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Dynamic Involvement of the Inducible Type of Nitric Oxide Synthase in Acid-Induced Duodenal Mucosal Alkaline Secretion in the Rat

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It has previously been shown that mucosal nitric oxide synthase (NOS) is involved in acid-induced duodenal mucosal alkaline secretion. The primary aim of the present study was to elucidate which isoform of NOS is responsible in rats. Immunohistochemistry showed that inducible NOS (iNOS) was constitutively expressed in villous epithelial cells. Exposing the duodenal mucosa to 10 mM HCl resulted in an increased duodenal mucosal alkaline secretion. This response was totally inhibited by intraluminal administration of a selective inhibitor of iNOS (L-N^6-1-iminoethyl-lysine). One hour after the acid exposure, western blot technique showed a marked increase in mucosal iNOS expression. A second acid exposure resulted in a further stimulation of alkaline secretion. These data suggest that exposure of the duodenal mucosa to HCl initiates an increased mucosal alkaline secretion, via NO synthesis mediated by iNOS located in the epithelial cells of the villi. In addition, luminal acid stimulates expression of iNOS.

KEY WORDS: acid; bicarbonate; duodenum; intestine; nitric oxide; NOS.

The ability of the duodenal mucosa to secrete bicarbonate into an unstirred layer of mucus covering the epithelium has been known for about two decades (1), and it has been convincingly shown that local acid exposure increases the mucosal alkaline output (2). Despite intense research, the regulation of such acid-induced duodenal mucosal alkaline secretion is still not fully understood. Prostaglandins, humoral factors, as well as local axon reflexes have been suggested to be involved (2). In addition, it has been shown that luminal acid induces duodenal mucosal synthesis of nitric oxide (NO), which in turn stimulates mucosal bicarbonate secretion (3). There are three principal isoforms of nitric oxide synthase (NOS) described, all of which catalyze the degradation of L-arginine to L-citrulline and NO (4): the endothelial type (eNOS), mainly present in the endothelium of vessels; the neuronal type (nNOS), located principally to nerves; and finally the inducible type (iNOS), which is expressed in several tissues and organs during inflammatory states. The eNOS and nNOS isoforms are constitutive and can be rapidly activated by changes in intracellular Ca^{2+} to produce NO for several minutes (4). They contrast to the inducible isoform, which, when expressed, is suggested to produce NO for

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several days even at the low Ca^{2+} levels found in resting cells (4). Nevertheless, the dogma that NOS is either constitutive or inducible has been challenged by several authors (4). It has been observed that expression of eNOS as well as nNOS can be upregulated (4) and that iNOS can be constitutively expressed to serve physiologic roles in uninflamed tissues, including epithelium of paranasal sinuses, airways, and kidneys (4, 5). The presence of constitutive nitric oxide synthases as well as inducible nitric oxide synthase has been shown in gastrointestinal tissues (6, 7), and it seems that the inducible isoform could be constitutively expressed in enterocytes even in the absence of inflammatory stimuli (8).

The aim of the present study was to elucidate which isoform of NOS is involved in the regulation of acid-induced duodenal mucosal alkaline secretion. Data were obtained indicating that iNOS is the principal isoform involved. A second aim was, therefore, to investigate if luminal acid exposure can induce an increased expression of iNOS.

MATERIALS AND METHODS

General. The project was approved by the Animal Ethics Committee of Göteborg University. Male Sprague-Dawley rats (Möllegaard Breeding Center Ltd., Ejby, Denmark), weighing 230–270 g, were kept in thermostatically controlled rooms with artificial light between 06:00 and 18:00 h. Experiments were performed on rats that were fasted overnight (12–16 h) with free access to tap water.

For induction of anesthesia, methohexital 50 mg/kg was given intraperitoneally. Free airways were ensured by a tracheal cannula. The left femoral artery was catheterized and connected to a Statham P23Dc pressure transducer (Statham, Hato Rey, Puerto Rico). The recorded pressure was integrated by a microcomputer and expressed as the mean arterial pressure over 5 min. The left femoral vein was cannulated for drug administration. Anesthesia was maintained with α-chloralose (50 mg/kg, given intravenously as a bolus injection, followed by continuous infusion at a rate of 25 mg/kg/hr). A slow isotonic intraarterial infusion (1 ml/hr) of 0.03 M $NaHCO_3$ containing 1.7% glucose (w/v) was given throughout the experiments in order to avoid dehydration and acidosis. A heating lamp and a heating pad, both thermostatically controlled, kept the body temperature at 38°C.

Abdominal Preparation. A pH-stat titration technique was used to measure the mucosal alkaline secretion from a duodenal segment. This technique has been described in detail elsewhere (9), but a brief summary will be given here. The abdomen was opened by a midline incision and a 1.5-cm duodenal segment (the proximal end 1 cm distal to the pylorus), with intact vascular supply and devoid of Brunner's glands (10), was isolated between two glass limbs connected to a reservoir. The reservoir was then filled with 150 mM NaCl, which was maintained at 38°C by a water jacket. The saline was recirculated through the duodenal segment by means of a gas lift (pure air, 400 ml/min). The common bile duct was catheterized approximately 5 mm proximal to the papilla of Vater in order to avoid contamination of the duodenal segment. Alkaline secretion into the luminal perfusate was continuously titrated to pH 7.4 with isotonic 0.02 M HCl under automatic control by a pH-stat device.

Immunohistochemistry. At the end of each experiment, the midportion of the duodenal segment under study was removed and fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 60 min at 4°C. The tissue was then frozen in liquid nitrogen and embedded in OCT compound (Sakura Finetek USA Inc., Torrance California). Sections of 8–10 μm were cut from the frozen tissue and mounted on slides. The slides were first preincubated with a 5% nonfat milk solution and then incubated with primary antibodies against different isoforms of NOS overnight at 4°C. Polyclonal antibodies against iNOS, eNOS, and nNOS were raised in rabbits (Affinity Bioreagents, Boulder, Colorado) and used at 1:800, 1:500, and 1:800, respectively. After careful rinsing three to five times with PBS (pH 7.4), biotin-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates Inc., Burmingham, Alabama) and FITC-conjugated Neutralite avidin (Southern Biotechnology Associates, Inc.) were used for detection of specific polyclonal NOS antibody binding. Negative controls were treated the same way, except for incubation with primary antibodies. Visualization of FITC was carried out by means of a Nikon Microphot FXA with a fluorescent unit (Nikon Corporation, Tokyo, Japan).

Morphological Examination of Mucosal Damage. Sections cut from frozen tissue were stained with hematoxylin–eosin, and subsequently examined by light microscopy (Dialux 20 EB, Leitz). Attention was paid to villus damage. As a damage index, the mean length of five villi per specimen was calculated from measurements with morphometric equipment (Freelance; Sight Systems, Newbury, England). The specimens were examined by an observer unaware of the experimental procedures.

Western Blot Analysis. The mucosa was scraped off and then frozen in liquid nitrogen and stored at −70°C. The frozen specimens were homogenized with a Polytron (PT-1200 C, Kinematica AG) in a PE buffer (10 mM potassium phosphate buffer, pH 6.8, and 1 mM EDTA) containing 10 mM 3-[(3-cholamidopropyl) dimethyl-ammonio] 1-propanesulfonate (CHAPS; Boehringer Mannheim, Mannheim, Germany), aprotinin (1 μg/ml, Boehringer Mannheim), leupeptin (10 μg/ml, Boehringer Mannheim), pepstatin (10 μg/ml, Boehringer Mannheim), and Pefablock (1 mg/ml, Boehringer Mannheim). The homogenate was then sonicated (2 × 15 sec) and centrifuged (10,000 *g* for 10 min at 4°C). The supernatant was analyzed for protein content by the method of Bradford (11) and stored at −70°C for further analysis.

Samples diluted in sodium dodecyl sulfate (SDS) sample buffer were heated at 70°C for 10 min before they were loaded (30 μg of total protein/lane) on a NuPAGE 10% Bis-Tris Gel (Novex, San Diego, California). One lane per gel was loaded with prestained molecular weight standards (SeeBlue, Novex). After electrophoresis, the proteins were transferred to a polyvinyldifluoride membrane (Amersham, Buckinghamshire, UK). The membrane was then incubated

with a specific polyclonal antibody against iNOS [a 21-kDa protein fragment corresponding to amino acids 961–1144 of mouse macrophage NOS was used as an immunogen (Transduction Laboratories, Lexington, Kentucky)]. An alkaline phosphatase-conjugated secondary goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, California) and CDP-Star (Tropix, Bedford, Massachusetts), as a substrate, were used to identify immunoreactive proteins by chemiluminescence. Finally, enhanced chemiluminescence (ECL) film (Amersham), was exposed to the membrane at room temperature, and the film was subsequently developed and scanned (Agfa Arcus II, Agfa Gevaert Group, Mortsel, Belgium). Semiquantification was performed using IPLab Gel software (Signal Analytics, Vienna, Virginia).

Experimental Protocol. Subsequent to surgery, the animals were left undisturbed for 30–60 min. During a 30-min control period, mucosal alkaline secretion and mean arterial pressure were measured and expressed as the mean value over 5-min periods. The duodenal segment was then exposed to an isotonic 10 mM HCl (pH 2) solution for 5 or 30 min. After the exposure, the chamber was rinsed several times with body-temperature isotonic NaCl and the experiment continued for another 60 min. One group of animals served as time controls and was subjected to isotonic NaCl for a period of 120 min.

Drugs. Methohexital (Brietal, Lilly Inc., Indianapolis, Indiana and α-chloralose (Sigma Chemicals Inc., St. Louis, Missouri) were used for anesthesia (for doses and administration routes see above). N^{G}-nitro-L-arginine methyl ester hydrochloride (L-NAME, Sigma Chemicals Inc.) and L-N^{6}-1-iminoethyl-lysine (L-NIL, Sigma Chemicals Inc.) were dissolved in saline for later intraluminal administration.

Statistics. Data within groups were analyzed by means of ANOVA for repeated measurements and a paired *t*-test. Comparisons between groups were performed by means of ANOVA and, when appropriate, an unpaired *t*-test. Presented changes in duodenal mucosal alkaline secretion were obtained from comparisons between the mean value of the 30-min control period and the mean value of the period 10–40 min after exposure. Immunoblot data between groups were compared using the nonparametric Mann-Whitney U-rank sum test. $P < 0.05$ was considered statistically significant. Data are given as means ± SEM.

RESULTS

Basal Conditions

Distribution of NOS in Duodenal Tissue. In animals killed immediately after they had been anesthetized ($N = 6$), immunohistochemistry demonstrated weak but distinct staining for iNOS in villous epithelial cells, especially at the tips of the villi, and western blot analysis confirmed the presence of iNOS protein (expressed as a 130-kDa band) in specimens from duodenal mucosal tissue (Figure 1A). On the other hand, eNOS immunoreactivity was restricted to vessels in the submucosa, and occasionally to vessels in the mucosa, but no eNOS could be detected in the villous epithelial cells (Figure 1B). nNOS immunoreactivity was observed in the muscularis externa and in adjacent submucosa. However, there was no villous tissue labeled positively to nNOS (data not shown). The same distribution pattern and immunofluorescence intensity for the isoforms of NOS were found in six animals subjected to 120-min exposure to isotonic NaCl, and the negative controls were dark in all specimens (data not shown).

Duodenal Mucosal Alkaline Secretion. Addition of the unselective NOS inhibitor L-NAME (0.3 mM, $N = 6$) or the specific iNOS inhibitor L-NIL (20 μM, $N = 6$) to the luminal perfusate did not significantly change basal mucosal alkaline secretion or mean arterial pressure as compared to six control animals (Figure 2).

Effects of Luminal Acid Exposure

Duodenal Mucosal Alkaline Secretion. The duodenal mucosal alkaline secretion was significantly increased by approximately 50% after 5 min luminal exposure to 10 mM HCl, made isotonic with NaCl (Figure 2, $N = 6$). Thirty minutes of exposure to isotonic 10 mM HCl raised the mucosal alkaline secretion to the same order of magnitude as did 5 min of exposure (Figure 2, $N = 6$). In the presence of intraluminal L-NAME (0.3 mM) or L-NIL (20 μM), the secretory response to 5 min of exposure to 10 mM HCl was abolished (Figure 2). NOS inhibition also prevented the secretory responses in animals subjected to 10 mM HCl for 30 min (data not shown). Six animals were subjected to repeated acid exposure, and a second 5-min exposure to 10 mM HCl 60 min after the first similar exposure resulted in a further stimulatory effect on the duodenal mucosal alkaline secretion ($P < 0.05$) (Figure 3).

Western Blot Analysis and Immunohistochemistry. In rats exposed to 10 mM HCl for 5 min and killed 60 min later, western blot analysis showed that duodenal mucosal expression of iNOS protein was significantly increased compared to time controls ($P < 0.05$). Exposure for 30 min to 10 mM HCl resulted in an expression of the protein that was not significantly different from that obtained in the group subjected to 5 min of acid exposure, and immunohistochemistry showed that there was a more intense iNOS immunoreactivity in the villous epithelial cells of animals exposed to acid than in control animals (Figure 4).

Histological Examination. Histological examination by means of light microscopy did not reveal any obvious mucosal damage in controls, animals subjected to 10 mM HCl for 5 min or in the group of rats

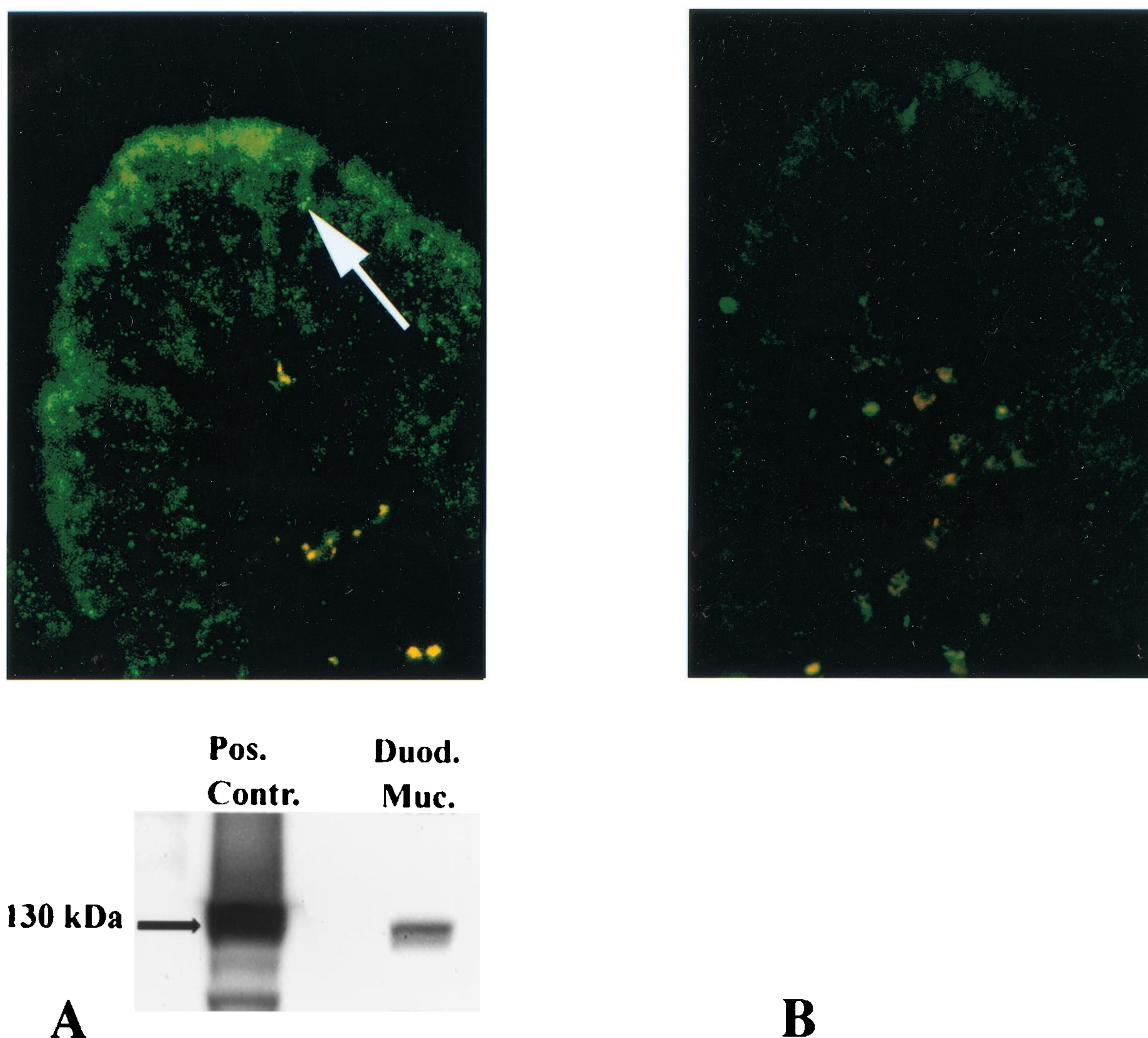


Fig 1. (A) Photomicrograph of immunofluorescence staining for iNOS in an untreated control rat. Staining is localized mainly to the enterocytes at the tip of the villus (upper panel, original magnification 400×). Lower panel: western blot analysis of iNOS in duodenal mucosal tissue (30 μg total protein) from a normal untreated rat. Lysate from stimulated mouse macrophage cells (RAW264.7) served as a positive control. (B) Photomicrograph of immunofluorescence staining for eNOS in an untreated control rat. No fluorescence signal can be detected in, or close to, the enterocytes (original magnification 400×).

exposed to 10 mM HCl for 30 min. The mean villus length was 643 ± 24 μm in the controls, 661 ± 23 μm in the 5-min acid exposure group, and 597 ± 20 μm in the 30 min acid exposure group. There were no significant differences between the groups.

DISCUSSION

The mediation of acid-induced duodenal mucosal alkaline secretion has been investigated in several species (2). It seems that the regulatory mechanisms are located close to the mucosal epithelium because lidocaine, applied at the serosal side of the duodenum at a dose shown in rat jejunum to diffuse to the submucosa (12), does not inhibit the response to acid in rats (13). Prostaglandins, humoral factors, as well as local axon reflexes involving capsaicin-sensitive neurons have been suggested as potential mediators (2, 13). Furthermore, recent studies have indicated a role for nitric oxide, synthesized close to or in the epithelium, in the regulation of the bicarbonate response (3, 14).

The present study confirms that acid-induced duodenal mucosal alkaline secretion is blocked by intraluminally administered L-NAME, a nonselective NOS inhibitor (14). The dose of L-NAME and the route of administration have been shown previously not to

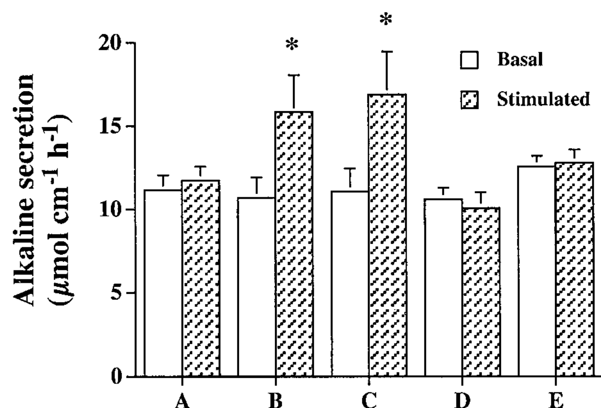


Fig 2. Effect of exposure to 10 mM HCl on duodenal mucosal alkaline secretion in different groups. Group A is controls subjected to sham stimulation (isotonic NaCl). Group B represents 5 min of exposure to acid, and group C represents 30 min of exposure to acid. Groups D and E show the effect of 5 min of acid exposure in animals receiving 0.3 mM of the unselective NO synthase inhibitor L-NAME intraluminally and 20 μM of the specific iNOS inhibitor L-NIL, respectively. Data shown are the mean values ± SEM of six experiments. *$P < 0.05$, significant difference from baseline.

affect the duodenal blood flow, supporting a juxtaepithelial site of action (14). To elucidate this finding further, we used immunohistochemistry to localize the isoforms of NOS in the duodenal tissue. A diffuse nNOS immunoreactivity was detected in the muscularis externa and in adjacent submucosa, most likely representing circular smooth muscle and neural plexa. However, no positive labeling was found in the mucosa of normal untreated rats. This is in line with previous studies, demonstrating that nerve fibers containing NOS are extremely rare in the mucosa/submucosa of the rat duodenum with the exception of NOS-containing nerve fibers closely attached to Brunner's glands (15, 16). The segment under study has been shown to lack Brunner's glands (10), and it therefore seems unlikely that nNOS is responsible for the proposed juxtaepithelial mediation of acid-induced duodenal mucosal alkaline secretion. Immunoreactivity, indicating the presence of eNOS protein, was detected principally in submucosal vessels and in a few mucosal vessels, thus confirming previous data (17, 18). No eNOS was observed in, or close to, the mucosal epithelium, which makes involvement of eNOS in acid-induced mucosal alkaline secretion less likely. Specimens stained for iNOS showed weak but distinct fluorescence signals in the mucosal epithelial cells, especially at the tips of the villi, and western blot analysis confirmed the presence of iNOS in the untreated rat duodenal mucosa. These results suggest that iNOS is the isoform involved in acid-induced duodenal mucosal alkaline secretion. Furthermore,

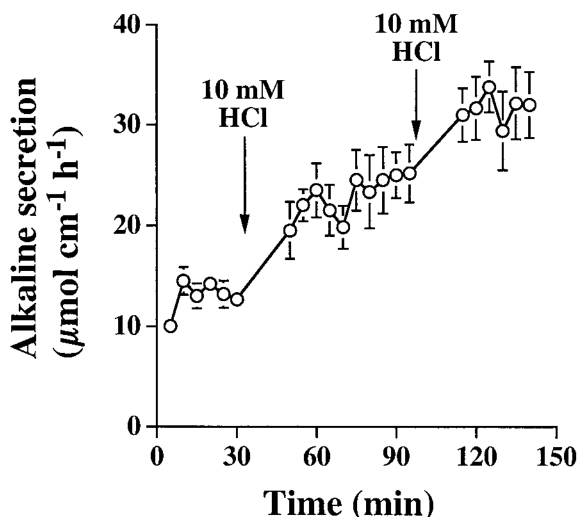
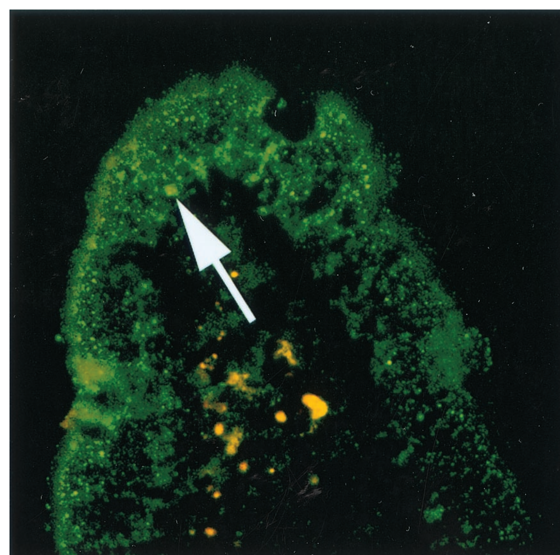


Fig 3. Effect of repeated 5-min exposure to 10 mM HCl on duodenal mucosal alkaline secretion. Data shown are the mean values ± SEM of six experiments.



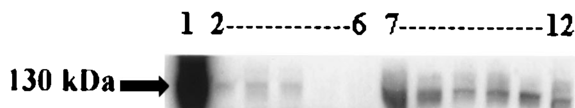


Fig 4. Upper panel: photomicrograph of immunofluorescence staining for iNOS in a rat exposed to 10 mM HCl for 30 min. Staining is localized mainly to the enterocytes at the tip of the villus (original magnification 400×). Lower panel: western blot analysis of iNOS in duodenal mucosal tissue (30 μg total protein) from normal untreated rats (lanes 2–6) and rats exposed to 10 mM HCl for 30 min (lanes 7–12). Lane 1 was loaded with lysate from stimulated mouse macrophage cells (RAW264.7) and served as a positive control.

the present study also shows that the selective iNOS inhibitor L-NIL prevents the secretory response to acid at a dose known from *in vitro* studies to effectively block inducible NOS, but not constitutive NOS (19, 20).

Low amounts of iNOS in the normal mucosal surface epithelium of the small intestine have been reported previously in several animal species, including the rat (8, 21–23). Unstimulated human duodenal enterocytes have also been reported to express iNOS protein (24). However, other studies show iNOS activity in tissues from rat small intestine only after exposure to inflammatory stimuli (25–27). There is also a study where no iNOS gene expression could be detected in digestive tissues of rats under normal conditions (28). One explanation for these divergent results is that iNOS expression varies depending on microenvironmental conditions, e.g., immunogens or other aggressors such as gastric acid. In line with such a hypothesis, we found in the present study that luminal acid is an inductive stimulus for iNOS expression. One hour after the duodenal mucosal acid exposure, there was a marked increase in iNOS expression compared to saline-exposed controls. No significant differences regarding the amount of iNOS could be detected by the immunoblot analysis between the two groups subjected to 5 and 30 min of acid exposure, respectively. Interestingly, increased levels of iNOS protein in response to acidic conditions have been demonstrated in macrophages (29). Thus, data suggest that a short-lasting luminal exposure to acid can induce an increased expression of mucosal iNOS within 60 min. The rapid induction of iNOS is surprising, but there are studies showing that increased amounts of iNOS mRNA in epithelial cells are present 10–30 min after stimulation (30, 31). A second exposure of the duodenal mucosa to acid, 60 min after the first exposure, resulted in a further stimulation of the mucosal alkaline secretion. Taken together, these findings strongly support a role for iNOS in the epithelial adaptation to luminal acidity.

It follows that the mucosal iNOS in the untreated controls may arise from preexperimental physiological exposure to gastric acid. However, because acute administration of a NOS inhibitor did not change basal mucosal alkaline secretion, it appears that the present epithelial iNOS is inactive under basal conditions and needs acidity, for example, to be activated. This is contradictory to the general opinion (4), but there are studies indicating that iNOS can be regulated even at the level of enzyme activity. Lovchik et al (32) have shown that iNOS can lie dormant in lung tissue of mice and become activated when the lung cells are isolated. Moreover, it has been shown both *in vivo* and *in vitro* that eNOS can be inactivated when attached to the caveolae (microdomains of the plasmalemma) coat protein caveolin, and a similar inhibitory action on iNOS activity was shown *in vitro* (33). The caveolin hypothesis is strengthened by the fact that monolayers of the human intestinal epithelial cell line T84 have been shown to contain caveolin (34). Physiologic stimuli may then induce a disruption of the caveolin-NOS interaction and thereby lead to NO release (33). Alternative regulatory mechanisms involve availability of L-arginine and necessary cofactors (4), or the ratio between L-arginine and endogenous NOS inhibitors (35). However, further studies are needed to elucidate the regulation of the enzyme activity of iNOS in the duodenal mucosa.

In summary, the present data suggest that luminal acidity activates duodenal mucosal alkaline secretion via a nitric oxide-dependent mechanism that includes iNOS located in the villous epithelial cells. The results further suggest that luminal exposure to acid can induce expression of iNOS, which allows for an upregulated response to a subsequently given stimulus, i.e., acid exposure.

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