Effects of endotoxin on gastric injury from luminal irritants in rats: potential roles of nitric oxide

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Effects of endotoxin on gastric injury from luminal irritants in rats: potential roles of nitric oxide. Am. J. Physiol. 275 (Gastrointest. Liver Physiol. 38): G449–G459, 1998.—The expression and function of inducible nitric oxide synthase (iNOS) in the stomach is unclear. This study assessed the effects of endotoxin on rat gastric iNOS expression and its role in gastric injury from luminal irritants. In conscious rats, a 5-h treatment with intraperitoneal lipopolysaccharide (LPS; 1–20 mg/kg) dose dependently increased gastric mucosal iNOS immunoreactivity and increased gastric luminal nitrate and nitrite accumulation (Griess reaction). LPS also increased gastric luminal fluid accumulation and reduced macroscopic gastric injury from gastric acidified ethanol. Aminoguanidine (45 mg/kg) did not prevent LPS-induced gastroprotection or gastric fluid accumulation. N^G-nitro-L-arginine methyl ester increased gastric luminal fluid and caused macroscopic gastric injury when given with LPS. Using an anesthetized preparation followed by removal of luminal fluid, LPS reduced gastric mucosal blood flow and exacerbated gastric injury from either acidified ethanol or acidified taurocholate, an effect that was negated by aminoguanidine. These data indicate that in conscious rats, the gastroprotective effect of endotoxin is dependent on constitutive NOS but not iNOS activity. However, the inducible isoform participates in the ability of endotoxin to exacerbate gastric injury from luminal irritants in the anesthetized rat.

THE CONSTITUTIVE ISOFORM of nitric oxide synthase (NOS) is important in maintaining gastric mucosal integrity (25). Some evidence suggests that the inducible isoform of this enzyme may play a similar role. Tepperman and Soper (24) demonstrated that administration of intravenous Escherichia coli lipopolysaccharide (LPS) augmented inducible NOS (iNOS) activity concurrent with a reduction in gastric injury from 40% ethanol. The gastroprotective action of LPS was inhibited by administration of the nonselective NOS inhibitor N^G-nitro-L-arginine methyl ester (l-NAME), and this effect was in turn abolished by pretreatment with the NOS substrate l-arginine. These results indicated that LPS renders the stomach more resistant to injury from a subsequent challenge with a luminal irritant. Moreover, although LPS clearly activates a variety of other factors simultaneously, the data from this study suggest that the protective action of LPS is mediated primarily through induction and activation of iNOS.

We and others (17, 30) have shown that endotoxin also substantially increases iNOS immunoreactivity in the rat small intestine. In the ileum, this increase in iNOS expression is accompanied by enhanced lipid peroxidation and morphological injury to the intestinal epithelium (17). Tepperman et al. (23) have similarly demonstrated that exposure of cultured rat intestinal epithelial cells to LPS results in enhanced iNOS activity and a reduction in cell viability. These observations would seem to imply that induction of this enzyme represents a mechanism of local intestinal damage. This being the case, one possible interpretation of the data reported by Tepperman and Soper (24) is that LPS causes mild irritation to the gastric mucosa through induction of iNOS and thereby elicits an adaptive cytoprotective response in the stomach (8, 28). However, Ferraz et al. (7) recently reported that endotoxin pretreatment enhanced the susceptibility of the stomach to damage and that this effect was negated by NOS inhibition with aminoguanidine, which is more selective for iNOS. Consequently, the role of iNOS in gastric injury models is unclear. It was our hypothesis that induction of this enzyme compromises gastric mucosal defense and has a deleterious effect in the stomach. Our first set of experiments was designed to determine the effects of LPS on gastric iNOS expression. The second set of experiments assessed whether LPS increased or decreased gastric mucosal resistance to injury from acidified ethanol. In a third study, the effect of NOS inhibition on these parameters was examined. The fourth study explored the potential role dilution and/or alkalization of the luminal irritant plays in the effects observed with LPS. The last study evaluated the effects of LPS on gastric mucosal blood flow.

MATERIALS AND METHODS

Animals and Experimental Model

Female Sprague-Dawley rats weighing ~200 g were used in all studies and were housed at constant room temperature with a 12:12-h light-dark cycle. All experiments were performed in rats deprived of food for 18–24 h but allowed free access to water up to the beginning of the studies. On the day of experimentation, all animals were randomly assigned to one of several groups. Sepsis was induced by intraperitoneal administration of LPS from E. coli 0111:B4, given in doses of 1 or 20 mg/kg body wt, whereas control rats received a comparable volume of saline (0.9%). Both doses of LPS have been previously demonstrated to increase iNOS immunoreactivity in the rat ileum 5 h after LPS administration (17). Consequently, 5 h after saline or LPS injection, rats were randomly assigned to one of several experimental protocols (described below). All experiments were approved by the University of Texas at Houston Animal Welfare Committee before studies were conducted.

Protein Extraction and Western Immunoblot Analysis

To estimate and compare the content of gastric iNOS, rats were anesthetized with an injection of 6 mg/kg ip xylazine...
and 70 mg/kg ip ketamine 5 h after receiving saline (n = 6), 1 mg/kg LPS (n = 5), or 20 mg/kg LPS (n = 6). Once satisfactory anesthesia had been achieved, a midline laparotomy was performed, and the stomach was immediately removed. The stomach was opened along the greater curvature, and the mucosa of the glandular portion of the stomach was gently rinsed with saline. The mucosa was blotted dry, scraped off the underlying muscularis externa and sersosa, snap-frozen in liquid nitrogen, and stored at -80°C before protein extraction and Western immunoblot analysis for iNOS. Protein in each sample was extracted by pulverizing the frozen tissue with the use of a mortar and pestle in a liquid nitrogen slurry. This sample was then added to 1 ml of lysis buffer (10 mM Tris, pH 7.4, 100 µM phenylmethylsulfonyl fluoride, and 1% SDS) and then subjected to two 15-s bursts of a Polytron (Vertashear). These samples were then transferred to Microfuge tubes and centrifuged for 10 min at 11,000 g. The supernatant was removed and added to sample buffer (125 mM Tris, pH 6.8, 2% SDS, 5% glycerol, 1% β-mercaptoethanol, and 0.003% bromphenol blue). Protein concentrations within each homogenate were determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL) before the sample buffer was added to the supernatant. The remaining proteins in these prepared homogenates were separated by SDS (7.5%)-PAGE, using 40 µg of protein per sample. Resultant proteins were electroblotted onto nitrocellulose paper and incubated for 1 h at room temperature in blocking solution (5% nonfat dried milk and PBS). The resultant blot was then washed twice in 0.1% Tween 20-PBS followed by a 1-h incubation with a specific polyclonal anti-iNOS antibody (1:2,000 dilution). Blots were then washed twice and incubated with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin as a secondary antibody (1:10,000 dilution) for 1 h. After two final washes, the immune complexes were visualized with the use of enhanced chemiluminescence detection. The resultant autoradiograph was then assessed semiquantitatively utilizing computer-assisted densitometry and reported as mean relative densitometric units.

To assess the hemodynamic effects of LPS in this experimental model, additional rats had carotid artery catheters placed the day before experimentation. Rats were anesthetized with an injection of 6 mg/kg ip xylazine and 70 mg/kg ip ketamine. A transverse neck incision was made and the carotid artery was isolated. A catheter (PE-50) was placed into the artery, tunneled to the back, and exteriorized at the base of the skull. A metal harness secured the line and allowed the rats free movement. The line was continuously perfused with 0.9% saline at a rate of 0.5 ml/h to prevent clotting. Rats were allowed to recover in individual cages with free access to water, but not food, for 18–24 h before experiments were conducted. On the day of study, rats received intraperitoneal LPS in doses of 1 mg/kg (n = 5) or 20 mg/kg (n = 5); control rats received a comparable volume of saline (n = 5). Mean arterial blood pressure was recorded continuously following these treatments and reported at baseline and 1, 3, and 5 h after the indicated treatments. These time points were chosen on the basis of previously published observations with LPS on the appearance of aortic iNOS immunoreactivity over time (10). After 5 h, rats were killed.

Effect of LPS on Gastric Injury From Acidified Ethanol

In this set of experiments, conscious rats were randomly selected to receive either intraperitoneal LPS in doses of 1 (n = 8) or 20 (n = 10) mg/kg body wt or a comparable volume of saline (control; n = 9). After a 5-h pretreatment period, all animals were given a 1-ml orogastric bolus of acidified ethanol (150 mM hydrochloric acid-50% ethanol). Because this concentration of alcohol in combination with hydrochloric acid results in reproducible macroscopic injury to the glandular portion of the stomach within 5 min of exposure (1, 14), rats were killed 5 min after administration of this injurious agent. In addition, four animals were given LPS (20 mg/kg) for 5 h followed by a 1-ml orogastric bolus of distilled water rather than acidified ethanol and killed 5 min later. Immediately after rats were killed, all stomachs were removed and the total area of macroscopic injury was quantified. This was accomplished by measuring the length and the width of each lesion with calipers to determine the surface areas involved with each lesion and then summing the individual surface areas to obtain the overall surface area injured for each stomach. Gross damage, when present, was confined to the glandular or acid-secreting portion of the stomach, so results were recorded as mean damage to this region in square millimeters ± standard error of the mean for each experimental group.

Morphological Analysis

In a separate set of experiments using a protocol similar to that used in the macroscopic injury studies and utilizing four animals per group, the morphological correlates of the resultant injury or protection were determined in animals pretreated with either intraperitoneal saline or LPS (20 mg/kg ip) for 5 h followed by a 1-ml orogastric gavage with distilled water or acidified ethanol. In these studies, animals were killed 5 min after receiving water or acidified ethanol. Immediately after rats were killed, the abdomen was opened, the gastroesophageal junction and pylorus were ligated, and 1.5 ml of 15% Karnovsky's fixative (9) injected through the nonglandular forestomach using a 27-gauge needle and syringe. Each stomach was rapidly removed and immersed in the same fixative for at least 24 h before processing for light microscopy. After fixation, each stomach was opened along the lesser curvature by an independent observer blinded to the protocol. Sections (2 x 10 mm) of the glandular epithelium were randomly excised from the glandular portion of each stomach. From these tissue blocks, hematoxylin and eosin-stained, paraffin-embedded sections were processed for routine light microscopy, using standard techniques. All slides were prepared such that the observer (D. H. Russell) assessing the extent of the resultant injury or protection was blinded to the experimental protocol. Only after the degree of damage had been assessed was the specimen decoded and the results collated. Tissue sections were evaluated as previously reported (14, 20). The criteria for assessing gastric mucosal damage have been published previously and are as follows: type I damage involves luminal surface mucous cells, type II damage involves luminal surface and gastric pit mucous cells, type III damage involves surface and gastric pit mucous cells as well as upper gland cells, and type IV damage consists of severe injury to all surface epithelium and all or most of the glandular epithelium.

NOS Inhibition Studies

Because it was shown that LPS dose dependently increased gastric iNOS immunoreactivity and prevented gastric injury from acidified ethanol (see results), further studies were conducted to examine the role of NO in this process. Using a similar protocol and a sample size of six rats per group, animals were given the NOS inhibitor aminoguanidine in doses of 15 or 45 mg/kg ip, concurrent with administration of LPS (20 mg/kg ip), whereas control animals received saline. These doses of aminoguanidine were chosen because they have been shown to primarily inhibit iNOS activity, as
opposed to the constitutive isoform of NOS in models of septic shock (32). In addition, some animals were given delayed administration of aminoguanidine (45 mg/kg ip) 2 h after LPS (n = 6) or saline (n = 4) administration. These latter groups were included because NOS inhibition during LPS pretreatment can have differing effects depending on whether it is given concurrently or later in the course of endotoxemia (11), i.e., at a time point when iNOS is clearly upregulated. In both sets of experiments, gastric injury was induced with acidified ethanol 5 h after LPS or saline administration, and macroscopic injury was assessed as previously described.

Because it was shown in this set of experiments that aminoguanidine failed to reverse or attenuate LPS-induced gastroprotection from acidified ethanol (see RESULTS), further studies were included to determine whether aminoguanidine was effective at inhibiting iNOS function in our model. This was accomplished by administering aminoguanidine (45 mg/kg ip) concurrently with LPS (20 mg/kg ip) or 2 h after LPS; control animals received saline. A sample size of six animals per group was used. Five hours after receiving LPS or saline, rats were killed and the stomach contents were aspirated via the forestomach. The entire volume of gastric juice contained within the stomach was removed and centrifuged immediately. The volume of supernatant was recorded, snap-frozen in liquid nitrogen, and stored at −70°C prior to quantification of gastric luminal nitrate and nitrite accumulation as an index of gastric mucosal NO synthesis (18). Nitrate reductase generated by anaerobic growth of E. coli was used to reduce nitrate to nitrite. The amount of nitrite generated was determined by a colorimetric method based on the Griess reaction with sulfanilic acid and N-(1-naphthyl)ethylenediamine hydrochloric acid. Optical density at 540 nm was read before and 20 min after addition of the chromagen, as previously reported (10, 13). Standards were prepared with nitrates and taken through the full assay procedure. Gastric luminal fluid was also assayed for protein content using the previously described BCA protein assay as well as for glucose as index of mucosal irritation (5). In addition, the electrolyte composition of the gastric fluid was examined by measuring the amount of sodium, potassium, chloride, and bicarbonate present. Glucose levels were quantified using the glucose hexokinase method (Boehringer Mannheim) as described by Bergmeyer et al. (2). Sodium, potassium, and chloride levels were quantified using the diluted ion-selective electrode (Boehringer Mannheim) technique (26). Bicarbonate levels were quantified using an automated clinical chemistry analyzer with reagents from Boehringer Mannheim, based on the phosphono-L-pyrourate carboxylase method described by Wilson et al. (31).

Because NOS inhibitors can have differential effects, we also examined the effects of LPS after administration of L-NAME, which has minimal isoform selectivity (22, 27). Accordingly, rats (n = 5/group) were given L-NAME (5 mg/kg) subcutaneously concurrent with LPS (20 mg/kg) administration; control rats received saline. Five hours later, rats were killed and gastric luminal fluid was removed. The volume was recorded and the fluid was assayed for protein, glucose, sodium, potassium, chloride, and bicarbonate content as described above. In this experiment, rats receiving L-NAME and LPS developed significant macroscopic gastric injury, which occurred in the absence of acidified ethanol. The extent of macroscopic gastric injury was determined as previously described. The dose of L-NAME chosen has been shown to restore mean arterial blood pressure in rats receiving this dose of LPS (10).

Role of Dilution in LPS-Induced Gastroprotection

Because it was shown that a 5-h pretreatment with LPS significantly increased gastric residual volume, an effect not negated by aminoguanidine (see RESULTS), we explored the possibility that LPS-induced gastroprotection may in part be due to dilution of the luminal irritant acidified ethanol. Thus further studies were undertaken in which LPS was given intraperitoneally in doses of 1 (n = 5) or 20 (n = 8) mg/kg for 5 h; control animals (n = 6) received intraperitoneal saline. Gastric residual volume was measured 5 h later as described in the aminoguanidine studies, and protein content and glucose content were measured. In addition, gastric luminal sodium, potassium, chloride, and bicarbonate content, as well as the serum concentrations of these electrolytes, were determined as previously described. Serum was prepared by withdrawing 1–2 ml of blood via a cardiac puncture at the completion of the experiment and then centrifuging the sample immediately. Serum was kept at 4°C and the electrolyte and glucose concentrations were determined as previously described. After completion of this experiment, the mean residual volume for LPS-treated animals was determined (1.6 ± 0.2 and 2.5 ± 0.6 ml for 1 and 20 mg/kg, respectively). Additional rats were randomly selected to receive either 1 ml of concentrated acidified ethanol or 1 ml of a diluted irritant, using a sample size of six animals per group. The diluted irritants were made by adding the mean residual volume (i.e., 1.6 or 2.5 ml) to 1 ml of acidified ethanol such that their final concentrations were 58 mM hydrochloric acid-19% ethanol and 43 mM hydrochloric acid-14% ethanol, respectively. Five minutes after administration of the injurious agents, rats were killed and macroscopic injury was determined.

The role of dilution and or neutralization of acidified ethanol was also examined by pretreating rats with saline (n = 5), 1 mg/kg LPS (n = 4), or 20 mg/kg LPS (n = 5) for 5 h and then administering a 1-ml orogastric bolus of acidified ethanol. Rats were killed 5 min after receiving the damaging agent, and the gastric fluid was aspirated. This fluid was then assayed for its alcohol concentration and its pH was measured using a standard pH probe (ORION 330). Ethanol levels were quantified on a clinical chemistry analyzer using the Boehringer Mannheim ethyl alcohol method (26).

The role of dilution was further assessed in conscious rats by utilizing a chronic gastric fistula preparation to remove gastric luminal fluid before the introduction of acidified ethanol. A chronic gastric fistula was prepared by anesthetizing rats with intraperitoneal xylazine (6 mg/kg) and ketamine (70 mg/kg). After adequate anesthesia had been obtained, a 1.5-cm abdominal incision was made and the forestomach (nonglandular portion) was identified. A gastrotomy was made and a silicon catheter (0.04 in. i.d.; SF Medical, Hudson, MA) was inserted with its tip proximal to the glandular stomach. A purse-string suture was used to secure it. The tube was brought out and tunneled to an exit point behind the head. The forestomach was sutured to the abdominal wall and the abdominal incision was closed. The catheters were occluded and then taped circumferentially to secure them. The animals were transferred to isolated cages and allowed to recover with full access to food and water. Seven days after the procedure, rats were fasted for 18–24 h (free access to water) for experimentation. On the day of experimentation rats were randomly selected to receive either LPS (20 mg/kg ip; n = 6) or saline (0.9%; n = 5) for 5 h. Gastric fistula tubes were allowed to drain during the pretreatment time. At the end of 5 h, the tubes were aspirated to ensure the stomach was empty of luminal fluid. Gastric injury...
was then induced with 1 ml of orogastric acidified ethanol, and macroscopic injury was assessed 5 min later as previously described. These groups were compared with a sham group of rats (abdominal incision, closure and 7-day recovery period; n = 6) as well as with a group of rats that did not undergo any operation (n = 6).

In still other studies, the effect of LPS on gastric injury from luminal irritants was examined with the use of an anesthetized preparation. This was accomplished by anesthetizing the animals 5 h after the indicated pretreatments with xylazine (6 mg/kg ip) and ketamine (70 mg/kg ip). After satisfactory anesthesia had been achieved, a midline laparotomy was performed, the gastroesophageal junction and pylorus were ligated, and the gastric contents were aspirated. After aspiration, 3 ml of acidified ethanol or 10 mM acidified taurocholate (10 mM taurocholic acid, 100 mM hydrochloric acid, 60 mM sodium chloride, 4 g polyethylene glycol, pH = 1.2) was introduced into the gastric lumen to induce gastric injury. This latter agent was also chosen as a luminal irritant because dilute bile acid results in less severe macroscopic injury. This latter agent was also chosen as a luminal irritant (29), rats were killed 10 min after receiving either injurious agent, and macroscopic injury was determined. In this set of experiments, the following pretreatment groups were studied: saline + acidified ethanol (n = 6), 20 mg/kg LPS + acidified ethanol (n = 6), saline + saline + bile acid (n = 8), saline + 20 mg/kg LPS + bile acid (n = 10), 45 mg/kg aminoguanidine + saline + bile acid (n = 4), and 45 mg/kg aminoguanidine + 20 mg/kg LPS + bile acid (n = 8).

**Blood Flow Studies**

The effect of LPS on gastric mucosal blood flow was examined by pretreating conscious rats with 20 mg/kg of LPS (n = 7) or 1 mg/kg of LPS (n = 6) for 5 h; controls received intraperitoneal saline (n = 6). At the end of the 5-h pretreatment period, rats were anesthetized with intraperitoneal xylazine (6 mg/kg) and ketamine (70 mg/kg), and gastric mucosal blood flow was determined using the laser-Doppler technique as previously reported (15). The stomach was exposed through a midline incision, and a catheter was introduced into the nonglandular forestomach to provide access for a Teflon-coated laser optic flow probe (Peri Flux PF 420, standard probe, 0.25-mm fiber separation). The flow probe was positioned to allow contact with the glandular portion of the stomach. After appropriate position was assured, the stomach was allowed to equilibrate for 15 min. After equilibration, mucosal blood flow to the stomach was recorded continuously with a laser-Doppler flow monitor (Peri Flux 4001 Master; Perimed, Järenfält, Sweden). Blood flow was monitored for a 20-min period and the animal was killed. Blood flow was reported in standard units according to Peri Flux guidelines.

**Chemicals**

Nitrocellulose filters were purchased from Schleicher Schuell (Keene, NH), and X-ray film (T-MAT) was purchased from Eastman Kodak (Rochester, NY). The enhanced chemiluminescence system for Western Immunoblot analysis was from Amersham (Arlington Heights, IL). The BCA protein assay was from Pierce. All other reagents, including LPS, aminoguanidine, L-NAME, and taurocholic acid, were of molecular biology grade and were purchased from Sigma Chemical (St. Louis, MO). Aminoguanidine was dissolved in 0.1 N hydrochloric acid and subsequently neutralized (pH 7.4) in 0.1 N sodium hydroxide. All other solutions were prepared in saline. The polyclonal antibody against iNOS utilized in Western immunoblotting analysis was prepared and developed by the Trauma Research Center at the University of Texas Medical School, Houston, TX. The efficacy of this antibody probe has been previously published (30).

**Statistics**

All values in the figures and text are expressed as means ± SE of n observations, where n is the number of animals examined. Statistical significance was determined using ANOVA followed by Fisher’s post hoc test. P < 0.05 was considered to be statistically significant.

**RESULTS**

LPS Enhances the Abundance of Gastric Mucosal iNOS Immunoreactivity

Using a site-directed polyclonal antibody to iNOS, the abundance of iNOS protein in gastric mucosal homogenates following saline or LPS pretreatment was examined without exposing the stomachs to an irritant. As depicted in Fig. 1, a 5-h pretreatment with LPS dose dependently increased iNOS immunoreactivity in the gastric mucosa compared with saline-treated controls. A small background amount of iNOS immunoreactivity was occasionally detected in saline-treated animals, however, it was not associated with much nitrate or nitrite accumulation (see Fig. 5). Furthermore, when densitometric analysis of all treatment groups was performed, both 1 and 20 mg/kg of intraperitoneal LPS resulted in significantly (P ≤ 0.006) more iNOS immunoreactivity (4.2 ± 0.7 and 12.2 ± 3.0 relative densitometric units) than saline-treated controls, clearly indicating that this protein is upregulated in gastric mucosa during endotoxemia.

The effects of LPS on mean arterial blood pressure at 1, 3, and 5 h after its administration are shown in Table 1. As demonstrated, 1 mg/kg of LPS did not result in
hypotension at any of the time points assessed. In contrast, 20 mg/kg of LPS caused a significant reduction in mean arterial blood pressure at all three time points. Blood pressure slightly increased over the last 2 h of the experiment after high-dose LPS administration, but blood pressure at 5 h was not significantly different from that measured at 1 or 3 h and was still significantly below baseline values.

### LPS Dose Dependently Reduces Gastric Injury From Acidified Ethanol in the Conscious, Intact Animal

Macroscopic findings. As shown in Fig. 2, LPS dose dependently attenuated gastric injury from acidified ethanol. In animals receiving saline, acidified ethanol resulted in macroscopic hemorrhagic lesion formation along the gastric folds that was confined to the glandular portion of the stomach. In comparison, in animals receiving the highest dose of LPS (20 mg/kg), there was almost a complete absence of macroscopic lesion formation. LPS (20 mg/kg ip for 5 h; n = 4) given alone, without exposing the stomach to acidified ethanol, did not result in any macroscopic lesions.

Morphological findings. Results of light microscopic evaluation of stomachs from saline- and LPS (20 mg/kg)-pretreated animals are demonstrated in Figs. 3 and 4. Animals treated for 5 h with saline followed by exposure to 1 ml of distilled water had an intact surface epithelium over underlying gastric pits and glandular cells characteristic of normal gastric epithelium (Fig. 3A). In contrast, animals receiving a 5-h treatment with LPS followed by exposure to water had superficial injury with some damage to surface epithelial cells (type I injury) and rare injury to cells of the upper gastric pits (type II injury), as shown in Fig. 3B. In this group of animals, no glandular cell injury was ever identified, but congestion was occasionally detected.

Exposure of the stomach to acidified ethanol for 5 min in saline-treated controls resulted in obliteration of the surface epithelium and loss of the mucosa in many areas down through the gastric pits (Fig. 4A). In areas corresponding to the visible hemorrhagic lesions, disruption of the epithelium could be seen extending for variable distances down the glands, typical of types III and IV injury. In such stomachs, the interstitium was often edematous and filled with fibrin and/or hemorrhage. Despite the severe nature of this damage, LPS-treated animals exposed to the identical damaging agent manifested a distinctly different pattern of morphological injury (Fig. 4B). The majority of injury was confined to the surface epithelial cells, with only superficial injury to the gastric pits, similar to that observed in animals receiving LPS followed by orogastric water.

Taken together, these studies demonstrated that LPS pretreatment to conscious rats not only prevented the visible gastric lesions from acidified ethanol but also provided a significant degree of morphological preservation in the face of a damaging luminal insult.

LPS-Induced Gastroprotection is not Reversed by Aminoguanidine

The effect of aminoguanidine on LPS-induced gastroprotection is shown in Table 2. As shown, the ability of LPS to prevent gastric injury from acidified ethanol was neither reversed nor attenuated by aminoguanidine at either dose, and delayed administration of aminoguanidine also had no effect. Thus further studies were conducted to determine whether aminoguanidine was effective at inhibiting NO formation in our model. Gastric luminal nitrate and nitrite accumulation was used as an index of gastric mucosal NOS activity, and LPS resulted in a significant increase in these NO metabolites compared with saline-treated animals (Fig. 5). This effect of LPS was in turn almost completely negated by aminoguanidine (45 mg/kg) given concurrently with LPS.

Interestingly, neither concurrent nor delayed administration of aminoguanidine significantly diminished the enhanced accumulation of fluid, protein, or glucose into the gastric lumen that was observed in all animals treated with LPS (Table 3). In addition, when the gastric luminal fluid was analyzed for its electrolyte content under these conditions, it was noted that LPS caused a significant increase in sodium, potassium, chloride, and bicarbonate content compared with controls, and aminoguanidine did not abolish these effects (Table 3).

In comparison, different results were obtained with L-NAME given concurrently with LPS to the conscious rat. L-NAME augmented LPS-induced gastric luminal...

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**Table 1. Mean arterial blood pressure determinations following intraperitoneal saline or LPS pretreatment in the conscious rat**

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>1 h</th>
<th>3 h</th>
<th>5 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>102±4</td>
<td>99±4</td>
<td>101±5</td>
<td>99±5</td>
</tr>
<tr>
<td>LPS (1 mg/kg)</td>
<td>100±4</td>
<td>99±5</td>
<td>103±6</td>
<td>105±7</td>
</tr>
<tr>
<td>LPS (20 mg/kg)</td>
<td>100±3</td>
<td>87±6*</td>
<td>82±6*</td>
<td>90±7*</td>
</tr>
</tbody>
</table>

*Values are means ± SE. LPS, lipopolysaccharide. *P < 0.05 vs. baseline, LPS (1 mg/kg), and saline.*
fluid accumulation and increased protein, glucose, sodium, potassium, chloride, and bicarbonate content (Table 3) when given simultaneously with LPS. L-NAME alone had no significant effect on these indexes compared with controls receiving saline. In addition, L-NAME reduced the pH of gastric luminal fluid compared with LPS (20 mg/kg) alone (7.25 ± 0.15 vs. 7.78 ± 0.17; P < 0.04), although the pH was still significantly alkaline compared with controls (pH = 4.12 ± 1.2). Moreover, LPS and L-NAME together caused macroscopic gastric injury before any introduction of a luminal irritant (LPS + L-NAME = 68 ± 21 vs. LPS + saline = 0 mm²; P < 0.001). Taken together, these NOS inhibition studies clearly demonstrate the differing effects these antagonists possess.

LPS Causes Dilution of Acidified Ethanol

It became quite obvious in the course of these experiments that a significant amount of fluid was accumulating in the gastric lumen of animals receiving LPS. We therefore addressed the potential role of dilution as an explanation for our findings with LPS. A 5-h pretreatment with LPS significantly (P < 0.05) increased the volume and the pH of gastric luminal fluid with both the 1 mg/kg (1.6 ± 0.2 ml; pH 7.74 ± 0.23) and the 20 mg/kg (2.5 ± 0.6 ml; pH = 7.78 ± 0.17) doses of LPS compared with rats receiving saline (0.26 ± 0.18 ml; pH = 4.12 ± 1.2). As depicted in Table 4, LPS also increased glucose accumulation into the gastric lumen and caused a significant increase in gastric luminal sodium, potassium, chloride, and bicarbonate content compared with saline-treated controls. After a 5-h pretreatment with LPS, serum electrolytes were not significantly different from controls, although the higher dose of LPS did significantly decrease serum glucose levels compared with controls (Table 4).

In the experiments examining the effect of diluted acidified ethanol on macroscopic injury to gastric mucosa, it was shown that diluted acidified ethanol caused injury that was similar in magnitude to that reported for animals receiving 1 or 20 mg/kg of LPS 5 h before exposure to concentrated acidified ethanol (Fig. 6). Furthermore, as shown in Table 5, animals receiving a 5-h pretreatment with either dose of LPS, followed by a 1-ml orogastric bolus of acidified ethanol, had alkalinization and dilution of the damaging agent compared with controls.

In the chronic gastric fistula preparation, saline-pretreated rats had 126 ± 26 mm² of macroscopic gastric injury from acidified ethanol compared with 114 ± 20 mm² in sham-operated rats. In chronic gastric fistula-prepared rats receiving LPS for 5 h, acidified
ethanol resulted in $96 \pm 22$ mm$^2$ of macroscopic damage. In rats not undergoing an operation, acidified ethanol caused $130 \pm 21$ mm$^2$ of macroscopic injury. There were no significant differences among the four pretreatment groups. Thus in the gastric fistula preparation in which luminal fluid was removed prior to introduction of acidified ethanol, LPS had no gastroprotective effect.

Aminoguanidine Negates LPS-Induced Exacerbation of Gastric Injury From Luminal Irritants in the Anesthetized Preparation

We next assessed the effect of LPS on gastric injury in an anesthetized preparation. In animals pretreated for

Table 2. Effect of aminoguanidine on LPS-induced gastroprotection from acidified ethanol in the conscious rat

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>Injury, mm$^2$</th>
</tr>
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<tbody>
<tr>
<td>Saline-saline</td>
<td>114 \pm 21</td>
</tr>
<tr>
<td>Saline-LPS$^b$</td>
<td>7 \pm 3$^a$</td>
</tr>
<tr>
<td>Aminoguanidine-saline$^c$</td>
<td>101 \pm 22</td>
</tr>
<tr>
<td>Aminoguanidine-LPS$^{3, c}$</td>
<td>15 \pm 6$^a$</td>
</tr>
<tr>
<td>Aminoguanidine-saline$^d$</td>
<td>122 \pm 19</td>
</tr>
<tr>
<td>Aminoguanidine-LPS$^{3, d}$</td>
<td>10 \pm 4$^a$</td>
</tr>
<tr>
<td>Aminoguanidine-saline$^{e}$</td>
<td>118 \pm 21</td>
</tr>
<tr>
<td>Aminoguanidine-LPS$^{3, e}$</td>
<td>17 \pm 8$^a$</td>
</tr>
</tbody>
</table>

Values are means \pm SE. $^*$P < 0.001 vs. counterpart. $^b$LPS (20 mg/kg ip) for 5 h. $^c$Aminoguanidine (15 mg/kg ip) given concurrently with saline or LPS for 5 h. $^d$Aminoguanidine (45 mg/kg ip) given concurrently with saline or LPS for 5 h. $^e$Aminoguanidine (45 mg/kg ip) given 2 h after LPS or saline and 3 h before 1 ml of orogastric acidified ethanol (150 mM HCl/50% ethanol).

Fig. 4. A: light micrograph (×400) of gastric mucosa from rat pretreated with saline for 5 h followed by exposure to 1 ml of orogastric acidified ethanol (150 mM HCl-50% ethanol) for 5 min, depicts severe damage to cells lining pits and upper glands of stomach. In this micrograph, pits are seen completely autolyzed and lifted up from glands, from which lining cells (arrows) can be seen sloughing from surrounding lamina propria. B: gastric mucosa from a rat given LPS (20 mg/kg ip) 5 h before exposure to acidified ethanol shows a relatively mild injury similar to that seen with LPS alone, consisting of surface cell vacuolization and upper pit disruption.

Fig. 5. Effect of NOS inhibition with aminoguanidine (45 mg/kg ip) given concurrently with either saline or LPS (20 mg/kg ip) for 5 h on gastric luminal nitrate and nitrite (NOx) accumulation without exposure to luminal irritants. Data are means \pm SE; n = 5 for all groups. $^*$P < 0.001 vs. vehicle/saline. $^{**}$P = 0.005 vs. vehicle/LPS.
Table 3. Effects of LPS on gastric luminal fluid electrolyte content and protein and glucose accumulation in the presence and absence of aminoguanidine or L-NAME in the conscious rat

<table>
<thead>
<tr>
<th>Group</th>
<th>Residual Volume, ml</th>
<th>Protein, mg</th>
<th>Glucose, mg</th>
<th>Na, meq</th>
<th>K, meq</th>
<th>Cl, meq</th>
<th>CO₂, meq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline-saline</td>
<td>0.25 ± 0.15</td>
<td>0.21 ± 0.04</td>
<td>0.03 ± 0.008</td>
<td>0.015 ± 0.002</td>
<td>0.005 ± 0.001</td>
<td>0.019 ± 0.002</td>
<td>0.004 ± 0.002</td>
</tr>
<tr>
<td>Saline-LPS</td>
<td>2.50 ± 0.60</td>
<td>1.52 ± 0.20</td>
<td>2.19 ± 0.45</td>
<td>0.290 ± 0.04</td>
<td>0.020 ± 0.003</td>
<td>0.258 ± 0.03</td>
<td>0.032 ± 0.05</td>
</tr>
<tr>
<td>Aminoguanidine-saline</td>
<td>0.30 ± 0.10</td>
<td>0.28 ± 0.09</td>
<td>0.03 ± 0.009</td>
<td>0.026 ± 0.03</td>
<td>0.009 ± 0.001</td>
<td>0.031 ± 0.001</td>
<td>0.004 ± 0.004</td>
</tr>
<tr>
<td>Aminoguanidine-LPS</td>
<td>2.20 ± 0.20</td>
<td>1.58 ± 0.20</td>
<td>1.50 ± 0.38</td>
<td>0.212 ± 0.003</td>
<td>0.015 ± 0.003</td>
<td>0.184 ± 0.029</td>
<td>0.027 ± 0.029</td>
</tr>
<tr>
<td>Aminoguanidine-LPS+L-NAME</td>
<td>2.10 ± 0.80</td>
<td>1.50 ± 0.20</td>
<td>1.41 ± 0.76</td>
<td>0.260 ± 0.10</td>
<td>0.020 ± 0.006</td>
<td>0.225 ± 0.09</td>
<td>0.030 ± 0.014</td>
</tr>
<tr>
<td>L-NAME-saline</td>
<td>0.38 ± 0.03</td>
<td>0.38 ± 0.03</td>
<td>0.01 ± 0.005</td>
<td>0.006 ± 0.001</td>
<td>0.008 ± 0.002</td>
<td>0.055 ± 0.001</td>
<td>0.002 ± 0.001</td>
</tr>
<tr>
<td>L-NAME-LPS</td>
<td>5.71 ± 0.60</td>
<td>5.00 ± 0.44</td>
<td>5.90 ± 0.89</td>
<td>0.67 ± 0.07</td>
<td>0.039 ± 0.004</td>
<td>0.604 ± 0.05</td>
<td>0.054 ± 0.013</td>
</tr>
</tbody>
</table>

Values are means ± SE. L-NAME, N G-nitro-arginine methyl ester. *P < 0.05 vs. saline-saline. †P < 0.05 vs. aminoguanidine-saline. ‡P < 0.05 vs. saline-LPS. §LPS (20 mg/kg ip) for 5 h. ¶Aminoguanidine (45 mg/kg ip) given concurrently with saline or LPS for 5 h. ¶Aminoguanidine (45 mg/kg ip) given 2 h after LPS or saline and 3 h before aspiration of gastric luminal fluid. †L-NAME (5 mg/kg sc) given concurrently with saline or LPS for 5 h.

DISCUSSION

This study demonstrated that a 5-h pretreatment with LPS results in increased expression and activity of iNOS in gastric mucosa. In the absence of a luminal irritant, LPS caused significant accumulation of bicarbonate-rich gastric luminal fluid and resulted in superficial microscopic gastric mucosal injury, but not macroscopic injury. The effect of LPS on gastric injury from luminal irritants differed depending on the precise conditions under which it was studied. In the conscious intact animal preparation, LPS dose dependently protected the gastric mucosa from acidified ethanol-induced gastric injury according to both macroscopic and morphological criteria. In contrast, with the use of an anesthetized preparation that required ligation of the pylorus and removal of gastric juice before introduction of a luminal irritant, LPS exacerbated gastric injury from dilute bile acid and acidified ethanol.

It is our contention that the apparent discrepancy between the conscious and anesthetized preparations is in part due to removal of accumulated gastric fluid in the latter model, whereas it is allowed to remain present in the former model. When this fluid is not removed and the stomach is challenged with a luminal irritant, dilution of the irritant must occur, thereby diminishing its ability to invoke gastric injury. This possibility is further substantiated by the finding that LPS was found to dilute the ethanol concentration and increase the pH of the luminal fluid after orogastric administration of acidified ethanol. Furthermore, diluted acidified ethanol resulted in gastric injury of a magnitude similar to that found in stomachs exposed to concentrated acidified ethanol after LPS pretreatment. In addition, when the gastric fistula preparation was used in conscious rats, thus not allowing gastric luminal irritants to remain within the stomach, the extent of injury was significantly reduced.

Table 4. Effects of LPS on serum and gastric luminal electrolyte content in the conscious rat

<table>
<thead>
<tr>
<th>Group</th>
<th>Glucose, mg</th>
<th>Na, meq</th>
<th>K, meq</th>
<th>Cl, meq</th>
<th>CO₂, meq</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>0.049 ± 0.012</td>
<td>0.120 ± 0.05</td>
<td>0.005 ± 0.002</td>
<td>0.12 ± 0.04</td>
<td>0.007 ± 0.003</td>
</tr>
<tr>
<td>LPS (1 mg/kg)</td>
<td>1.210 ± 0.45*</td>
<td>0.260 ± 0.04*</td>
<td>0.016 ± 0.001*</td>
<td>0.21 ± 0.05*</td>
<td>0.031 ± 0.003*</td>
</tr>
<tr>
<td>LPS (20 mg/kg)</td>
<td>1.760 ± 0.59*</td>
<td>0.260 ± 0.06*</td>
<td>0.021 ± 0.004*</td>
<td>0.22 ± 0.05*</td>
<td>0.030 ± 0.007*</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. saline. †P = 0.03 vs. saline.
nal fluid to accumulate, LPS pretreatment did not result in gastroprotection. Nevertheless, another possible explanation for our findings in the anesthetized preparation is that gastric mucosal blood flow was influenced by anesthesia and, in fact, high-dose, but not low-dose, LPS reduced blood flow in this setting. However, Ferraz et al. (7) demonstrated that LPS in doses that were unlikely to reduce blood flow exacerbated gastric injury from topically applied 20% ethanol in an ex vivo gastric chamber preparation, which also requires that the animals be anesthetized. In that study, the role of dilution was eliminated because the model chosen requires that the stomach be opened and placed into a chamber after a 6-h pretreatment with endotoxin. Under these conditions, the gastric fluid would be removed and thus would not be available to dilute a subsequent challenge from a topically applied damaging agent. As in our present study using the anesthetized preparation, LPS was found to exacerbate, not prevent, gastric injury (7). Ferraz et al. (7) did not examine the effect of LPS on gastric injury in conscious rats.

The retention of fluid in the gastric lumen during endotoxemia could be due to gastric stasis secondary to delayed gastric emptying, although the protein shedding and accumulation of glucose into the gastric lumen, as well as the morphological injury observed after LPS treatment, would suggest that it results from mucosal irritation (5). Furthermore, the luminal alkalinization after LPS is also consistent with mild damage to the gastric epithelium (21). The accumulation of fluid within the gastric lumen also appears to represent a protective response, enabling the stomach to withstand the challenge of potentially noxious substances it might be exposed to, such as bile or acid. Along these lines, Esplugues et al. (6) recently demonstrated that endotoxin results in inhibition of gastric acid secretion and that this response represents a protective reflex. Given this possibility, our findings could have clinical significance. For example, during sepsis, there is often an associated ileus (3), and duodenogastric reflux of bile in this clinical scenario is not an uncommon event (19). In the critical care setting, in which the septic patient often requires ventilatory support and is prone to develop erosive stress gastritis (4), the patients frequently have nasogastric decompression of gastric juices. This being the case, our data would suggest that this clinical practice may not necessarily be beneficial to the patient and may in fact predispose them to the development of erosive gastritis.

It is noteworthy that aminoguanidine did not reverse the accumulation of fluid within the gastric lumen yet significantly diminished accumulation of gastric luminal nitrates and nitrites after LPS administration. As a result, significant gastroprotection from acidified ethanol-induced gastric injury was still observed in LPS-pretreated animals when the conscious preparation was used. In comparison, when the luminal fluid was removed and the stomach was challenged with an irritant in the anesthetized preparation, there was clearly an exacerbation of gastric injury identified in animals receiving LPS. The ability of LPS to augment gastric injury from dilute bile acid under these conditions was in turn negated by the concurrent administration of aminoguanidine, implicating a role for iNOS in this response. However, the data also suggested that iNOS in all probability does not play a role in either the accumulation of fluid within the gastric lumen observed during endotoxemia or the gastroprotective

Table 5. Effects of a 5-h LPS pretreatment on gastric luminal fluid pH and alcohol concentration after a 1-ml orogastric bolus of acidified ethanol in the conscious rat

<table>
<thead>
<tr>
<th>Group</th>
<th>pH</th>
<th>Alcohol, g/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>2.1±0.1</td>
<td>1.5±0.07</td>
</tr>
<tr>
<td>LPS (1 mg)</td>
<td>3.5±0.4*</td>
<td>1.1±0.09†</td>
</tr>
<tr>
<td>LPS (20 mg)</td>
<td>3.9±0.3*</td>
<td>0.8±0.10†</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P ≤ 0.008 vs. saline. †P ≤ 0.003 vs. saline.
actions of LPS in conscious rats, because aminoguanidine did not attenuate either action. Interestingly, the nonselective NOS inhibitor L-NAME enhanced gastric luminal fluid accumulation and significantly increased gastric luminal electrolyte, protein, and glucose content when given concurrently with LPS. Moreover, the combination of L-NAME and LPS together resulted in significant macroscopic gastric injury without any subsequent exposure to a luminal irritant. These findings suggested that LPS-induced gastroprotection in conscious rats is dependent on the constitutive isoform of NOS and is independent of fluid accumulation if this isoform is inhibited.

Our findings with NOS inhibition are consistent with those made by Takeuchi et al. (22), in which the role of NO in regulating gastric acid secretion in the damaged stomach was examined. Their study demonstrated that the mild irritant 20 mM taurocholate caused luminal alkalinization and inhibition of acid secretion and resulted in adaptive cytoprotection in the rat. More importantly, acid secretion was actually enhanced in the stomach after exposure to taurocholate in the presence of L-NAME, an effect that was reversed by L-arginine. In stomachs not injured with taurocholate, L-NAME had minimal effects on gastric secretion (22), similar to the effects we observed with L-NAME in the absence of LPS. As in our study with LPS, aminoguanidine did not modify any gastric secretory responses to taurocholate. The results of Takeuchi et al. (22) suggested that when the stomach is injured (i.e., taurocholate) both acid-inhibitory and acid-stimulatory pathways are activated. Their results further suggested that the constitutive isoform of NOS has some influence on the acid-stimulatory pathway in the damaged stomach because the increased acid response to injury with taurocholate was unmasked in the presence of L-NAME (22). In our study LPS clearly caused mild injury to the surface epithelium of the stomach. Inhibition of the constitutive isoform of NOS with L-NAME under these conditions would then be expected to result in enhanced fluid accumulation, presumably from increased acid secretion via the acid-stimulatory pathway.

Our data may shed some light on a previous study by Tepperman and Soper (24), who first demonstrated that LPS results in gastroprotection in the conscious intact animal model. Their findings generated a lot of excitement and interest in iNOS as a potential cytoprotective mechanism because the protective response associated with LPS was blunted by the administration of L-NAME given concurrently with LPS. When given early in sepsis (before iNOS upregulation occurs), NOS inhibition suppresses the constitutive isoform of NOS (11). Blockade of this isoform would be expected to decrease the resistance of the stomach to alcohol-induced gastric injury, as found by Tepperman and Soper (24). Their study did not assess other NOS inhibitors. Additionally, accumulation of gastric luminal fluid was not addressed, nor was the effect of delayed administration of L-NAME. Inclusion of this latter group is important if one considers the findings reported by Lazo et al. (11). In that study, delayed administration of NOS inhibitors at a time point when iNOS upregulation was clearly present resulted in a reduction in LPS-induced vascular injury to the ileum and colon. In comparison, when L-NAME was given concurrently with LPS, an exacerbation of LPS-induced injury to these organs was observed (11). In our conscious rat model, aminoguanidine, which is more selective for iNOS (27, 32), did not reverse LPS-induced gastroprotection or reduce the accumulation of gastric juice. On the other hand, L-NAME given simultaneously with LPS caused macroscopic gastric injury without subsequent exposure to a luminal irritant. Together, our own observations and those of others indicate that inhibition of NOS can have either beneficial or deleterious effects on gastric injury, depending on which isoform is being inhibited and the precise conditions under which the study occurs.

In conclusion, this study demonstrated that during endotoxemia, the expression of iNOS is increased in the gastric mucosa. In conscious animals, LPS caused accumulation of bicarbonate-rich fluid within the gastric lumen and diminished the magnitude of gastric injury from acidified ethanol. Neither effect was influenced by NOS inhibition, but in the presence of L-NAME LPS lost its gastroprotective action. In comparison, when gastric luminal fluid that accumulated after LPS administration was removed in an anesthetized preparation, the stomach was rendered more susceptible to injury from a subsequent challenge with a luminal irritant. The ability of LPS to exacerbate gastric injury from damaging agents under these conditions was in turn markedly diminished by iNOS inhibition. Collectively these findings suggest that dilution of luminal irritants may play a role in gastric mucosal defense during sepsis, provided that the constitutive isoform of NOS is active. However, upregulation of gastric iNOS does not appear to represent a gastroprotective mechanism and could in fact increase the susceptibility of the stomach to damage under certain conditions.

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REFERENCES


