The importance of mucus layers and bicarbonate transport in preservation of gastric juxtamucosal pH

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Phillipson, Mia, Christer Atuma, Johanna Henriksnäs, and Lena Holm. The importance of mucus layers and bicarbonate transport in preservation of gastric juxtamucosal pH. Am J Physiol Gastrointest Liver Physiol 282: G211–G219, 2002.—Mucus thickness is suggested to be related to mucosal protection. We therefore investigated the importance of the removable mucous layer and epithelial bicarbonate transport in preservation of the gastric juxtamucosal pH (pHjm) during luminal acid. Anesthetized rats were prepared for intravital microscopy of the gastric mucosa, and pHjm was measured with pH-sensitive microelectrodes. The mucus was either left intact (IM) or removed (MR) down to the firmly attached mucous layer, and HCl (pH 1) was applied luminally. Removal of the loosely adherent mucus layer did not influence the pHjm during luminal acid (pentagastrin: IM/MR 7.03 ± 0.09/6.82 ± 0.19; pentagastrin + indomethacin: IM/MR 6.89 ± 0.20/6.95 ± 0.27; ranitidine: IM/MR 2.38 ± 0.64/2.97 ± 0.62), unless prostaglandin synthesis and acid secretion were inhibited (ranitidine + indomethacin: IM/MR 2.03 ± 0.37/1.66 ± 0.18). Neutral pHjm is maintained during endogenous acid secretion and luminal pH 1, unless DIDS was applied luminally, which resulted in a substantially decreased pHjm (1.37 ± 0.21). Neutral pHjm is maintained by a DIDS-sensitive bicarbonate transport over the surface epithelium. The loosely adherent mucous layer only contributes to maintaining pHjm during luminal pH 1 if acid secretion and prostaglandin synthesis are inhibited.

pH-sensitive microelectrodes; 4,4’-diisothiocyanostilbene-2,2’-disulfonic acid; rat

The gastric mucosa produces degrading factors such as gastric acid and proteolytic enzymes. To maintain mucosal integrity, an effective defense system is required. The first line of defense against acid, the mucus-bicarbonate barrier, is one of the least-understood components and is at present being focused on by several research groups.

We have previously shown that a pH gradient with a neutral pH at the surface epithelial cells is maintained in the mucus covering the corpus mucosa down to a luminal pH of 2 in acid-secreting and nonsecreting mucosae (25). One explanation of this gradient is that bicarbonate, secreted from the surface epithelial cells into the mucus gel, neutralizes back-diffused acid and that endogenous acid traverses the mucus only in distinct “channels” leading from the gland openings toward the lumen (9, 16). Bicarbonate production in the surface epithelial cells is stimulated by endogenous prostaglandins (10, 11, 27). Furthermore, Teorell (28) demonstrated that for each proton secreted from the parietal cell, one bicarbonate ion is released from the basolateral membrane of the parietal cell to the capillaries leading to the surface epithelium. This blood-borne transport of bicarbonate during acid secretion is probably important for the maintenance of a neutral surface pH (19, 26) when the luminal pH is low (pH 1).

In the Necturus antrum, a luminal mucous layer has been shown to be necessary for keeping the juxtamucosal and intracellular pH neutral in the presence of luminal acid (20). Infection by Helicobacter pylori and the use of ulcerogenic drugs (nonsteroidal anti-inflammatory drugs) inhibit mucin synthesis and secretion and decrease the thickness of the mucous layer (4, 12, 15, 17). On the basis of these and other studies (7), the hypothesis that mucus thickness is correlated to mucosal protection has been proposed.

We have recently found it possible to separate the mucous layer covering the corpus mucosa into two different layers, in addition to loose mucus in the gastric lumen (3). The most luminal of the two layers, the loosely adherent mucus, can be removed by suction or by rubbing with a cotton tip, whereas the inner layer, the firmly adherent mucus, cannot be removed by applying this physical strain. The physical properties and physiological importance of the different layers are unknown, as is their composition and possible differences between them in permeability to acid.

In this study, we investigated the importance of the loosely adherent mucous layer for the pH at the epithelial surface [juxtamucosal pH (pHjm)] during inhibition or stimulation of acid secretion (the latter resulting in production and transport of bicarbonate from the parietal cells to the microcirculation supplying the surface epithelial cells) in the presence of a luminal pH of 1. The influence of prostaglandin-stimulated bicarbonate production on the pHjm was also investigated.

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To determine the route by which bicarbonate traverses the epithelial cells, an inhibitor of the apical Cl⁻/HCO₃⁻ exchanger DIDS was used.

MATERIALS AND METHODS

Animal preparation. Male Sprague-Dawley rats (Charles River, Uppsala, Sweden) weighing 160–290 g were used for the experiments. They were kept under standardized conditions (21–22°C, 12:12-h light/dark) in cages with mesh bottoms and had free access to tap water and pelleted food (Ewos, Södertälje, Sweden). The animals were deprived of food, but not water, for 17–20 h before they were anesthetized with thiobutabarbital sodium (Inactin, 120 mg/kg ip). Spontaneous breathing was facilitated by a short PE-200 cannula placed in the trachea, and the body temperature was maintained at 37.5 ± 0.5°C by means of a heating pad controlled by a rectal thermistor. A PE-50 cannula containing heparin (12.5 IU/ml) dissolved in isotonic saline was placed in the right femoral artery to monitor blood pressure. The right femoral vein was catheterized for administration of Ringer solution (in mM: 120 NaCl, 2.5 KCl, 0.75 CaCl₂, and 25 NaHCO₃) either alone or with pentagastrin (40 μg·kg⁻¹·h⁻¹), which was given as a continuous intravenous infusion starting 30 min before the acid periods. The mucosal layer was left intact (IM). In group II (n = 8), acid stimulation was stimulated as in group I, and the outer, loosely adherent mucous layer was removed (MR) by gentle suction immediately before the acid (pH 1) was administered topically. In groups III (n = 6, IM) and IV (n = 5, MR), indomethacin (3 mg/kg, diluted in saline) was given intravenously as a bolus dose 1 h before the acid periods, in addition to pentagastrin stimulation. In groups V (n = 5, IM) and VI (n = 6, MR), acid secretion was inhibited with ranitidine (1 mg/kg iv given as a bolus), administered at least 30 min before the acid periods. In groups VII (n = 6, IM) and VIII (n = 5, MR), both indomethacin and ranitidine were given as described above. In group IX (n = 5), acid secretion was stimulated with pentagastrin. The loosely adherent mucous layer was removed 30 min later, and DIDS was applied luminally (0.5 mM) for 15 min before the acid periods.

Chemicals. The following chemicals and drugs were used: thiobutabarbital sodium (Inactin; Research Biochemicals International, Natick, MA), heparin (KabiVitrum, Stockholm, Sweden), silicone glue (Kebo Lab, Stockholm, Sweden), proton cocktail (hydrogen ion Ionophore II-Cocktail; Fluka, Buchs, Switzerland), 1 M HCl (Titrisol; Merck, Darmstadt, Germany), tributylchlorosilane (Fluka). HEPES (Sigma, St. Louis, MO), pentagastrin (Peptavlon; ICI Pharmaceuticals, Macclesfield, England), Indomethacin (Confortid; Dumex, Copenhagen, Denmark), ranitidine (Zantac; Glaxo Wellcome, Miltonndal, Sweden), and DIDS (Sigma).

Statistics. The results are expressed as means ± SE. Differences in pHᵢₑₘ within the same group and between groups of animals were evaluated statistically by ANOVA in medians (Mann-Whitney test). Differences in acid secretion were evaluated by ANOVA (single-factor factorial, nonrepeated measures followed by Fisher protected least significant differences test). Differences were regarded as significant when P < 0.05. All statistical calculations were performed on a Macintosh computer with the software Statview II SE Graphics (Abacus Concepts, Berkeley, CA).

RESULTS

Figure 1 shows the mean values for pHᵢₑₘ and for pentagastrin-stimulated acid secretion in groups I and II. pHᵢₑₘ was neutral during the control period in both

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groups (see Table 1). When HCl at pH 1 was applied luminally in group I (n = 7, IM), pH\textsubscript{jm} immediately decreased to 6.1 ± 0.1, but 4 min later it had returned to >7. The pH\textsubscript{jm} remained at a slightly but significantly lower level as long as the acid was in the lumen. In group II (n = 8, MR), acid (pH 1) was applied after the outer adherent mucus layer had been removed.

Table 1. Juxtamucosal pH in treatment groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Preacidic Period</th>
<th>During Luminal Acid, pH 1</th>
<th>Postacidic Period</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Penta IM</td>
<td>7.45 ± 0.14</td>
<td>7.03 ± 0.09</td>
<td>7.49 ± 0.10</td>
</tr>
<tr>
<td>II Penta MR</td>
<td>7.53 ± 0.10</td>
<td>6.82 ± 0.19</td>
<td>7.45 ± 0.15</td>
</tr>
<tr>
<td>III Penta + Indo IM</td>
<td>7.49 ± 0.10</td>
<td>6.89 ± 0.20</td>
<td>7.47 ± 0.10</td>
</tr>
<tr>
<td>IV Penta + Indo MR</td>
<td>7.61 ± 0.12</td>
<td>6.95 ± 0.27</td>
<td>7.48 ± 0.11</td>
</tr>
<tr>
<td>V Ran IM</td>
<td>7.44 ± 0.21</td>
<td>2.38 ± 0.64</td>
<td>6.69 ± 0.43</td>
</tr>
<tr>
<td>VI Ran MR</td>
<td>7.44 ± 0.13</td>
<td>2.97 ± 0.62</td>
<td>7.25 ± 0.30</td>
</tr>
<tr>
<td>VII Ran + Indo IM</td>
<td>6.98 ± 0.13</td>
<td>2.03 ± 0.37</td>
<td>7.11 ± 0.06</td>
</tr>
<tr>
<td>VIII Ran + Indo MR</td>
<td>7.19 ± 0.18</td>
<td>1.66 ± 0.18</td>
<td>6.07 ± 0.49*</td>
</tr>
<tr>
<td>IX Penta + DIDS MR</td>
<td>7.35 ± 0.11</td>
<td>1.37 ± 0.21</td>
<td>5.78 ± 1.06</td>
</tr>
</tbody>
</table>

Values are means ± SE. pH at the juxtamucosal surface before acid was instilled in the gastric lumen (preacidic period at 15 min), 5 min after instillation of acid pH 1 in the lumen, and 10 min after replacement of acid with saline (postacidic period at 45 min) are shown. Penta, pentagastrin; Indo, indomethacin; Ran, ranitidine; IM, intact mucus; MR, mucus removed. *P < 0.05 vs. group with the same treatment and intact mucus.

An immediate transient reduction of the pH\textsubscript{jm} to 5.6 ± 0.7 was noted, followed by a steady-state pH\textsubscript{jm} at a level slightly but significantly lower than the control level; this was maintained throughout the acidic period. The pH\textsubscript{jm} values did not differ significantly between groups I and II at any time during the experiment. When the acid was washed away in both groups, the pH\textsubscript{jm} returned to values not different from the control levels.

Basal acid secretion was significantly higher in group II (0.29 ± 0.13 μeq·min\(^{-1}·cm\(^{-2}\)) than in group I (0.08 ± 0.01 μeq·min\(^{-1}·cm\(^{-2}\)). Even during stimulation of acid secretion with pentagastrin, there was a tendency toward higher acid secretion (significant at 0–15 min) in group II compared with group I. Differences in acid output might be due to seasonal differences since the experiments were conducted groupwise at different times.

Figure 2 shows the mean values for pH\textsubscript{jm} and for acid secretion in the pentagastrin- and indomethacin-treated groups III (IM) and IV (MR). The pH\textsubscript{jm} was neutral during the control period in both groups (see Table 1). When acid at pH 1 was applied topically in group III (n = 6, IM), the pH\textsubscript{jm} immediately stabilized at a slightly but significantly lower level (pH 6.9 ± 0.2), which was maintained throughout the acidic period.
In group IV (n = 5, MR), an immediate transient initial dip of the pHjm to 5.9 ± 1 was observed in response to the presence of acid in the lumen, followed by return to a level slightly but significantly lower than during the control period. The pHjm values in groups III and IV did not differ significantly at any time between each other or between the groups without indomethacin pretreatment (I and II). Basal acid secretion before pentagastrin stimulation was 0.12 ± 0.05 μeq·min⁻¹·cm⁻² in group III and 0.14 ± 0.04 μeq·min⁻¹·cm⁻² in group IV.

Figure 3 shows the mean values for pHjm and for the acid secretion in the groups given ranitidine (V, n = 5, IM; VI, n = 5, MR). The pHjm was neutral in both groups during the control period (see Table 1). In the presence of luminal acid at pH 1, the epithelial surface was acidified to pH 2.2 ± 0.5 in group V (6 min after acid application, IM) and to 1.8 ± 0.2 in group VI (20 min after acid application, MR). These pHjm levels were significantly lower than in the pentagastrin-stimulated groups I–IV. When the acid was washed away, the pHjm returned to neutral or near-neutral values within 10 min, significantly different from the control level before acid and the pentagastrin-treated groups (I–IV) only at two time points in group V. Groups V and VI did not differ significantly from each other at any time.

In the groups in which acid secretion and prostaglandin synthesis were inhibited (VII, n = 6, IM; and VIII, n = 5, MR) the initial pHjm (Fig. 4) was slightly lower (significant at one point) than in the pentagastrin-stimulated groups (I–IV; see Table 1). When acid at pH 1 was applied luminally in group VII (IM), the pHjm decreased to 2.0 ± 0.3 (10 min after acid application). This was significantly lower than in the pentagastrin-stimulated groups I–IV but not compared with the ranitidine-treated groups V and VI. The pHjm remained at a significantly lower level compared with groups I–IV throughout the experiment.

In group VIII (MR), the pHjm dropped to a lowest level of 1.4 ± 0.1 (15 min after acid application) in the presence of acid in the lumen, which was significantly
lower than in the pentagastrin-stimulated groups (I–IV) and compared with groups V–VI with inhibition of acid secretion. When the acid was washed away, the pHjm was still significantly lower than in the other groups. Compared with group VII, which also received ranitidine and indomethacin, removal of the loosely adherent mucous layer resulted in a nonsignificantly lower pHjm in the presence of luminal acid and a significantly slower reestablishment of the pH gradient after the acidic period. This indicates that the loosely adherent mucus is important only if acid secretion and prostaglandin synthesis are inhibited.

In group IX (n = 5, Fig. 5), acid secretion was stimulated with pentagastrin and the mucosa was pre-treated with DIDS (0.5 mM) before acid challenge. The loosely adherent layer was removed before DIDS was applied to minimize interactions between DIDS and the mucus. The pHjm was initially neutral (see Table 1) but decreased to a lowest level of 1.4 ± 0.2 (5 min after acid application) in the presence of luminal acid at pH 1. This value is significantly lower than in the other pentagastrin-stimulated groups (I–IV). When the acid had been washed away, the pHjm slowly returned within 20 min to a neutral value.

DISCUSSION

The mucous layer continuously covers the gastric mucosa (1) and is believed to contribute to the preepithelial defense mechanisms in different ways. In the Necturus antrum, a luminal mucous layer has been shown to be necessary for keeping the pHjm, and intracellular pH neutral in the presence of luminal acid (20). In accordance with this, an unstirred layer has been shown in which the pH gradient is formed by bicarbonate secreted from the epithelial cells, neutralizing back-diffused acid (22, 25). Moreover, we have earlier studies suggesting that acid secretion is restricted to
penetrating the mucous layer from its site of formation to the stomach lumen in channels, thereby limiting the acidification of the mucous layer (16).

In an in vivo study in the rat corpus, an inverse relation between mucous gel thickness (measured by alternately focusing on the fluorescent cell surface and the gel-lumen interface) and intracellular acidification in the surface epithelium was found (7). This indicates that a thicker mucous layer would protect the underlying cells from back-diffused acid. We have recently found that the adherent gastric mucous layer can be divided into two different layers (3). The most luminal of these two layers, which we have designated the loosely adherent layer, can be removed by suction or mechanically removed with a cotton tip, leaving a thinner, still continuous, firmly adherent layer attached to the mucosa. The thicknesses of the different layers can be measured with micropipettes inserted into the mucus. The total mucus thickness (loosely adherent plus firmly adherent) was 189 ± 11 μm, and the thickness of the firmly adherent layer (measured directly after removal of the loosely adherent layer) was 80 ± 5 μm (3). The physical properties and physiological importance of the different layers are still unknown.

One purpose of the present study was to investigate the importance of the loosely adherent mucous layer in maintaining the basal pH_m during HCl (pH 1) applied in the lumen. The results clearly show that the loosely adherent layer is of minor importance in maintaining the pH_m, since pH at the epithelial cell surface was not altered when the loosely adherent layer was removed before acid pH 1 was instilled into the lumen. This seems plausible, since the loosely adherent layer most probably is removed from the mucosal surface by in-

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**Fig. 4.** Results from ranitidine- and indomethacin-treated rats (group VII, n = 6; and group VIII, n = 5); see Fig. 1 for details. Calculations for significance were performed by the Mann-Whitney test. *P < 0.05 vs. values before acid exposure, †P < 0.05 vs. pentagastrin-stimulated and indomethacin-treated groups (I–IV); §P < 0.05 vs. ranitidine-treated groups (V and VI) with a corresponding mucus status; ‡P < 0.05 between groups VII and VIII.
gested food, which also stimulates acid secretion. The function of the loosely adherent layer might be to lubricate food particles and bind bacteria. In earlier experiments in which the pH gradient was measured through the entire mucous layer (firmly and loosely adherent), the depth of this gradient in the mucus during acid secretion (∼100 μm closest to the epithelial surface, luminal pH 2 or 3) was found to correspond quite well with the thickness of the firmly adherent mucous layer (3, 15, 25). This supports the finding from the present study that the loosely adherent layer is not important for preserving a pH gradient.

Our results also support recent observations by Chu et al. (5) in a study in which the pH in the mucous layer and the mucus thickness were measured with a confocal microscope by use of different fluorescent dyes. They found that a mucous gel between 25- and 75-μm-thick provided the most alkaline surface epithelium during superfusion with pH 3 and that if the mucous gel was thicker than 100 μm it did not provide greater...
surface protection. However, they found an inverted pH gradient in the mucous gel covering the rat corpus mucosa during pentagastrin stimulation, with pH 5 in the lumen and pH 3.5 at the cell surface, suggesting that acid diffused from its site of secretion toward the lumen. Discrepancies between their results and ours have been thoroughly discussed in a previous article (16).

The results from the present study convincingly show that the pH gradient is better preserved in an acid-secreting stomach than in a resting one, which is in agreement with earlier reports (18, 26). Thus during endogenous acid secretion the gastric microcirculation supplies the surface epithelial cells with the bicarbonate needed for juxtamucosal neutralization. When DIDS was applied luminally and acid secretion was stimulated with pentagastrin, the pH_{jm} decreased dramatically when acid (pH 1) was applied in the lumen. This indicates that bicarbonate is transported transcellularly through a DIDS-sensitive mechanism. In vitro experiments have revealed that gastric bicarbonate secretion is dependent on luminal chloride ions, indicating the presence of an apical Cl⁻/HCO₃⁻ exchanger (8). Bicarbonate enters the surface epithelial cells in cotransport with sodium (6, 24). DIDS is an inhibitor of the Cl⁻/HCO₃⁻ exchanger as well as of Na⁺/HCO₃⁻ cotransport. In the parietal cell, a basolateral Cl⁻/HCO₃⁻ exchanger is found, and inhibition of this transporter inhibits acid secretion (14). In the concentration used, DIDS applied luminally to the gastric mucosa in this study seemed to inhibit at least the apical Cl⁻/HCO₃⁻ exchanger in the surface epithelial cells. Since the acid secretion was not reduced after DIDS treatment, no major amount of the inhibitor appeared to have entered the circulation.

It has been shown that inhibition of prostaglandin synthesis with indomethacin decreases bicarbonate secretion and that prostaglandin 16,16-dimethyl E₂ stimulates the alkali secretion in the fundic mucosa (9, 21). Moreover, inhibition of the prostaglandin synthesis with indomethacin in the presence of a luminal pH of 2 resulted in a decrease in the pH_{jm} compared with that in a control group (23). In accordance with these findings, when acid secretion and prostaglandin synthesis were both inhibited in the present study (group VII), the pH at the cell surface was slightly reduced in the control situation before acid was applied in the lumen. However, in our model, the pH_{jm} was not further reduced in the presence of luminal pH 1 by inhibition of prostaglandin synthesis, whether acid secretion was stimulated or inhibited. It is possible that the strong acid that penetrates down to the surface epithelial cells when acid secretion is inhibited conceals a small fraction of bicarbonate secretion that is prostaglandin-dependent. However, these results indicate that gastric prostaglandin-dependent bicarbonate secretion plays only a minor role in neutralizing back-diffused acid.

When inhibition of acid secretion and of prostaglandin synthesis was combined with removal of the loosely adherent mucous layer (group VIII), a lower pH_{jm} was seen in this group in the presence of luminal acid and the recovery to a neutral pH after removal of luminal acid was significantly delayed. This could, however, be due to thinning of the firmly adherent mucous layer induced by indomethacin, which we have found in a preliminary study (15). Thus not only the blood-borne load of bicarbonate but also the prostaglandin-dependent bicarbonate secretion is reduced and the firmly adherent layer is thinner, resulting in inadequate maintenance of the pH gradient. Further studies are required to elucidate the importance of the thickness of the firmly adherent layer in maintaining the pH_{jm}.

A reduction in pH_{jm}, as we have shown here occurs during inhibition of acid secretion and with pH 1 in the lumen. Although the stomach does not normally experience a significant acid load during inhibition of acid secretion, the present study suggests that it would be more susceptible than normal to luminal acid if this was ingested. However, in this study when acid was applied for 20 min, this treatment did not seem to damage the mucosa since the pH returned to the values before acid challenge when the acid was changed to saline. We have previously shown that ⁵¹Cr-EDTA clearance from blood to gastric lumen increases during luminal pH 1 in animals not stimulated to produce acid (26). This permeability increase is abolished if acid secretion is stimulated with impromidine or pentagastrin. The increase in permeability, however, is reversed as soon as the luminal acid is changed to saline, which contradicts the theory that a sustained damage to the mucosa has occurred. Thus exposure to acid pH 1 for 20 min does not induce sustained damage or sustained reduction in pH_{jm}. We can, however, not draw any conclusions about what effect a longer exposure to luminal acid would have on pH_{jm} or epithelial damage.

In conclusion, the loosely adherent mucous layer does not seem to be important in maintaining the pH_{jm} in the presence of luminal acid (pH 1). Hence an ~80-μm-thick mucous layer (the firmly adherent layer) is adequate for stabilizing a pH gradient with a neutral value at the cell surface while the pH in the lumen is 1. DIDS-sensitive bicarbonate transport from the alkaline blood during acid secretion is essential for maintenance of a neutral pH_{jm}. Prostaglandin-stimulated bicarbonate secretion seems to play only a minor role and only when acid secretion is inhibited and the loosely adherent mucous layer is removed.

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