether the increase in HCO₃⁻ transport was caused by increased HCO₃⁻ conductance and/or by Cl⁻/HCO₃⁻ exchange.

Thus the results suggest that both NHE2 and NHE3 contribute to human duodenal intestinal Na⁺ absorption. Although this was observed in rabbit ileum, in which both apical NHE2 and NHE3 contributed approximately equally to basal Na⁺ absorption (27), in other species one or another NHE may predominate in a given intestinal segment. For instance, in chicken small intestine and colon, NHE2 and not NHE3 accounts for the majority of basal Na⁺ absorption (6). Although the contribution of apical NHE2 versus NHE3 in human intestinal Na⁺ or proton-induced CO₂ absorption has not been compared in other intestinal segments, both are present in the brush border of villus cells in human jejunum, ileum, and colon, although in the colon NHE2 message is considerably larger (7).

Given the importance of understanding the regulation of duodenal HCO₃⁻ secretion in health and disease, it now becomes important to recognize that regulation of both HCO₃⁻ secretory and absorptive processes must be considered and examined separately. Moreover, in carrying out studies of HCO₃⁻ secretion, experiments must be done in a way that the HCO₃⁻ absorptive and secretory processes can be measured separately.

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