Inducible nitric oxide synthase is involved in acid-induced gastric hyperemia in rats and mice

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The gastric mucosa is frequently exposed to various potentially injuring agents such as acid, proteolytic enzymes, ingested agents, and bacteria. Maintaining gastric mucosal integrity requires an effective mucosal defense. The gastric mucosal blood flow is an important component of the defense, as it dilutes, neutralizes, and transports away noxious substances that have penetrated the luminal barriers such as the mucus layer, the bicarbonate in the mucus, and the tight junctions of the epithelia. The gastric mucosal blood flow also provides the gastric epithelial surface cells with bicarbonate needed for neutralization of back-diffused acid and maintenance of a pH gradient in the mucus layer (32, 36).

When acid, in combination with a barrier-breaking substance (e.g., ethanol or sodium taurocholate), is applied luminally to the gastric mucosa, gastric mucosal blood flow increases (2, 25). This increase in gastric blood flow can be blocked with a nonselective nitric oxide synthase (NOS) inhibitor (26).

Nitric oxide (NO) has been reported to influence different components of gastric mucosal defense, including the mucosal blood flow (19, 37). In mammals, three isoforms of NOS, encoded by different genes, have been identified (22). The constitutively expressed enzymes can be divided into isozymes associated with neurons (nNOS, type I) and isozymes present in the endothelium lining the vasculature (eNOS, type III). The inducible NOS (iNOS, type II) needs a stimulus (cytokines, lipopolysaccharides) for expression in specific cell types, e.g., macrophages, neutrophils, endothelial cells, and epithelial cells. It is generally believed that the constitutively expressed isozymes are responsible for the normal physiological effects of NO, whereas iNOS is activated in different pathophysiological states (37).

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

We have recently found in rats that gastric mucosal blood flow increased when HCl, pH 1 (not combined with a barrier breaker), was applied luminally (15, 36). Permeability measured as $^{51}$Cr-EDTA clearance from blood to gastric lumen increased slightly during luminal pH 1 but was reversed as soon as the luminal acid content was changed to saline; consequently, no visible lesions were detected. These results are in contrast to those when HCl was given with a barrier breaker resulting in hemorrhagic lesions (2, 25, 26). It is not known if the...
mechanisms behind these two types of hyperemia are identical.

In the present study, we wanted to investigate the mechanisms regulating the hyperemia in response to luminal acid, without the addition of a barrier breaker, a situation mimicking a between-meal condition. We hypothesized that the hyperemia was initiated by synthesis of NO in the gastric epithelial cells in response to luminal acid alone. To investigate which isofrom of NOS that might be involved in acid-induced hyperemia, rats treated with specific nNOS and iNOS inhibitors and mice lacking the gene coding for either iNOS or nNOS were used.

METHODS

Animal Preparation

All animals were kept under standardized conditions of temperature (21–22°C) and illumination (12:12-h light-dark). They were kept in cages with mesh bottoms and had free access to tap water and pelleted food (Ewos, Södertälje, Sweden).

Rats. Sprague-Dawley male rats (B&K Universal, Stockholm, Sweden) weighing 170–250 g were deprived of food, but not water, for 17–20 h before they were anesthetized by an intraperitoneal injection of thiobutabarbitual sodium (Inactin, 120 mg/kg body wt; Research Biochemicals International, Natick, MA). Spontaneous breathing was facilitated by a short PE-200 cannula placed in the trachea, and 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; KabiVitrum, Stockholm, Sweden) 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 glucose). The chamber was filled over the mucosa, exposing ~1.2 cm² of the rat gastric mucosa and 0.28 cm² of the mouse mucosa through the hole. The mucosal chamber did not touch the mucosa, to not 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 constant intervals of 10 min and titrated (Autoburette ABU 91; Radiometer, Copenhagen, Denmark) with 10⁻³ M NaOH or 10⁻³ M HCl to the initial pH of the saline and is presented as microequivalents secreted in the chamber per minute per square centimeter.

Blood Flow Measurements

Laser-Doppler flowmetry (LDF; Periflux Pf 2, Pf3, or Pf 4001; Perimed, Stockholm, Sweden) was used for blood flow measurements in all experiments. The laser light is guided to the tissue by an optical fiber (standard probe, diameter = 0.7 mm), and the backscattered light is 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 (1).

Mucosal Permeability

To investigate the damaging effect of 155 mM acid (HCl) on the rat gastric mucosa, mucosal permeability was determined by measuring the clearance of ⁵¹Cr-labeled EDTA (⁵¹Cr-EDTA; NEN Chemicals, Du Pont, Boston, MA) from blood to lumen. 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 (3, 7, 13). After completion of surgery and ~60 min before the start of the experiment, 50–75 μCi were injected as a bolus dose (0.5 ml) followed by a continuous intravenous infusion of ⁵¹Cr-EDTA (10–30 μCi/ml in the Ringer solution) at a rate of 1.0 ml/h. Three to four 0.2-ml blood samples were drawn...
during the experiment at a time interval of at least 30 min. The first one was taken 60 min after the injection of $^{51}$Cr-EDTA. After each blood sample withdrawal, the blood volume loss was compensated for by injection of a 10% Ficoll 400 solution or 7% bovine albumin (Sigma Chemical, St. Louis, MO) in saline. The blood sample was centrifuged, and 50 μl of the plasma were removed for measurements of radioactivity [counts/min (cpm)]. The gastric mucosa was covered with isotonic saline that 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; Pharmacia, Uppsala, Sweden). In each experiment, the various blood plasma $^{51}$Cr-EDTA activities were plotted against time, and a straight line was drawn between the two nearest values. Each clearance value was calculated by dividing each individual luminal cpm value by a corresponding plasma cpm value. If there was <10% deviation between the different blood plasma cpm values, a mean plasma cpm per milliliter value was calculated and used for all clearance samples. The part of the stomach that had been exposed in the chamber was cut out and weighed after the experiment. Clearance is expressed as milliliters per minute per 100 grams wet tissue weight.

**Clearance**

\[
\text{Clearance} = \frac{\text{lumen sample (cpm/ml) \times sample volume (ml) \times 100}}{\text{plasma (cpm/ml) \times tissue weight (g) \times time (min) }}
\]

**RNA Isolation and cDNA Synthesis**

Total RNA was isolated from scrapings of the mouse gastric mucosa with an RNasy Mini kit (Qiagen; KEBO, Spånga, Sweden) and digested with RNase-free DNase, as recommended by the supplier. The concentration and purity of total RNA were determined by measuring the optical density at 260 and 280 nm. The ratio of absorbance at 260 to 280 nm was 1.8–2.0. cDNA synthesis was performed with the Reverse Transcription System (Scandinavian Diagnostics Services, Falkenberg, Sweden) using 1 μg total RNA/20 μl cDNA synthesis reactions [5 mM MgCl₂, 1× RT buffer [10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100], 1 mM each dNTP, 1 U/μl recombinant RNasin RNase inhibitor, 15 U/μl avian myeloblastosis virus RT, and 0.5 μg oligo(dT)₁₅ primer]. The reactions were incubated for 60 min at 20°C followed by 5 min at 99°C and were stored at −20°C. Before usage, the cDNA was diluted in water (see below).

**Real-Time RT-PCR**

The LightCycler FastStart DNA Master SYBR Green I was used for quantitative analyses of the generated cDNA. This technique uses SYBR Green I (Roche Biochemicals, Stockholm, Sweden), a fluorescent dye specific for the double-stranded DNA that binds to the amplified PCR product, and it is possible to detect and quantify the amount of PCR product at each cycle. The amplification was combined with a melting curve analysis. The cDNA sequences for the genes of interest were obtained from GenBank, and the primer sequence was designed by Dr. Andreas Nietsche (TIB MOLBIOL, Berlin, Germany) as follows: forward primer 5’-CAGCTGGGCGTGTAACACCTT-3’ and reverse primer 5’-CATTGGAAAGGAGCCTTCTGG-3’. To avoid amplification of genomic DNA, the primer sets were designed to span intron-exon borders. PCR amplifications were performed in a total volume of 20 μl containing 2 μl cDNA sample, 2 μl LightCycler-FastStart DNA Master SYBR Green I, 0.5 μM of each primer, and 2.5 mM MgCl₂. For each reaction, the polymerase was activated by a preincubation at 95°C for 10 min, and amplification was then performed at 45 cycles of switching between 95°C for 15 s, 60°C for 10 s, and 72°C for 15 s followed by melting point analysis from 65 to 95°C. The results were represented as threshold cycle values, C_T values, i.e., the estimated amplification cycle number when the fluorescence exceeds a specified threshold value (10, 14). All of these template samples were run in duplicate. Control cDNA from gastric mucosa cells was diluted (×4, ×16, ×64, and ×256) and run in the PCR amplification to create standard curves by plotting C_T values vs. the dilution of the cDNA templates. These standard curves were then used to control the efficacy of the PCR, i.e., twofold amplification for each cycle. Negative controls in which no template was present and samples from cDNA synthesis lacking RT were added to the PCR to screen for possible contamination and genomic amplification.

**Calculation of Relative Quantification Values**

The relative quantification values were calculated according to the manufacturer’s description (Roche Biochemicals). The C_T represents the PCR cycle at which an increase in fluorescence above a baseline signal can be detected. The C_T value was used to calculate the amount of PCR product compared with the internal control, glyceraldehyde-6-phosphate dehydrogenase (G-6-PDH). The C_T value for G-6-PDH was subtracted from the iNOS C_T value to calculate the mean change in C_T in each experiment group.

**Experimental Protocol**

**Blood flow measurement and luminal acid (groups 1–9).** The anesthetized and operated rats (n = 23) were given at least 1 h to achieve a steady state before the experiment started. They were divided into four groups. In group 1, HCl in a concentration of 155 mM was administered topically for 20 min after a control period of 50 min. The acid was washed away and changed to saline for a further 30-min period.

In group 2, Nω-nitro-l-arginine (l-NNA; Sigma Chemical, Stockholm, Sweden) was given in a dose of 10 mg/kg iv bolus followed by 3 mg·kg⁻¹·h⁻¹ continuous intravenous infusion throughout the experiment. When blood flow was stabilized (30 min after l-NNA injection), HCl in a concentration of 155 mM was administered topically on the mucosa for a 20-min period. The luminal acid exposure was followed by a 30-min period of saline.

In group 3, the selective inhibitor of iNOS, l-Nω-(1-imino-ethyl)lysine (l-NIL; Alexis, Lüfelfingen, Switzerland), was administered in the same dose as used for l-NNA (10 mg/kg iv bolus followed by 3 mg·kg⁻¹·h⁻¹ continuous iv infusion). Later (50 min), 155 mM HCl was administered luminally for two consecutive 10-min periods, followed by a 30-min control period.

**Group 4** received the nNOS inhibitor, S-methyl-l-thiocitrulline (SMTC; 10 mg/kg iv bolus followed by 3 mg·kg⁻¹·h⁻¹ continuous iv infusion; Alexcis), and luminal acid (HCl, 155 mM) was administered 50 min later for two consecutive 10-min periods, followed by a 30-min control period.

In groups 5–9, the mice [iNOS-sufficient (+/+)] and -deficient (−/−) and nNOS-sufficient (+/+), heterozygous (+/−), and -deficient (−/−), n = 29] were left 1 h after surgery to achieve a steady state, before the control period of 20 min started. HCl (155 mM) was then applied luminally for two consecutive 10-min periods, followed by another 20-min control period. In nNOS groups 7–9, when the experiment was terminated, the stomach was cut out, opened, rinsed, and dried with a paper towel before being weighed.
the bolus dose and the start of the continuous infusion of 10-min periods. Values are expressed as means during the HCl applications, when it was calculated for EDTA clearance was calculated for 15-min periods except mM), receiving the same treatment as mice (group 11, n = 3), iNOS control mice (+/+), group 11 (n = 3), were killed without anesthesia (spinal translocation), and the gastric mucosa was scraped immediately for mRNA isolation. In groups 12–14, the mice were operated using the same experimental protocol as in groups 5–9. In these groups (nos. 12–14) mRNA was isolated at 40 min (see Fig. 5). In group 12 [n = 3, iNOS(+/+)], the mucosa was exposed to saline throughout the experiment, and mRNA was isolated at 40 min when acid normally was changed to saline (see Fig. 5). In groups 13 [n = 5, iNOS(+/+)] and 14 [n = 4, iNOS(−/−)], acid was applied luminally the last 20 min before mRNA was isolated from the mucosa.

Blood flow (LDF) is presented as a percentage of control. Control values are the mean values of the 10-min period before topically applied HCl (155 mM). Vascular resistance was calculated from the mean arterial blood pressure divided by the blood flow. Venous pressure was assumed to be zero, and portal venous pressure was not taken into consideration. Vascular resistance, like the blood flow, is presented as a percentage of control.

Mucosal permeability (group 10). The clearance of 51Cr-EDTA from the blood to the gastric lumen was investigated in rats (n = 5) before, during, and after luminal acid (155 mM), receiving the same treatment as group 1. After (50 min) the bolus dose and the start of the continuous infusion of 51Cr-EDTA, luminal saline was changed to 155 mM acid for two consecutive 10-min periods, followed by saline. 51Cr-EDTA clearance was calculated for 15-min periods except during the HCl applications, when it was calculated for 10-min periods. Values are expressed as means ± SE.

Real-time RT-PCR; iNOS (groups 11–14). iNOS control mice (+/+), group 11 (n = 3), were killed without anesthesia (spinal translocation), and the gastric mucosa was scraped immediately for mRNA isolation. In groups 12–14, the mice were operated using the same experimental protocol as in groups 5–9. In these groups (nos. 12–14) mRNA was isolated at 40 min (see Fig. 5). In group 12 [n = 3, iNOS(+/+)], the mucosa was exposed to saline throughout the experiment, and mRNA was isolated at 40 min when acid normally was changed to saline (see Fig. 5). In groups 13 [n = 5, iNOS(+/+)] and 14 [n = 4, iNOS(−/−)], acid was applied luminally the last 20 min before mRNA was isolated from the mucosa.

Statistical Methods

The results are expressed as means ± SE. Statistical significance was determined with ANOVA for repeated measurements, followed by the Fisher’s protected least significant difference test. The differences were regarded as significant at $P < 0.05$. All statistical calculations were performed on a Macintosh with the software Statview II SE Graphics (Abacus Concepts, Berkeley, CA).

RESULTS

HCl Topically in Rats (Group 1)

Gastric mucosal blood flow was increased significantly by 48 ± 13%, during the acid period (Fig. 1, n = 6), and vascular resistance decreased. Mean arterial blood pressure (mmHg) and acid output were stable during the entire experiment. When acid was washed away, the blood flow returned to the basal level. The increased acidity in the chamber 10–20 min after HCl topically probably depends on acid not being totally removed. This can also be seen in Figs. 2 and 3.

L-NNA + HCl Topically in Rats (Group 2)

Administration of the unspecific NOS inhibitor L-NNA resulted in an elevated but stable blood pres-

Fig. 1. Mean arterial blood pressure (MAP, mmHg), gastric mucosal blood flow [laser-Doppler flowmetry (LDF), %], vascular resistance (R, %), and acid secretion (H+, μeq·min⁻¹·cm⁻²) in control rats (n = 6). LDF and R are presented as percentages of the control period, time 45–50 min. HCl (155 mM) was applied luminally. During the acid application period and 10 min after, acid secretion measurements were not performed because of disturbances from the applied acid. The higher acidity after luminal acid probably depends on remaining acid in the mucosal chamber. Values are expressed as means ± SE. *P < 0.05 compared with the control period before acid application.

Fig. 2. MAP (mmHg), LDF (%), and H+ (μeq·min⁻¹·cm⁻²) in Nω-nitro-L-arginine (L-NNA)-treated rats (n = 6). LDF and R are presented as percentages of the control period, time 45–50 min. HCl (155 mM) was applied luminally. Acid secretion was not measured during HCl application and the following 10 min. The higher acidity after luminal acid probably depends on remaining acid in the mucosal chamber. Values are expressed as means ± SE. *P < 0.05 compared with the control period before acid application.
s sal blood flow could be seen in response to luminal acid. Again, acid secretion was not altered by SMTC.

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**iNOS**(+/+) and **−/−** Mice + HCl Topically (Groups 5 and 6)

Mean arterial blood pressure was stable at similar levels in the iNOS wild-type mice and in the group in which the iNOS gene had been inactivated. In wild-type mice (Fig. 5, n = 6), gastric blood flow increased by ~30% during luminal acid; meanwhile vascular resistance decreased. In the iNOS(−/−) group (n = 6), the blood flow increase was reduced and significantly elevated only at one time point (13 ± 6%) during luminal acid, and the decrease in resistance was completely abolished.

**nNOS**(+/+), **−/+**, and **−/−** Mice + HCl Topically (Groups 7, 8, and 9)

Mean arterial blood pressure did not differ whether the nNOS gene was partly (+/−, n = 7) or completely depleted (−/−, n = 6) compared with the positive control (+/+, n = 4). A small decrease in blood pressure was seen during the experiment (Fig. 6). The blood pressure decrease might be because these mice were more stressed than the mice in the iNOS group and required higher doses of isoflurane for induction of anesthesia. The nNOS offspring were housed together independently of their type (+/+, −/−, or −/−), and

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Fig. 3. MAP (mmHg), LDF (%), and H⁺ (μeq·min⁻¹·cm⁻²) in L-N⁶-(1-iminoethyl)lysine (L-NIL)-treated rats (n = 6). LDF and R are presented as percentages of the control period, time 45–50 min. HCl (155 mM) was applied luminally. The higher acidity after luminal acid probably depends on remaining acid in the mucosal chamber. Values are expressed as means ± SE. *P < 0.05 compared with the control period before acid application.

Fig. 4. MAP (mmHg), LDF (%), and H⁺ (μeq·min⁻¹·cm⁻²) in S-methyl-l-thiocitrulline (SMTC)-treated rats (n = 5). LDF and R are presented as percentages of the control period, time 45–50 min. HCl (155 mM) was applied luminally. Acid secretion was not measured during HCl application and the following 10 min. Values are expressed as means ± SE. *P < 0.05 compared with the control period before acid application.
iNOS is involved in acid-induced gastric hyperemia

DISCUSSION

In this study, luminal acid (155 mM HCl) applied on the gastric mucosa caused a hyperemia that was blocked by nonselective inhibition of NOS and by selective inhibition/inactivation of the gene coding for iNOS. nNOS, however, does not seem to be important for this gastric mucosal defense system.

A large amount of NOS is present in the gastric surface epithelial cells. The role of this epithelial NOS in gastric defense mechanisms has not been investigated earlier. An alert defense system is crucial in the stomach, since the mucosa is constantly challenged not only by ingested pathogens but also by endogenous secreted acid (theoretically 155 mM) and degrading enzymes. Earlier studies have shown that, during a luminal pH of 1, the pH at the epithelial surface decreases to approximately pH 1.5 and gastric mucosal blood flow increases (32, 36). In the present study, the involvement of the gastric epithelial NOS in this hyperemic response to intraluminal acid was investigated.

The mechanism behind acid-induced gastric mucosal hyperemia has been elucidated in studies where acid, in combination with a barrier-breaking substance (e.g., ethanol or sodium taurocholate), increases gastric mucosal blood flow and causes hemorrhagic lesions when

Fig. 5. MAP (mmHg), LDF (%), and R (%) in control mice (+/+, n = 6) and inducible nitric oxide synthase (iNOS) knockout mice (iNOS deficient (−/−), n = 6). LDF and R are presented as percentages of the control period, time 15–20 min. HCl (155 mM) was applied luminaly. Values are expressed as means ± SE. iNOS(+/+) iNOS sufficient. *P < 0.05 compared with the control period before acid application.

since the nNOS(−/−) mice have been reported to be more aggressive (24) this could have influenced all of them. The blood flow increase in response to acid did not differ between the groups, nor did the effect of luminal acid on vascular resistance.

The wet weights of stomachs (presented as % respective mouse weight × 1,000) showed that the nNOS(−/−) mice had a significantly larger stomach (1.11 ± 0.08) than +/− (0.74 ± 0.03) or +/+ (0.74 ± 0.12) mice. This has been reported previously and was shown to be the result of pylorus hypertrophy (20).

Mucosal Permeability (Group 10)

In this group of rats (n = 5), blood-to-lumen clearance of 51Cr-EDTA increased significantly during luminal acid (155 mM HCl) from 0.13 ± 0.02 ml·min⁻¹·100 g tissue⁻¹ before to 0.24 ± 0.04 ml·min⁻¹·100 g tissue⁻¹ after 20 min of luminal acid. This increase was reversed as soon as the acid was changed to saline (0.19 ± 0.03 ml·min⁻¹·100 g tissue⁻¹ 0–15 min after acid exposure and 0.15 ± 0.03 ml·min⁻¹·100 g tissue⁻¹ 15–30 min after acid exposure), indicating that no permanent damage of the gastric mucosa had occurred.

Real-time RT-PCR: iNOS (Groups 11–14)

The results of iNOS gene expression, using real-time PCR, are presented in Table 1. iNOS mRNA expression was found in the gastric mucosa of iNOS(+/+ ) mice [group 11 (n = 3), group 12 (n = 3), and group 13 (n = 5)]. The iNOS was thus expressed constitutively, but the expression did not change by the anesthesia, gastric preparation, or luminal acid when examined up to 130 min after induction of anesthesia. In the iNOS(−/−) mice, iNOS mRNA was not even found after 45 PCR cycles (n = 4).

DISCUSSION

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Fig. 6. MAP (mmHg), LDF (%), and R (%) in neuronal nitric oxide synthase (nNOS) mice (+/+ , n = 4; heterozygous mice (+/−), n = 7; and −/−, n = 6). LDF and R are presented as percentages of the control period, time 15–20 min. HCl (155 mM) was applied luminaly. Values are expressed as means ± SE. *P < 0.05 compared with the control period before acid application.
applied luminally to the gastric mucosa (2, 25). This treatment activates sensory afferent nerves, leading to a release of calcitonin gene-related peptide (CGRP) in the vicinity of the submucosal arterioles (25). CGRP acts on the vascular endothelium lining these vessels, resulting in generation of NO (19). 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 nonselective NOS inhibitor (26). 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 result in a significant increase in the susceptibility of the gastric mucosa to damage (17, 25, 26).

In this study, acid alone caused the hyperemia. No macroscopic lesions were formed, and the increase in mucosal permeability of $^{51}$Cr-EDTA seen during luminal acid was reversed as soon as the acid was changed to saline. These results are probably because of a lesser acidification of the gastric epithelium compared with the acid as was changed to saline. The analysis was carried out using LightCycler real-time RT-PCR with SYBR Green. The threshold cycle value ($C_t$) was used to calculate the amount PCR product compared with the internal control, G-6-PDH (the $C_t$ value for G-6-PDH was subtracted from $iNOS C_t$ value to calculate mean change in ($\Delta C_t$) in each experimental group).

Table 1  
Relative quantification of $iNOS$ mRNA in gastric mucosa of $iNOS(+/-)$ mice

<table>
<thead>
<tr>
<th>Group No.</th>
<th>C</th>
<th>$\Delta$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>J1 Control</td>
<td>G-6-PDH</td>
<td>$iNOS$</td>
<td></td>
</tr>
<tr>
<td>Unanesthetized</td>
<td>24.3 ± 0.3</td>
<td>38.2 ± 1.1</td>
<td>13.9 ± 0.8</td>
</tr>
<tr>
<td>J2 Saline</td>
<td>G-6-PDH</td>
<td>$iNOS$</td>
<td></td>
</tr>
<tr>
<td>Anesthetized</td>
<td>22.7 ± 0.1</td>
<td>37.5 ± 0.3</td>
<td>13.9 ± 0.3</td>
</tr>
<tr>
<td>J3 HCl (155 mM)</td>
<td>G-6-PDH</td>
<td>$iNOS$</td>
<td></td>
</tr>
<tr>
<td>Anesthetized</td>
<td>24.4 ± 0.5</td>
<td>38.4 ± 0.4</td>
<td>14.0 ± 0.5</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE for $n$ animals; $iNOS$, inducible nitric oxide synthase; $iNOS (+/-)$, $iNOS$ sufficient; G-6-PDH, glyceraldehyde-6-phosphate dehydrogenase. $iNOS (+/-)$ mice were divided into 3 different groups in respect to treatment. $iNOS$ mRNA expression could not be detected in the $iNOS$-deficient mice. The analysis was carried out using LightCycler real-time RT-PCR with SYBR Green. The threshold cycle value ($C_t$) was used to calculate the amount PCR product compared with the internal control, G-6-PDH (the $C_t$ value for G-6-PDH was subtracted from $iNOS C_t$ value to calculate mean change in ($\Delta C_t$) in each experimental group).

The involvement of epithelial $NOS$ in the hyperemia in response to gastric luminal acid has not been studied, since only nonselective $NOS$ inhibitors have been used. These inhibitors block both the enzymes in the surface epithelial cells and the enzymes located in the endothelium, and, when the $eNOS$ is inhibited, no dilation of the vessels would occur by luminal acid even though the afferent nerves would be activated and CGRP released. Accordingly, in the present study, we also found inhibition of the acid-induced hyperemia with a nonselective $NOS$ inhibitor.

Earlier reports suggested the epithelial $NOS$ activity to be more $nNOS$ dependent than $eNOS$ dependent, and no mucosal $iNOS$ has been found during nonpathological conditions (5, 33). Hence, we tried to selectively inhibit $NOS$ with the purported specific inhibitor SMTC. When SMTC was given to rats in this study, the blood flow increase in response to luminal acid was decreased. In addition, arterial blood pressure was increased transiently. Acute inhibition of $NOS$ should not affect the mean arterial blood pressure (30). Although SMTC is at least 10 times more specific for $NOS$ than $eNOS$ (9), intravenous administration of SMTC has been reported to increase arterial blood pressure, probably because of a direct $eNOS$ inhibitory effect on the vascular smooth muscle tone (11). In the present study, as described above, the blood pressure increased directly after SMTC treatment, thus suggesting an inhibitory effect not only on $NOS$ but also on $eNOS$. We therefore studied mice in which the $NOS$ gene was inactivated, to avoid specificity problems of the inhibitors.

The $NOS$ mice showed a similar increase in blood flow in response to luminal acid as the normal littermates (+/+, +/−), indicating that $NOS$ is not involved in the gastric hyperemia in response to luminal acid. Mean arterial blood pressure decreased during the experiment in the different (+/+, +/−, and −/−) $NOS$ mice. The blood pressure decrease might be because these mice were stressed and required slightly higher doses of isoflurane for induction of anesthesia. The $NOS$ mice have been reported to display increases in aggression (24), which might have influenced all of them, since littermates were housed together independently of their type.

When the specific $iNOS$ inhibitor 1-NIL was given intravenously to rats, the blood flow increase in response to luminal acid was attenuated. The mean arterial blood pressure was not altered by the drug, indicating that the inhibitor did not inhibit $eNOS$. In $iNOS$ mice, the hyperemia in response to luminal acid was also attenuated. Thus these results suggest that $iNOS$ is involved in the gastric hyperemia in response to luminal acid and that $iNOS$ has a protective role in the gastric defense in this context.
In earlier studies, the presence of iNOS has not been demonstrated in the gastric mucosa (23, 33). However, we found iNOS mRNA expression using real-time RT-PCR in mouse gastric mucosa at a level not influenced by anesthetia, preparation of the gastric mucosa, and luminal acid. These results indicate a possible posttranscriptional regulation of iNOS activity different from the regulation occurring in the macrophages. Posttranscriptional regulation of iNOS has been suggested earlier (8, 31), as levels of the enzyme argininosuccinate synthetase, the rate-limiting step when citrulline is converted back to arginine (via argininosuccinate), influence NO output. Tetrahydrobiopterin, an NOS cofactor, has also been reported to be an important regulator of NOS function (35). A regulation of the NOS cofactor, has also been reported to be an important regulator of NOS function (35). A regulation of the enzyme posttranscriptionally could explain the findings of constant levels of iNOS mRNA independently of treatment (the gastric preparation or application of luminal acid). In the epithelial cells of the respiratory tract (in airways and paranasal sinuses; see Refs. 12 and 27), esophagus (6), and parts of the small intestine (duodenum and ileum; see Refs. 16 and 17) and colon (29), iNOS is being expressed in a seemingly constitutive way during normal conditions. This could reflect that bacteria, viruses, and fungi, etc., constantly challenge these organ systems. The high level of tonic expression of iNOS and NO production found in airways and paranasal sinuses has special relevance to airway defense mechanisms (21, 34) and possibly also to gastrointestinal defense mechanisms.

In conclusion, this study shows that iNOS is involved in the acid-induced gastric hyperemia. We also found iNOS mRNA expression in the gastric mucosa under basal conditions, indicating a regulation at a posttranscriptional level.

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