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Acid Transport through Gastric Mucus

Review based on the doctoral thesis
Acid Transport Through Gastric Mucus;
A Study in vivo in Rats and Mice

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ABSTRACT

The gastric mucosa is frequently exposed to endogenously secreted hydrochloric acid of high acidity. Gastric mucosal defense mechanisms are arranged at different levels of the gastric mucosa and must work in unison to maintain its integrity.

In this work, several mechanisms underlying gastric mucosal resistance to strong acid were investigated in anesthetized rats and mice. The main findings were as follows:

Only when acid secretion occurred did the pH gradient in the mucus gel withstand back-diffusion of luminal acid (100 mM or 155 mM HCl), and keep the juxtamucosal pH (pHjm) neutral. Thus, with no on-going acid secretion and low luminal pH, the pH gradient was destroyed.

Bicarbonate ions, produced concomitant with hydrogen ions in the parietal cells during acid secretion and transported by the blood to the surface epithelium, were carried transepithelially through a DIDS-sensitive transport. Prostaglandin-dependent bicarbonate secretion seemed to be less important in maintaining a neutral pH_{jm}.

Removal of the loosely adherent mucus layer did not influence the maintenance of the pH_{jm}. Hence, only the firmly adherent mucus gel layer, approximately 80 µm thick, seemed to be important for the pH_{jm}.

Staining of the mucus gel with a pH-sensitive dye revealed that secreted acid penetrated the mucus gel from the crypt openings toward the gastric lumen only in restricted paths (channels). One crypt opening was attached to one channel, and the channel was irreversibly formed during acid secretion.

Gastric mucosal blood flow increased on application of strong luminal acid (155 mM HCl). This acid-induced hyperemia involved the inducible but not the neural isoform of nitric oxide synthase. These results suggest a novel role for iNOS in gas-

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tric mucosal protection and indicate that iNOS is constitutively expressed in the gastric mucosa.

INTRODUCTION

The gastric epithelium is regularly exposed to the endogenous aggressive factors acid and degrading enzymes such as pepsin. How the stomach can resist autodigestion, i.e., remain safe and sound when forming a harmful environment for bacteria and inducing enzymatic degradation of proteins, has intrigued physiologists for centuries. Dr Claude Bernard wrote in 1856 that the mucus coating the stomach rendered it impermeable to the acidic gastric juice, as if it were a porcelain vase (1, 2). The question of how it achieves this protective effect has remained unanswered until recently.

In addition to the harsh intrinsic environment, the stomach also faces ingested bacteria, food and drink and sometimes also ulcerogenic drugs, and has to function as a barrier to protect the interior from the external environment.

Acid secretion and regulation

Hydrochloric acid (HCl) is produced and secreted by the parietal cells located in the gastric glands in the corpus part of the stomach. The acid activates the proteolytic enzyme pepsin, creates a bacteriotoxic environment, and initiates degradation of proteins. Histamine, gastrin and acetylcholine stimulate acid secretion and are reported to potentiate the effects of one another (3). Histamine is secreted by the enterochromaffin-like (ECL) cells that are present in the oxyntic glands and diffuses to the H_2 receptors located on the basolateral membrane of the parietal cells. Gastrin is produced by the gastrin (G) cells in the antrum, and is transported to the corpus in the systemic circulation. Gastrin stimulates acid secretion mainly by stimulating the ECL cells to secrete histamine, although gastrin receptors (cholecystokininB; CCKB) have also been found on the parietal cells (4). Acetylcholine is released from the vagal nerves and activates G cells, ECL cells and parietal cells, but its efficacy in stimulating acid secretion varies greatly between species and this effect in humans is very weak. H_2-receptor antagonists inhibit acid secretion, indicating that histamine plays a key role in the stimulation of acid secretion.

When the parietal cells are activated to produce acid, intracellular tubulovesicles are translocated to the apical membrane, thereby increasing the area of the secretory membrane, and the transporter H^+/K^+-ATPase is activated. When stimulated, the parietal cell can secrete an acid with a concentration of 155 mM, i.e., with a pH of Ξ0.8 (5). For every hydrogen ion formed, one bicarbonate ion is created through the enzyme carbonic anhydrase. To prevent alkalinization of the parietal cell during acid secretion, basolateral extrusion of bicarbonate is important. This occurs mainly through the Cl^-/HCO_3^- exchanger. The bicarbonate enters the bloodstream and can be measured as an alkaline tide in arterial blood and in urine after a meal or histamine stimulation of acid secretion (5, 6).

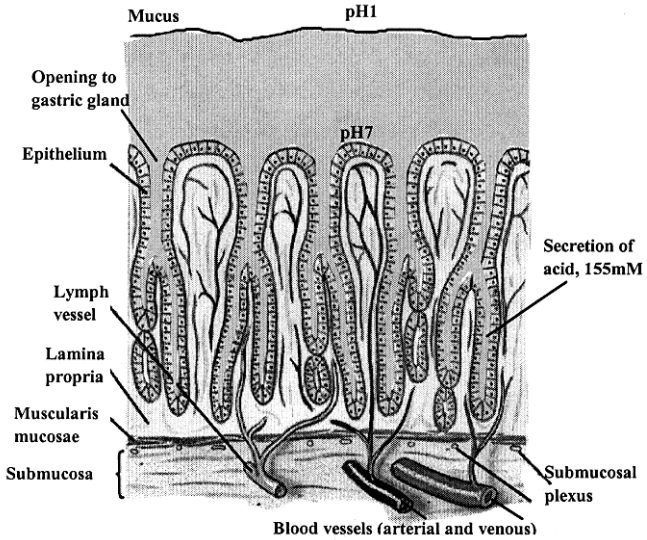

Fig. 1. Schematic drawing of a cross-section of the gastric corpus mucosa with adhering mucus layer and blood supply. N.B. acid must pass through the mucus to the gastric lumen.

To exert its function in the gastric lumen, the acid must pass through the mucus layer (Fig. 1). How this occurs without acidification of the mucus layer itself has not previously been established. The hydrostatic pressure that has recently been measured in the lumina of rat gastric glands might be the driving force for acid transport through the mucus layer (7). This pressure increases from approximately 12 to 17 mmHg on stimulation of acid secretion.

Gastric mucosal defense

The defense mechanisms of the gastric mucosa are crucial for the maintenance of an effective barrier and for preventing the stomach mucosa from digesting itself. The defense is arranged at different levels, which work in concert for effective protection.

The preepithelial level or the first line of defense consists of the mucus layer and bicarbonate secreted into the mucus, creating a pH gradient within the mucus.

The epithelial level consists of intercellular tight junctions and proton and bicarbonate transport systems.

The postepithelial level consists mainly of an effective blood flow and the gastrointestinal autonomic nervous system, the enteric nervous system (ENS).

The preepithelial level

The mucus layer forms a continuous coat over the gastric epithelium. Bicarbonate is secreted from the epithelium into the mucus layer, where it neutralizes acid that is back-diffused from the lumen of the stomach and forms a pH gradient, with a higher pH at the epithelial cell surface (8, 9).

The mucus layer

A continuous layer of mucus gel, secreted by the surface epithelial cells and the mucous neck cells, covers the gastric mucosa. The mucus gel serves as a physical barrier and molecules with the size of pepsin cannot penetrate through diffusion. However, hydrogen ions are able to diffuse through the gel, although their diffusion is approximately four times slower than that through an unstirred layer of equivalent solution (10).

The mucus consists of 5% high molecular weight glycoproteins (10^3 kDa) (11), called mucins, and 95% water together with electrolytes and small amounts of lipids and proteins, including immunoglobulins. The glycoproteins are the gel-forming components of the mucus and consist of a protein core that has highly glycosylated regions. The carbohydrate side chains bound to the protein vary in structure and might influence the physical properties of the mucin. The nonglycosylated regions of the protein core are rich in cysteine and can form inter-mucin disulfide bridges, which create the polymeric structure of the gel by linking the mucins together.

The surface epithelial cells secrete mucins of the MUC5AC type and the mucous neck cells secrete MUC6 mucins. Whether the different mucins exhibit different physical behaviors and physiological functions is still unknown. It has recently been found possible to separate the mucus layer covering and adherent to the corpus mucosa into two different layers, in addition to the loose mucus in the gastric lumen (12). The most luminal of the two layers, the loosely adherent mucus, can be removed by suction or by rubbing with a cotton tip, while the inner layer, the firmly adherent mucus, cannot be removed by this physical means. The physical properties and physiological importance of the different layers are unknown, as is their composition, and it is not known whether they differ in permeability to acid.

The thickness of the mucus layers depends on the secretion of mucins and the degree of erosion and proteolytic degradation of the layers. Mucus secretion is stimulated by agents such as prostaglandins and nitric oxide, whereas the mucus layer is degraded by pepsin (13, 14).

Bicarbonate secretion

The surface epithelial cells secrete bicarbonate into the mucus gel. The bicarbonate neutralizes back-diffused acid and creates a pH gradient in the mucus layer, with a neutral pH at the cell surface when the luminal pH is low. Bicarbonate can be produced from carbon dioxide and water in the gastric mucosal surface epithelial cells by the enzyme carbonic anhydrase (13). Furthermore, Teorell (15) 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 of the mucosa. The capillaries are arranged along the gastric glands and are directed toward the surface epithelium. Thus, during acid secretion, bicarbonate will be transported by the blood to the surface epithelium, where it will be available for transport across the surface epithelial cells into the mucus.

Vagal stimulation (16), prostaglandins (17, 18, 19, 20, 21), gastric distension, and

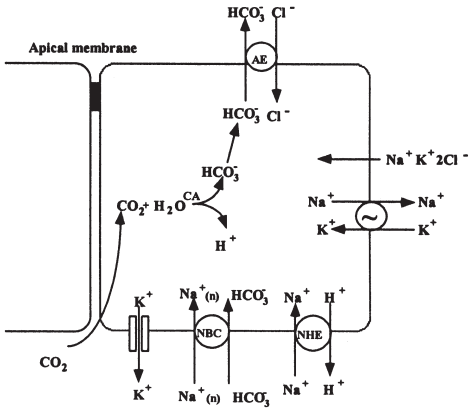

Fig. 2. Schematic drawing of the ion transporters in the gastric surface epithelial cell.

acid in the gastric lumen all increase gastric bicarbonate secretion. Experiments in vitro have shown that gastric bicarbonate secretion is dependent on luminal chloride ions, indicating the presence of an apical Cl^-/HCO_3^- exchanger (22). However, the route by which bicarbonate traverses the surface epithelium during acid secretion has not yet been established.

pH gradient in the mucus gel

A millionfold proton concentration gradient can exist between the gastric lumen and the blood, and pH gradients have been found in the mucus layer covering the gastric mucosa by means of inserted pH-sensitive electrodes (8, 9). The gradient succeeds in keeping the epithelial surface neutral (juxtamucosal pH, pH_{jm}) while in the lumen pH is 2 in both acid-secreting and non-secreting mucosae (9).

Naturally, bicarbonate secretion is necessary for the creation of a pH gradient. Moreover, the mucus layer is crucial for the existence of a pH gradient, since it consists of an unstirred layer in which neutralization of back-diffused acid by secreted bicarbonate can occur. In the Necturus antrum, a luminal mucus layer was shown to be necessary for keeping the juxtamucosal and intracellular pH neutral in the presence of luminal acid (23). The existence of a pH gradient in the gastric mucus during acid secretion is a physiological paradox, since acid, secreted into the gastric glands, has to pass through the mucus gel to reach the gastric lumen.

The epithelial level

Gastric mucosal permeability and cellular transporters

The mucosa of the gastric corpus is a tight epithelium and under normal conditions it is relatively impermeable to transport of luminal contents, including water. The apical cell membrane of gastric surface epithelial cells has a low permeability to hydrogen ions (24, 25), and the tight junctions connecting the surface cells to each

other are even less conductive for ions than is the cellular pathway (26). The paracellular pathway contributes only to approximately 25% of the total tissue conductance. Cellular transporters that transport bicarbonate in a luminal direction and protons toward the buffering blood are important for the viability of the surface cells. Ion transporters located in the gastric surface epithelial cells are illustrated in Fig. 2. Gastric epithelial cells express the Na^+/H^+ exchanger NHE_2 basolaterally. This exchanger is activated when the extracellular pH increases, and might be involved in transporting bicarbonate transcellularly (27).

The postepithelial level

Gastric mucosal blood flow

The gastric mucosal blood flow is an important part of the defense, as the circulating blood dilutes, neutralizes and carries away noxious substances that have managed to overcome the more luminal barriers. The blood stream also has an important function in transporting oxygen, nutrients and gastric hormones to the different mucosal cell types.

The mucosal capillaries are arranged along and in close proximity to the gastric glands (28). This architecture is of special relevance for the oxygen-consuming parietal cells and for the bicarbonate transport from the acid-secreting parietal cells to the surface epithelium. At the level of the gastric epithelium, the capillaries form a honeycomb network around the openings of the gastric pits. Hence, the distance between the capillaries and the surface epithelial cells is minimized. Progressing toward the gastric epithelial surface, the capillaries become increasingly fenestrated, which facilitates transport across the capillary membrane. The capillaries empty into collecting mucosal venules oriented perpendicular to the luminal surface.

The gastric mucosal blood flow is regulated at the level of the submucosal arterioles and is under the intricate control of the central and enteric nervous systems, autocrine and paracrine regulation of hormones and growth factors, and mucosal production of eicosanoids. For example, gastric hyperemia caused by mucosal acidification by luminal acid and barrier-breaking substances (e.g., ethanol or sodium taurocholate) is due to activation of sensory afferent nerves, leading to release of calcitonin gene-related peptide (CGRP) in the vicinity of the submucosal arterioles and generation of nitric oxide (NO) (29). Impairment of this neurally mediated hyperemic response through disruption of the sensory afferent nerves, antagonism of CGRP receptors, or blockade of NO synthesis, results in a significant increase in the susceptibility of the gastric mucosa to damage (30), indicating the importance of a sufficient mucosal blood flow for the preservation of the mucosal barrier.

Nitric oxide and mucosal blood flow

Nitric oxide has been reported to influence different components of gastric mucosal defense, such as mucosal blood flow, mucus secretion and mucosal permeability (31). NO is produced together with L-citrulline from the amino acid L-arginine and molecular oxygen under enzymatic catalysation. In mammals, three isoforms of the

NO-synthesizing enzyme, nitric oxide synthase (NOS), encoded by different genes, have been identified (32). The constitutively expressed enzyme is Ca^{2+}-dependent and can be divided into isoforms associated with neurons (nNOS, type I) and isoforms present in the endothelium lining the vasculature (eNOS, type III). NO produced in the endothelial cells diffuses to the underlying vascular smooth muscle cells, where it stimulates soluble guanylate cyclase, leading to elevated cGMP levels, and relaxation of the vascular smooth muscle. The inducible NOS (iNOS, type II) is Ca^{2+}-independent and needs a stimulus (cytokines, lipopolysaccharides) for expression in specific cell types, e.g., macrophages, neutrophils, and endothelial and epithelial cells. It is generally believed that the constitutively expressed isoforms are responsible for the normal physiological effects of NO, whereas iNOS is activated in different pathophysiological states (31).

The gastric mucosal surface cells have been shown to contain a large quantity of NOS that resembles the nNOS isoform (33). The involvement of this epithelial NOS in gastric mucosal defense has not yet been investigated.

AIMS

The overall aim of this investigation was to further elucidate the mechanisms underlying gastric mucosal resistance to strong acid. More specifically, the following questions were addressed:

How does endogenously secreted acid penetrate the mucus gel without disrupting the pH gradient?

Which gastric mucosal defense mechanisms are crucial for the maintenance of the juxtamucosal pH when luminal pH is 1?

Which NOS isoform is involved in the acid-induced hyperemia?

MATERIALS AND METHODS

The *rats* Male Sprague-Dawley or F1 hybrids of Lewis and DA, (150–290g) were anesthetized with an i.p. injection of Inactin® (120 mg kg^{-1} bw). Spontaneous breathing was facilitated by a short cannula placed in the trachea.

The *mice* (C57BL/6×129SvEv controls or mice with a deactivated gene for iNOS or nNOS) was induced by spontaneous inhalation of isoflurane (Forene(, Abbott Scandinavia AB, Kista, Sweden). The inhalation gas was administered continuously through a breathing mask (Simtec engineering) and contained a mixture of 40% oxygen, 60% nitrogen, and Ξ2.2% isoflurane. A cannula containing heparin (12.5 IU ml^{-1}) dissolved in isotonic saline was placed in a carotid artery to monitor blood pressure. Ringer's solution was administered intravenously (1 ml h^{-1} (femoral vein, rat) or 0.35 ml h^{-1} (jugular vein, mouse), to prevent dehydration, 120 mM NaCl, 2.5 mM KCl, 0.75 mM $CaCl_2$ and 25 mM $NaHCO_3$).

The body temperature was maintained at 37.5±0.5°C by means of a heating pad controlled by a rectal thermistor. The preparation of the gastric mucosa for intravital

microscopy has been described previously (34). Briefly, exteriorization of the mucosa through a midline abdominal incision was followed by an incision along the greater curvature in the forestomach. The animal was placed on a Lucite table with a part of the corpus of the stomach loosely draped over a truncated cone in the center of the table, with the mucosal surface facing upwards. A "mucosal chamber" with a hole in the bottom corresponding to the position of the cone, was fitted over the mucosa, exposing approximately 1.2 cm^2 of the rat gastric mucosa and 0.28 cm^2 of the mouse mucosa through the hole. The mucosal chamber did not touch the mucosa, in order not to impair blood flow, and the edges of the hole were sealed with silicon grease. The chamber was filled with 5 ml (rat) or 3 ml (mouse) of unbuffered 0.9% saline, maintained at 37°C by means of circulating warm water in a jacket in the bottom of the chamber. The saline was replaced at regular intervals of 10–15 min and titrated (Autob¸rette ABU 91) to the initial pH of the saline and is presented as microequivalents secreted into the chamber per minute and cm^2 ($\mu Eq\ min^{-1}\ cm^{-2}$).

The animals were allowed to rest for at least one hour after completion of the surgical procedures, and the experiments were not commenced until the mean arterial blood pressure, blood flow and acid secretion were stabilized.

All experiments were approved by the Uppsala University Ethical Committee for Animal Experiments.

Juxtamucosal pH

The juxtamucosal pH was measured with hydrogen ion-selective microelectrodes, inserted into the mucus gel at an angle of 30-40° to the mucosa by means of a micromanipulator, and placed just above the surface epithelium under supervision through a stereomicroscope. To investigate the importance of blood-borne bicarbonate originating from the parietal cells during acid secretion, pH_{jm} was measured in rats during topical application of 100 mM HCl, low acid secretion (not stimulated / ranitidine inhibited acid secretion, 1 mg kg^{-1}, iv), stimulated acid secretion (pentagastrin 40 µg $kg^{-1}\ h^{-1}$) or a continuous iv infusion of $NaHCO_3^-$ (5 mmol $kg^{-1}\ h^{-1}$, iv). The influence of endogenous prostaglandins on pH_{jm} was investigated by pretreatment with indomethacin (3 mg kg^{-1}, iv). To investigate the route by which bicarbonate passes through the cells, the apical Cl^-/HCO_3^- exchanger was inhibited with DIDS (4,4´-diisothiocyanostilbene-2,2´-disulfonic acid, 0.5 mM luminally for 15 min) during stimulated acid secretion.The significance of the loosely adherent mucus layer on pH_{jm} was studied by removing this layer before luminal acid was applied.

Glass tubing (borosilicate tubing with omega dot, OD 1.2 mm, ID 0.9 mm) was pulled with a pipette puller (pp-83) to a tip diameter of 1–3 µm. The microelectrodes were siliconized at 200°C with tributylchlorosilane and stored at 100°C. They were filled up to a distance of approximately 300 µm from the tip with a proton cocktail (hydrogen ion Ionophore II-Cocktail). The remaining part of the electrode was filled with HEPES buffer at pH 7.4, connected by an Ag/AgCl wire to a dual differential electrometer with a high input impedance (FD223), and put in a

pipette holder (MEH3SF 1.2). The reference electrode was filled with 3 M KCl, connected by an Ag/AgCl wire to the ground of the electrometer, and placed in the saline covering the gastric mucosa. To eliminate electrical disturbances, the experiments were performed in a Faraday cage.

The electrodes were calibrated before and after the experiments in iso-osmolar solutions (310 mOsm) with a pH of 1.5–8 at 37°C.

Mucosal permeability

Mucosal permeability of the corpus mucosa during topical application of 100 or 155 mM HCl, was determined by measuring the clearance of ^{51}chromium-labeled EDTA (^{51}Cr-EDTA) from blood to gastric lumen (35). The technique appears to provide a highly reproducible measure of mucosal integrity and has the advantage that each animal can serve as its own control (36, 37, 38). In control rats, pentagastrin or impromidine (histamin H_2 receptor agonist) acid secretion stimulated rats (40 µg kg^{-1} $^{h-1}$ or 500 µg kg^{-1} h^{-1} respectively) or rats receiving continuous infusion of $NaHCO_3^-$ (5 mmol kg^{-1} h^{-1}, iv), 50–75 µCi ^{51}Cr-EDTA was injected as a bolus dose (0.5 ml), followed by a continuous intravenous infusion of ^{51}Cr-EDTA (10–30 µCi ml^{-1} in the Ringer solution) at a rate of 1.0 ml h^{-1}. Four 0.2 ml blood samples were drawn during the experiment at intervals of approximately 30 min. After each blood sample withdrawal, the blood volume loss was compensated for by injection of a 10% Ficoll 400 solution in saline. The blood sample was centrifuged and 50 µl of the plasma was removed for measurements of radioactivity (counts per minute, cpm). The gastric mucosa was covered with isotonic saline, which was replaced every 15 min. The luminal solution and the blood plasma were analyzed for ^{51}Cr activity in a gamma counter (1282 Compugamma CS). In each experiment the various ^{51}Cr-EDTA activities in blood plasma were plotted against time and a straight line was drawn between the two nearest values. Each clearance value was calculated by dividing each individual effluent cpm value by a corresponding plasma cpm value. Clearance was calculated as:

$$\text{Clearance} = \frac{\text{lumen sample (cpm ml}^{-1}) \times \text{sample volume (ml)} \times 100}{\text{plasma (cpm ml}^{-1}) \times \text{tissue weight (g)} \times \text{time (min)}}$$

and is expressed as ml min^{-1} 100 g^{-1} wet tissue weight.

Blood flow measurements

Laser-Doppler flowmetry (LDF, Periflux Pf 2, Pf3 or Pf 4001) was used for blood flow measurements. The laser light is guided to the tissue by an optical fiber (standard probe, diameter = 0.7 mm) and the backscattered light picked up by a pair of fibers of the same size. With this technique blood flow is determined as a voltage output or perfusion units. Blood flow was recorded continuously throughout the experiments from the mucosal side of the stomach, with the probe 0.5–1 mm above the surface in the saline solution. Validation of the accuracy of the LDF technique for the gastrointestinal application has been performed earlier (39). To investigate if

the effect of luminal acid (HCl 100 mM or 155 mM) on mucosal blood flow was dependent on acid secretory state / blood-borne bicarbonate, LDF was measured in rats with low acid secretion, stimulated acid secretion (pentagastrin, 40 µg kg^{-1} h^{-1} or impromidine, 500 µg kg^{-1} h^{-1}) or low acid secretion with continuous infusion of $NaHCO_3^-$ (5 mmol kg–1 h–1, iv). To investigate the role of endogenous NO production in this hyperemia, and to clarify which NOS that was involved, rats received 10 mg kg^{-1} i.v. bolus followed by 3 mg kg^{-1} h^{-1} continuous iv infusion of L-NNA (N^{ω}-nitro-L-arginine, unspecific NOS inhibitor), L-NIL (L-N6-(1-iminoethyl)-lysine, specific iNOS inhibitor) and SMTC (S-methyl-L-thiocitrulline, specific nNOS inhibitor). The influence of topical application of 155 mM HCl on mucosal blood flow was also investigated in control mice or genetically manipulated mice with inactivated gene for either iNOS or nNOS.

Visualization of acidic channels in the mucus gel

A camera (Canon Ftb, with film Kodak Ektachrome 320T) was connected to the stereomicroscope (Leica MZ12) and the gastric mucosa was transilluminated with light from a 150 W light source guided by fiberoptics. The pH-sensitive dye Congo red (1 mM, blue below pH 3 and red above pH 5.2), was applied topically to stain the mucus gel in control rats, acid secretion stimulated rats (pentagastrin 40 µg kg^{-1} h^{-1} iv), acid secretion partly inhibited (omeprazole 400 µmol kg^{-1} bg daily for seven consecutive days) and acid secretion completely inhibited (omeprazole 200 µmol kg^{-1} bg every 8th hour for seven consecutive days). Thirty minutes after application, the Congo red was rinsed off and replaced by saline.

Interactions between Congo red and samples of the two different mucus layers were investigated by spectrophotometric measurements (Lambda 2) at 500 nm.

Real-time RT-PCR

RNA was isolated from scrapings of the mouse gastric mucosa (iNOS+/+ or –/–) to investigate if iNOS expression was detectable and possibly upregulated by anesthesia, surgery or luminal acid (HCl, 155 mM). cDNA synthesis was performed with the Reverse Transcription System. The LightCycler FastStart DNA Master SYBR Green I was used for quantitative analyses of the generated cDNA. Calculations were performed as follows: The CT represents the PCR cycle at which an increase in fluorescence above a baseline signal can be detected. The CT value was used to calculate the amount of PCR product in comparison with the internal control, G6PDH. The CT value for G6PDH was subtracted from the iNOS CT value to obtain the mean O-CT in each experimental group.

Statistical analysis

The results are expressed as means ± SE. For statistical evaluations of differences between data within a group, analysis of variance (ANOVA) for repeated measures was used, while ANOVA for multiple comparisons was performed when comparing data between groups. ANOVA was followed by Fisher's protected least-significant

difference test. Differences in pH_{jm} within the same group and between groups of animals were evaluated statistically by analysis of variance in medians (Mann-Whitney test). To compare single values, Student's t-test for paired or unpaired data was used. All statistical calculations were performed with the software Statview II™ SE Graphics (Abacus Concepts Inc). The differences were regarded as significant if $p<0.05$.

RESULTS AND DISCUSSION

The gastric mucosa is continuously exposed to high acidities and requires efficient defense mechanisms working in unison for its preservation. The ambition with the present studies was to increase the understanding of how the gastric mucosa is able to resist the strong luminal acid to which it is exposed on a regular basis. More specifically, pre- (juxtamucosal pH, mucus layer, acid and bicarbonate transport), and postepithelial defense mechanisms (mucosal blood flow), as well as the permeability of the gastric mucosa, were investigated.

What is the pH at the epithelial cell surface during acid secretion and exposure to strong luminal acid?

A mucus layer wherein a pH gradient can be formed covers the entire gastric mucosa. The pH gradient protects the gastric mucosa against back-diffusing acid from the lumen. Earlier studies of the pH gradient and the pH at the gastric epithelial cell surface (pH_{jm}) were generally conducted in a non-acid-secreting situation, and a total collapse of the gradient was observed when the luminal pH decreased below 1.4 (8, 40). However, luminal acidity is a result of endogenous acid secretion, and investigation of the pH gradient during stimulation of acid secretion therefore seems highly relevant. In addition, acid secretion has been found to have a protective effect on mucosal resistance against luminal acid, since a smaller fall in potential difference was found in acid-secreting than in non-acid-secreting mucosae following topical application of acid (41). Results from our laboratory showed the existence of a pH gradient during acid secretion in the presence of luminal acid at pH 2 (9).

Acid secretion is stimulated by the smell of food or even by thinking of food (the cephalic phase, vagus activation). During the cephalic phase, the stomach might be empty, with no luminal contents to buffer the produced acid, resulting in a dramatic drop of luminal pH. In addition, in between meals, the gastric lumen pH can be very low, even though acid secretion is not stimulated (42). We mimicked these conditions and applied acid with a pH of 1 luminally in control rats and in rats in which acid secretion was inhibited or stimulated (43, 44). We found that in the control rats, with low acid secretion, pH_{jm} decreased significantly to approximately 1.6 ± 0.2 during application of luminal pH 1 (Fig. 3). When acid secretion was inhibited with ranitidine and HCl at pH 1 was applied topically, a similar drop of pH_{jm} to 2.2 ± 0.5 was observed (not shown in the figure). In both situations, pH_{jm} returned to neutrality when the acid was changed to saline, which is not consistent with initiation of a

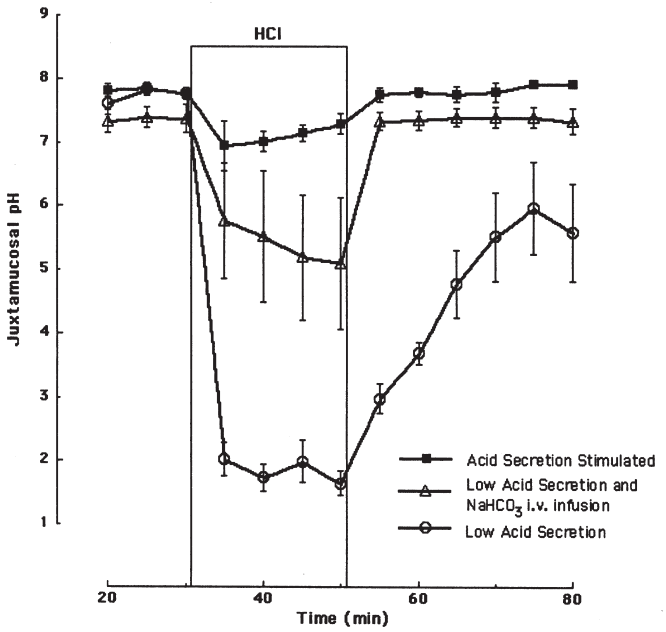

Fig. 3. Juxtamucosal pH before topical application of 100 mM HCl, in the presence of this acid, and after its removal in rats with low acid secretion ○, stimulated acid secretion ■ or low acid secretion and i.v. infusion of $NaHCO_3$ △. Values are means ± SE of 5-min periods.

sustained injury of the mucosa. However, during pentagastrin-stimulated acid secretion the pH of the gastric epithelial cell surface was neutral even when HCl in a concentration of 100 mM was applied topically (Fig. 3). It seems reasonable to conclude that ongoing acid secretion is a prerequisite for preservation of a functional intra-mucus pH gradient in the presence of luminal pH 1 and that a blood-borne alkaline tide provides the gastric epithelium with the HCO_3^- needed for preservation of a neutral pH_{jm}.

Bicarbonate transport

Bicarbonate needs to be persistently secreted from the surface epithelial cells to meet and neutralize back-diffused acid. Gastric bicarbonate secretion is difficult to measure during acid secretion, since the set-up has to be CO_2 impermeable. Because of this inconvenience, bicarbonate secretion in the stomach has most often been measured during inhibition of acid secretion. In the studies presented here bicarbonate secretion was estimated indirectly by measuring the pHjm at the epithelial cell surface in both acid-secreting and non-secreting mucosae.

The alkaline tide

When acid secretion is stimulated, the blood passing the parietal cells on its way to the surface epithelium is alkalinized by bicarbonate. The arrangement of the capil-

laries along the gastric glands is optimal for transporting this alkaline tide. As discussed earlier, we found that on-going acid secretion is required for preservation of a neutral pHjm in the presence of luminal pH 1, indicating that the blood-borne bicarbonate is important for neutralization of back-diffused acid in the mucus. In accordance with this finding, Kivilaakso (45) reported that iv infusion of $NaHCO_3$ causing high-HCO_3^- metabolic alkalosis significantly decreased the incidence of acid-induced mucosal injury. Respiratory alkalosis of similar degree had no protective effect against luminal acid, indicating that it is HCO_3^- and not the alkalinity per se that is important.

When HCO_3^- was infused intravenously in rats with no ongoing acid secretion, pH_{jm} was only slightly reduced by topical application of acid at pH 1 (Fig. 3). The above results confirm that blood-delivered bicarbonate is involved in preserving the pH gradient during acid secretion. However, the pH gradient was not as efficient as in the acid-secreting groups, probably because the HCO_3^- concentration was not as high as it is locally in the mucosal capillaries during endogenous acid secretion. In dogs, the concentration of arterial bicarbonate increased by 5 mM after feeding (46), and the local concentration of bicarbonate in the gastric mucosa must be much higher. However, the basal pH_{jm} before application of luminal acid was independent of the acid secretory status, indicating that the alkaline tide per se did not stimulate a basal bicarbonate secretion (43, 44).

Prostaglandin-stimulated bicarbonate secretion

How important is the prostaglandin-dependent bicarbonate secretion for the maintenance of pH_{jm}? When acid secretion and prostaglandin synthesis were both inhibited, we found that the pH at the cell surface was slightly reduced in the control situation before acid was applied in the lumen (43). However, after topical application of HCl (155 mM), inhibition of prostaglandin synthesis did not further reduce pH_{jm}, 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. These results indicate that gastric prostaglandin-dependent bicarbonate secretion is involved in basal bicarbonate secretion when no acid secretion occurs, but does not seem to be important in neutralizing back-diffused acid during acid secretion.

DIDS-sensitive bicarbonate transport

Our results convincingly show that the pH gradient is better preserved in an acid-secreting stomach than in a resting one (43, 44). Thus, during endogenous acid secretion the gastric microcirculation supplies the surface epithelial cells with the bicarbonate needed for juxtamucosal neutralization. When DIDS had been applied luminally and acid secretion stimulated with pentagastrin, pH_{jm} decreased dramatically when HCl at pH 1 was applied topically in the lumen (to pH 1.4, Fig. 4) (43). This indicates that bicarbonate is transported through a DIDS-sensitive mechanism. Experiments in vitro have shown that gastric bicarbonate secretion is dependent on

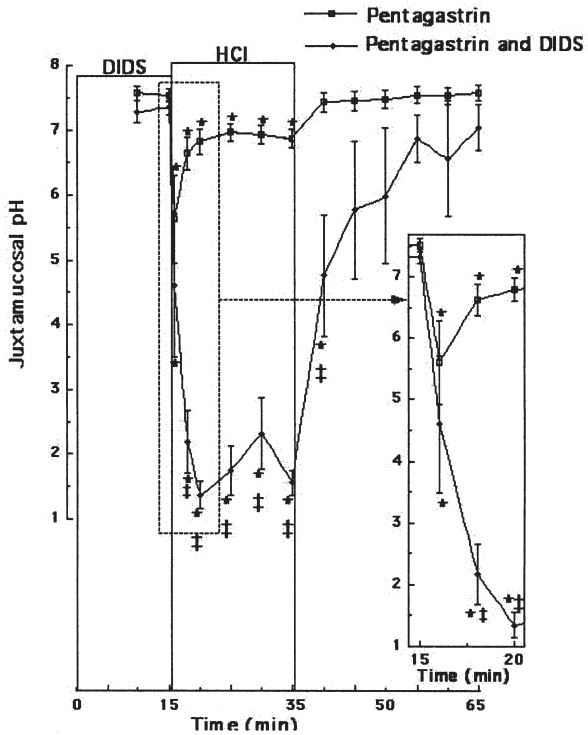

Fig. 4. Juxtamucosal pH before topical application of 100 mM HCl, in the presence of this acid, and after its removal in pentagastrin □ and pentagastrin plus DIDS ◆ treated groups. Values are means ± SE of 5-min periods (except for the first 5 min after acid application, during which one 1-min period followed by two 2-min periods were used).

luminal chloride ions, indicating the presence of an apical Cl^-/HCO_3^- exchanger (22). Bicarbonate has been reported to enter the surface epithelial cells basolaterally in cotransport with sodium (47, 48). DIDS is an inhibitor of the Cl^-/HCO_3^- exchanger as well as of Na^+/HCO_3^- cotransport. In the parietal cell, a basolateral Cl^-/HCO_3^- exchanger has been found and inhibition of this transporter inhibited acid secretion (49). Since the acid secretion was not reduced after DIDS treatment, no major amount of the inhibitor appeared to have entered the circulation. Thus, DIDS applied luminally to the gastric mucosa probably mainly inhibited the apical Cl^-/HCO_3^- transporter in the surface epithelial cells. However, it is also possible that DIDS might influence the paracellular transport of HCO_3^-, which normally is very restricted (13).

How important are the mucus layers?

As early as in 1856 Claude Bernard stressed the importance of the mucus covering the gastric mucosa in the defense against the acidic gastric juice. Today we know that the mucus layer covers the gastric mucosa as a continuous coat (11), and it is

believed that it contributes to the preepithelial defense mechanisms in different ways. The pH gradient that has been demonstrated in the mucus layer is probably dependent on the unstirred layer created within the mucus itself, in addition to secreted bicarbonate, neutralizing back-diffused acid (8, 9). It has been difficult histologically to study the mucus, since it easily becomes dehydrated and eroded during the preparation. In our in vivo model, we have previously observed two separate mucus layers covering the gastric mucosa, a loosely adherent layer that is easily removed by suction, and a layer that firmly adheres to the gastric epithelium (12). The latter layer cannot be removed by suction or rubbed off with a cotton tip. The thicknesses of the different layers can be measured with micropipettes inserted into the mucus. With this method the total mucus thickness (loosely adherent plus firmly adherent) was found to be 189±11 μm and the thickness of the firmly adherent layer (measured directly after removal of the loosely adherent layer) was 80±5 μm (12). An increase in thickness is a normal defensive response to luminal insult, and it is generally believed that the thicker the mucus, the better the protection (11).

How removal of the loosely adherent mucus layer influenced pH_{jm} in the presence of HCl, pH 1, in the lumen was investigated (43). The results clearly show that the loosely adherent layer is of minor importance in maintaining the pH_{jm}, since removal of this mucus layer caused no further changes of pH_{jm}, compared to the control group. This seems plausible, since the loosely adherent layer most probably is removed from the mucosal surface by ingested food, which also stimulates acid secretion. One 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 mucus layer (firmly and loosely adherent), the depth of this gradient in the mucus during acid secretion (Ξ100 μm closest to the epithelial surface, luminal pH 2 or 3) was found to correspond quite well with the thickness of the firmly adherent mucus layer (9, 12, 52). This supports the finding from the present study that the loosely adherent layer is not important for preserving a pH gradient. The only situation where we found that the loosely adherent layer influenced pH_{jm} was when both acid secretion and prostaglandin synthesis were inhibited (43). Under these conditions removal of the loosely adherent mucus layer resulted in a slower recovery to a neutral pH after application of luminal acid. This could indicate that in a non-acid secretion situation, prostaglandin-stimulated bicarbonate secretion contributes to neutralization of back-diffused acid. However, we have found that the thickness of the inner firmly adherent mucus layer is increased after topical treatment with PGE_2, and correspondingly decreased after pretreatment with indomethacin (52). Hence, the remainder of the firmly adherent mucus layer is perhaps too thin to establish an efficient neutralization zone for acid and bicarbonate. Further studies are required to elucidate the importance of the thickness of the firmly adherent mucus layer in maintaining the pH_{jm}.

On the basis of these results, the simplified assumption that the thicker the mucus layer, the better the protection needs to be revised and efforts should be directed toward understanding the regulation and contents of the firmly adherent mucus layer.

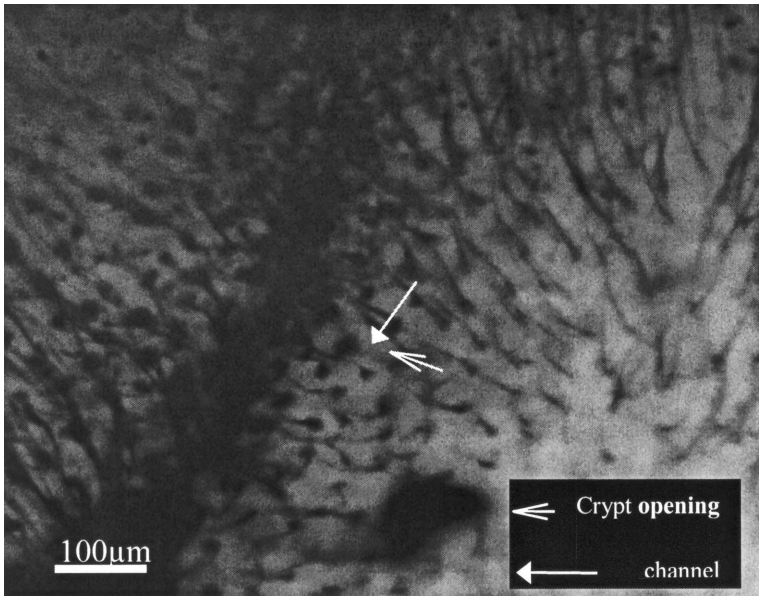

Fig. 5. Blue colored crypt openings with attached channels in the mucus covering an acid secreting gastric mucosa seen from above.

How is acid transported through gastric mucus?

Acid is secreted into the lumen of the gastric glands and has to pass through the mucus layer to reach the gastric lumen. Since we invariably found a neutral or slightly alkaline pH at the epithelial cell surface during acid secretion, the acid must have penetrated the mucus layer from the site of production to the lumen of the stomach without acidifying the epithelial cell surface. Thus, unrestricted diffusion as a major acid transport mechanism can be ruled out.

In a previous study, acidic spots in the mucus gel during acid secretion were observed after staining the mucus with the pH-sensitive dye Congo red (blue, pH<3; red, pH>5.2) (53). We have now for the first time identified channels for transport of acid through the mucus layer covering the gastric mucosa after staining the mucus with Congo red (Fig. 5) (54). This treatment revealed blue-colored crypt openings with attached thread-like channels with an outer diameter of 5–7 µm in the mucus during acid secretion. These channels are most probably created by high intraglandular pressure (7), pushing acid and mucus from the gland lumen into the firmly adherent mucus layer and leaving a path with a structure different from that of the surrounding gel. The existence of channels transporting glandular secretions to the gastric lumen would also explain how the large molecules pepsinogen/pepsin and intrinsic factor traverse the mucus layer, since diffusion is restricted for molecules of their size (11, 13).

A channel occasionally became attached to the micropipette and could be pulled out of the mucus layer, indicating that the wall of the channel has a firm configura-

tion (54). After the mucus had been stained with Congo red, the channels, could be seen to be pushed in front of the microelectrode or moved sideways. In accordance with these observations, we have in all our measurements of pH gradients and pH_{jm} only once been able to penetrate a channel with a microelectrode and record a low pH during acid secretion (9, 43, 44). The structure of the channel wall is not known. However, in the corpus part of the stomach, as discussed above, two cell types secreting different mucins have been identified, surface mucous cells and mucous neck cells (MUC5AC and MUC6, respectively) (50, 51). The two types of mucins differ considerably in their histochemical properties (50). In absorbance experiments on samples of the different mucus layers, we found that Congo red interacted significantly more with the loosely adherent layer than with the firmly adherent one (54). In addition, Congo red was concentrated at the channel structures or adhered to the channels, irrespective of the state of acid secretion. Thus we hypothesize that the loosely adherent mucus layer and the channel wall consist mainly of the same mucins and that mucus from mucous neck cells is pushed out from the crypts through the channels, forming the loosely adherent layer. In addition, the channel in itself might consist of mucus from the mucous neck cells. Until proper antibodies directed toward the protein core of the rat mucins become available, this question remains speculative.

Studies of acid transport through the mucus have been conducted by many researchers for several decades and resulted in various explanatory models. Schreiber and Scheid (55) suggested a model for transport of protons from the gland to the lumen, where acid was bound to and buffered by the mucus in the glands and was then transported together with the continuously formed mucus toward the lumen. In their model, pepsinogen was also transported within the mucus layer, and when converted into pepsin should degrade the mucus and release the acid. Their model is based upon the acid secretion rate in vitro, which is much lower than the acid secretion measured in vivo (56), and an unrealistically high mucus secretion would therefore be required for buffering and binding secreted acid. Further studies performed by Chu et al. (57) suggested that acid diffuses from its site of secretion toward the lumen since, with an inverted confocal microscope and pH sensitive dyes, they found a reversed pH gradient with pH 5 in the lumen and pH 3.5 at the cell surface during pentagastrin stimulation.

When acid is injected under pressure into mucus in vitro (58), a phenomenon called viscous fingering occurs provided the mucus has a pH above 4. This is a process in which a fluid of lower viscosity, injected into one of higher viscosity, penetrates rather than displaces the stationary solution. Translated into our model, where we have found a high pressure in the gastric glands (7) and a neutral pH in the mucus at the epithelial cell surface (9, 43, 44), the channels we have observed in vivo could represent the viscous fingers demonstrated earlier in vitro. Bhaskar et al. (59) have also found an increase in mucus viscosity as the pH was lowered, which was reversed when the pH increased. However, our channels seem to be independent of pH, as they are irreversible structures also observed when no acid secretion occurs.

Influence of luminal acid on gastric mucosal blood flow and mucosal permeability

We found that when acid secretion was not stimulated, topical application of HCl, pH 1, resulted in a 75% blood flow increase and a threefold increase in mucosal permeability (44). Compared to the baseline value, the blood flow was still significantly higher 20 minutes after the luminal acid had been changed to saline. When $NaHCO_3$ was given i.v. or acid secretion was stimulated with pentagastrin or impromidine, the gastric mucosal blood flow was only increased 25–35% by topical application of HCl, pH 1, and the increase was reversed as soon as the luminal acid was changed to saline. The mucosal permeability was not altered by topical application of HCl, pH 1, in these groups. Again, blood-borne bicarbonate seemed to be vital in the mucosal protection against luminal acid. Combined with the results of the pH_{jm} measurements, these findings indicate that when the concentration of the luminal acid overcomes the bicarbonate secretion needed for neutralization of the acid in the mucus gel, the epithelial surface becomes acidified and hydrogen ions can diffuse into the mucosa as the gastric permeability increases. The increase in mucosal blood flow was significantly greater when low pH_{jm} and increased permeability were detected.

Earlier studies have shown that, in combination with a barrier-breaking substance (e.g., ethanol or sodium taurocholate), acid increases the gastric mucosal blood flow and causes hemorrhagic lesions when applied luminally to the gastric mucosa (29, 60). The mechanism underlying this acid-induced gastric mucosal hyperemia involves activation of sensory afferent nerves, leading to release of CGRP in the vicinity of the submucosal arterioles (29). CGRP acts on the vascular endothelium lining these vessels, resulting in generation of NO (30). NO diffuses to the underlying vascular smooth muscle cells, where it stimulates soluble guanylate cyclase, leading to elevated cGMP levels and relaxation of the vascular smooth muscle. This increase in gastric blood flow can be blocked with a non-selective NOS inhibitor (61). In addition, impairment of the neurally mediated hyperemic response through disruption of the sensory afferent nerves, antagonism of CGRP receptors, or blockade of NO synthesis, results in a significant increase in the susceptibility of the gastric mucosa to damage (29, 61, 62). We found that the gastric mucosal blood flow was increased by luminal acid alone, without the addition of a barrier-breaker. This hyperemia was greater when no acid secretion occurred. Whether the mechanism underlying this acid-induced hyperemia also involves CGRP-releasing nerves is not known. How these nerves are activated to release CGRP when no mucosal acidification takes place is also unknown.

The role of inducible NOS in the gastric hyperemia in response to luminal acid

We found that iNOS is involved in the gastric hyperemia occurring in response to luminal acid and that iNOS has a protective role in the gastric defense in this context (63). Our results demonstrated that application of luminal acid (HCl, 155 mM) on the gastric mucosa caused a hyperemia that was blocked in iNOS –/– mice (Fig.

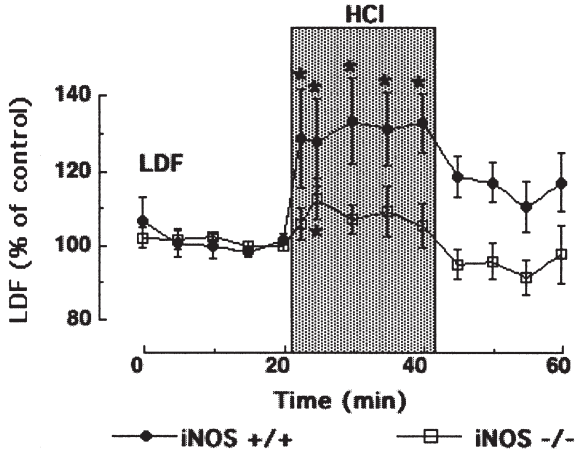

Fig 6. Gastric mucosal blood flow (LDF%) in control mice (+/+, n=6) and iNOS knockout mice (–/–, n=6) presented as percent of control period, time 15–20 min. HCl (155 mM) was applied luminally. Values are expressed as mean ± SE. * $p<0.05$ compared with the control period before acid application.

6) and by selective inhibition of iNOS (L-NIL treated rats). The hyperemia in response to luminal acid was not altered in nNOS –/– mice or by selective inhibition of nNOS (SMTC treated rats). Earlier studies have failed to reveal the presence of iNOS in the gastric mucosa under normal conditions (33, 64). However, using real-time RT-PCR in mouse gastric mucosa we found iNOS mRNA expression at a level not influenced by anesthesia, preparation of the gastric mucosa, or luminal acid (63). These results indicate the possibility of posttranscriptional regulation of iNOS activity that differs from the regulation occurring in macrophages. Interestingly, iNOS has been found to exist in a constitutive way (or to be constantly induced) in other organ systems that are exposed to the exterior, and therefore would function as an effective barrier inhibiting the entrance of unwanted agents into the system. In the epithelial cells of the respiratory tract (in airways and paranasal sinuses) (65, 66), the high levels of tonic expression of iNOS and NO production have special relevance for airway defense mechanisms (67, 68). iNOS is also expressed in a seemingly constitutive way under normal conditions in the esophageal epithelium (69), in parts of the small intestine (duodeneum and ileum) (70, 62), and in occasional patches in the colon (71), and is possibly involved in the mucosal defense. The constant presence of iNOS in these organ systems may reflect the regular challenge by for example bacteria, viruses and fungi.

CONCLUSION

In summary, the resistance of the gastric mucosa to strong acid is dependent on a network of defense mechanisms cooperating at different levels. A neutral pH_{jm} is

dependent on the alkaline tide supplying a sufficient amount of bicarbonate and probably also by a firmly adherent mucus layer. Acid secretion is transported in restricted channels through the mucus, enabling pH_{jm} to remain neutral even during ongoing acid secretion. Luminal acid increases gastric mucosal blood flow through iNOS activation.

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