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Direct Detection of Nitric Oxide and its Roles in Maintaining Gastric Mucosal Integrity Following Ethanol-induced Injury in Rats

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In gastric mucosal injury, nitric oxide (NO) plays both cytoprotective and cytotoxic roles, and the NO level is one determinant of these dual roles. We employed electron paramagnetic resonance (EPR)-spectrometry combined with an NO-trapping technique to directly evaluate NO production in ethanol-induced gastric injury in rats. The rat stomach, mounted on an *ex vivo* chamber, was perfused with ethanol (12.5 and 43%), and NO levels in mucosal tissues were measured during perfusion. Luminal nitrite/nitrate (NO_x) content, mucosal blood flow, area of mucosal injury, transmucosal potential difference (PD), and luminal pH were simultaneously monitored with/without pre-administration of the NO synthase inhibitor, N^G-nitro--L-arginine methyl ester (L-NAME). NO levels in the gastric tissue increased during ethanol perfusion, and luminal NO_x levels increased after the perfusion, accompanying an increase in the area of mucosal injury and changes in physiological parameters. Preadministration of L-NAME aggravated the gastric mucosal damage and suppressed increases in mucosal blood flow in a dose-dependent manner. These results demonstrate that endogenous NO produced in ethanol-induced gastric injury contributes to maintenance of mucosal integrity via regulation of mucosal blood flow.

Keywords: Nitric oxide; Chambered stomach; Ethanol; Gastric mucosal integrity; Electron paramagnetic resonance

INTRODUCTION

It is now recognized that nitric oxide (NO) is an endogenous mediator of vascular tone, a neurotransmitter in both the peripheral and central nervous systems, and an effector molecule in the immune system.[1–3] NO is endogenously synthesized from L-arginine by NO synthases (NOSs), which exist in various organs and tissues in mammals.[4–6] In the gastric mucosa, two types of constitutive NO synthases (cNOSs) have been discovered by means of immunohistochemical techniques: neuronal NO synthase (nNOS), localized in chief cells and mucosecretory cells of the gastric epithelium, and endothelial NO synthase (eNOS), localized in endothelia of the submucosal arterioles and muscularis mucosae.[7–10] Inducible NO synthase (iNOS), which is expressed in states of inflammation and immune activation, has not been detected in normal rat stomach.[11] Additionally, a non-enzymatic NO production pathway appears to be active in the stomach, in which dietary nitrate is reduced to nitrite by the tongue surface bacteria and the nitrite is then carried into the stomach and reduced to NO under luminal acidic conditions.[12–15] Endogenous stomach NO, produced through these enzymatic and non-enzymatic pathways, has been demonstrated to be involved not only in the regulation of gastric mucosal blood flow (GMBF), but also in the modulation of acid secretion,[16,17] mucus secretion[18,19] and gastric motility.[20]

The pathophysiological effects of NO in gastric injury have been extensively evaluated using

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the intragastric application of ethanol or various agents such as taurocholic acid,[21,22] HCl,[23] or aspirin.[24] In an ethanol-induced gastric mucosal injury model in rodents, the condition was worsened by pretreatment with an NO synthase inhibitor.[25,26] Conversely, perfused ethanol has also been shown to scavenge gastric tissue-derived NO through the nitrosation of ethanol.[27] Similarly, the administration of a low dose NO donor into the stomach reduced the area of gastric mucosal damage,[28,29] while a high dose exacerbated the same damage.[30] These results suggest that the biological effects of NO might be strongly influenced by endogenous NO production levels, and that NO may have dual roles in gastric mucosal injury, i.e. cytoprotective and cytotoxic. To fully explore this, it is important to determine the exact amount of endogenously produced NO. However, this is problematic because NO is a highly reactive and short-lived radical, making direct detection difficult.

To elucidate the contribution of NO to the physiology and pathophysiology of ethanol-induced gastric mucosal injury in rats we employed an *ex vivo* gastric chamber system, and continuously monitored macroscopic mucosal damages and physiological parameters, such as GMBF, potential difference (PD) between the gastric lumen and the abdominal cavity, and the pH of the perfusates. NO production in the gastric mucosa and lumen was evaluated directly, using an electron paramagnetic resonance (EPR) NO trapping technique,[31] and indirectly, by measuring luminal NO_x (nitrite plus nitrate) concentrations by a Griess-reaction assay method with high performance liquid chromatography (HPLC).[32] All these measurements were performed under both conditions with or without the intraperitoneal administration of an NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME). The increase in intragastric NO levels could be correlated to changes in the area of the hemorrhagic gastric mucosal injury and to other physiological parameters. The contribution of endogenously produced NO in the stomach to maintain gastric mucosal integrity via regulation of GMBF is discussed.

MATERIALS AND METHODS

Animal Preparation

Male Sprague–Dawley rats, weighing 200–250 g (Charles River, Ibaraki, Japan) were deprived of food for 22 h but had free access to tap water. They were kept in individual cages at a controlled temperature (23°C). Anesthesia was induced with urethane (1.25 mg/kg) administered intraperitoneally (i.p.). All procedures related to animal care described herein were in accordance with the criteria outlined in the guideline for animal experimentation by the Japanese Association for Laboratory Animal Science, 1987.

Chamber Preparation and Protocol

In this study, we used a lucite chamber (Tamura-seisakusho, Kyoto, Japan) system which was applied to *ex vivo* gastric perfusion model in rats.[33–35] The base and rim of the chamber were made of acrylic resin. The surgical procedures followed those of Takeuchi *et al*.[35] After a laparotomy along the median line of the rat's abdomen, the lower esophagus and pylorus were ligated. The gastric lumen was exposed through a median incision at the greater curvature, mounted on the basal part of the lucite chamber and covered with the plastic rim. The area of the exposed gastric mucosa measured 3.14 cm^2. Normal saline (2 ml) was filled to the plastic rim. Normal saline was used for perfusion, at the rate of 0.6 ml/min, through a silicone rubber tube (diameter 1.0 mm), delivered by two peristaltic pumps (Atto Co. Ltd, Tokyo, Japan). A minimum of 60 min (60–120 min) was allowed to stabilize the chambered stomach systems after the surgical procedure. The values measured in this stabilized state were defined as the basal levels. GMBF, PD between the gastric lumen and the abdominal cavity, and the pH of the perfused luminal normal saline were monitored continuously and simultaneously with a digital multi-channel analyzer (LEG1000; Nihon-kohden, Tokyo, Japan) to evaluate the stability of the gastric mucosa. After the system was stabilized, the NOS inhibitor was administered intraperitoneally, followed by perfusion of ethanol (12.5 or 43% in normal saline) at a rate of 0.6 ml/min for 30 min. Then, via the peristaltic pump, normal saline was used to reperfuse.

Direct Measurement of NO by EPR Spectrometry

NO produced in the rat's stomach was measured before and 10 and 30 min after perfusion of ethanol (43%), using an NO trapping technique with EPR spectroscopy.[31,36–38] DETC·$3H_2O$ solution (400 mg/kg) and Fe–citrate mixture (40 mg/kg of $FeSO_4$·$7H_2O$ and 200 mg/ml of sodium citrate) were injected intraperitoneally and subcutaneously, respectively. Fe–DETC complex thus internally formed could trap endogenously produced NO to yield NO–Fe–DETC complex.[36] Thirty minutes after the NO trapping agent was injected, the stomach was removed under deep anesthesia. The glandular mucosa at the side of greater curvature was selectively resected and minced. Each sample was taken up by a 1-ml plastic syringe and extruded into a glass capillary tube (75 mm in length; 46 μl inside volume), into a quartz tube (outer diameter, 5 mm).

EPR spectra were recorded at ambient temperature with a spectrometer (TE-200; JEOL, Tokyo, Japan). The instrument settings were: center field, 331 mT; field scan, 4 mT; sweep time, 4 min; time constant, 0.3 s; modulation amplitude, 0.32 mT; modulation frequency, 100 K; microwave power, 60 mW. To improve the signal-to-noise ratio in the EPR spectrum of NO–Fe–DETC in tissues, the spectrum obtained was an average of five accumulations. The amplitude of the signal, which was proportional to the amount of NO, was obtained by measuring the peak-to-peak height of the lower field side signal in a three-line spectrum. The NO adduct concentration of the Fe–DETC complex was estimated by comparing it with the signal height of a standard solution of a chemically synthesized NO complex.[39]

NO_x (NO_2^- + NO_3^-) Assay of the Perfused Luminal Fluid

Perfused luminal fluid was collected in a polypropylene micro-centrifuge tube (0.6 ml) every 10 min, and centrifuged at 3000 rpm for 15 min. The supernate of the collected fluid (10 μl) was subjected to NO_x analysis. Nitrite and nitrate were assayed by HPLC combined with the Griess-reagent-flow reaction system (Model ENO 10; Eicom, Kyoto, Japan), in which a cadmium column was employed for the reduction of nitrate to nitrite. The detection limit of NO_x was approximately 0.3 pmol. The combined concentration of nitrite and nitrate was then quantified. The luminal NO_x levels decreased with an increase in the flow rate of perfusion. Normal saline, 12.5% ethanol, and 43% ethanol contained NO_x of 0.25 ± 0.10 μM (NO_2^-, 30%; NO_3^-, 70%). No contamination of NO_x from the materials of the chamber and tube was detected in the perfusion of either the normal saline or the ethanol.

Measurement of GMBF

GMBF was measured continuously using a laser Doppler flowmeter (ALF21R; Advance, Tokyo, Japan) with an optic probe (diameter, 1 mm) that had been placed gently on the gastric mucosa of the anterior wall of the corpus. The flowmeter can scan 1 mm in depth from the surface of the gastric mucosa on which the optic probe is in contact. This method is very sensitive to noise, so it is important to be careful not to move the end of the optic probe. GMBF basal levels were 10–20 ml/min/100 g.

Evaluation of Hemorrhagic Gastric Mucosal Injury

The stomach was removed immediately under deep anesthesia and pinned to a rubber board so that it could be photographed using a digital camera. The area of mucosal injury with macroscopic hemorrhage was analyzed using NIH Image Freeware (v. 1.58), and the result was evaluated for the percentage of total glandular mucosa within a chamber.

Measurement of PD Between Gastric Lumen and Abdominal Cavity

The PD between the gastric lumen and abdominal cavity was measured by an electrometer (7555; Yokogawa, Tokyo, Japan) using two agar bridges, one of which was set into the lumen and the other into the abdominal cavity after injecting normal saline (3 ml).

Measurement of Luminal PH

The exit silicone tube (diameter, 1 mm; length, 35 cm) was connected directly to a flow-type pH glass electrode, so the pH of the perfused luminal fluid was continuously measured by a pH meter (F-21; Horiba, Kyoto, Japan). The pH of normal saline, which ranged from 6.0 to 7.3, scarcely affected that of the perfusate.

Chemicals Used

Urethane (Tokyo-kasei, Tokyo, Japan) was dissolved (25%, w/w) in normal saline (Otsuka, Tokyo, Japan). This solution was administered intraperitoneally at a volume 5 ml/kg. Ethanol (HPLC grade) (Kanto Chem., Tokyo, Japan) was diluted to 12.5 and 43% (v/v) with normal saline. L-NAME hydrochloride (Sigma, St. Louis, USA) and N^G-nitro-L-arginine (L-NNA) (Dojindo, Kumamoto, Japan) were dissolved in normal saline and administered intraperitoneally. The source of the NO trapping agent, iron sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$) and sodium citrate (Wako Chem., Osaka, Japan) were dissolved in a normal saline. Sodium *N*,*N*-diethyldithiocarbamate trihydrate ($DETC \cdot Na \cdot 3H_2O$) (Aldrich, Milwaukee, USA) were dissolved in normal saline.

Statistics

Data are expressed as the mean ± SE of the values from five rats from each group. Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Values of $p < 0.05$ were considered significant.

RESULTS

Direct Measurement of NO by EPR Spectrometry

NO produced in the gastric tissue was measured before and during perfusion of ethanol (43%) into

the chambered stomach system. As shown in Fig. 1, the signal intensity from the NO adduct increased during perfusion of ethanol. This increase was suppressed by pretreatment with the NOS inhibitor L-NAME. Before perfusion of ethanol, the NO levels were 0.14 ± 0.02 nmole/g-tissue/30 min. The NO levels increased slightly to 0.17 ± 0.04 nmole/g-tissue/30 min after 10 min of perfusion. After 20 and 30 min of perfusion, the levels rose significantly to 0.24 ± 0.02 and 0.25 ± 0.06 nmole/g-tissue/30 min ($p < 0.01$), respectively (Fig. 2). Preadministration of NOS inhibitor L-NAME (10 mg/kg, i.p.) significantly suppressed the increase of NO levels in the gastric tissues during the perfusion of ethanol (43%), although after L-NAME pretreatment but prior to perfusion, the NO level was slightly but insignificantly decreased.

Changes in Luminal NO_x Levels Before and After Perfusion of Ethanol

As shown in the shaded area of Fig. 3, the luminal NO_x levels decreased sharply by 70–90% during ethanol perfusion. Such a drop can be caused by the formation of ethylnitrite, which is a product of the reaction of NO with ethanol and is undetectable in the assay based on the Griess reaction.[27] The luminal NO_x levels recovered immediately after reperfusion of the saline and reached 115% after 10 min of saline reperfusion. Luminal NO_x concentrations were 2.65 ± 0.75 μM before perfusion of ethanol. These reached 3.16 ± 0.74 μM after 10 min of perfusion of ethanol. Two ethanol concentrations of 12.5 and 43% had an insignificant influence on the changes in luminal NO_x levels before and after ethanol perfusions. The suppression

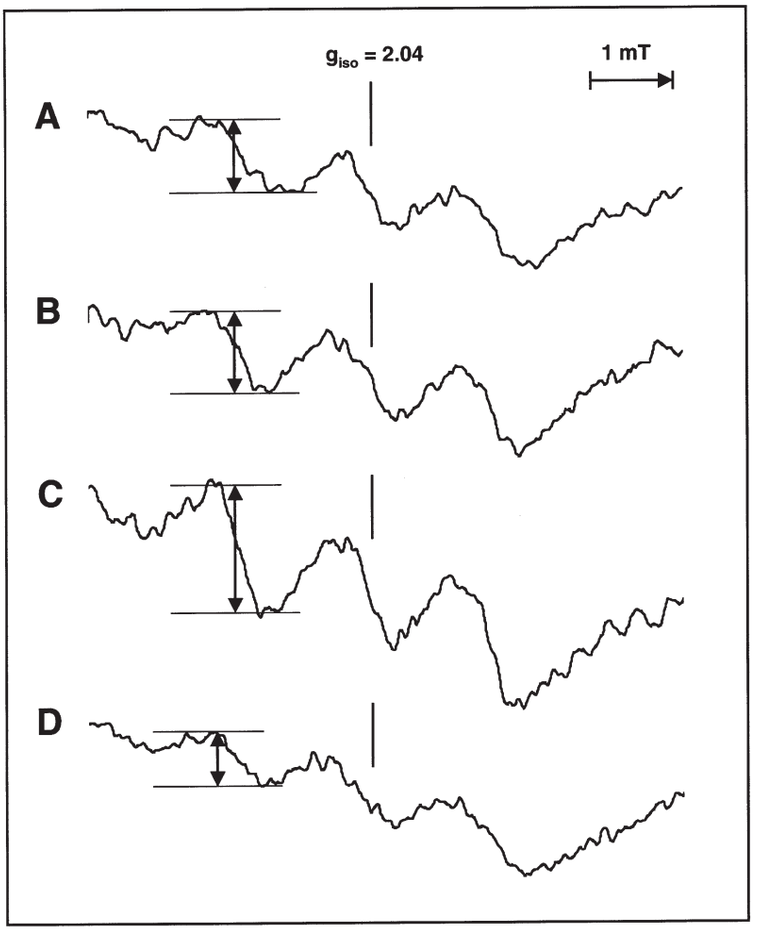

FIGURE 1 The X-band EPR spectra of the nitric oxide adduct of Fe–DETC (NO–Fe–DETC) complex observed at ambient temperature in the mucosal tissues resected from the rat stomach mounted on the lucite chamber. The NO-trapping agent (Fe–DETC complex) was injected 30 min before taking measurements. (A) EPR spectrum, before perfusion of the ethanol; (B, C) EPR spectra, 10 and 30 min after perfusion of ethanol (43%), respectively; (D) EPR spectrum, 30 min after perfusion of ethanol (43%) with the preadministration of L-NAME (10 mg/kg). The spectrum was obtained with an average of five accumulations. The signal height, which is proportional to the amount of NO generated, was obtained by measuring the peak-to-peak height of lower field side signal (arrows) in the three-line spectrum. The instrument settings were as follows: center field, 331 mT; field scan, 4 mT; sweep time, 4 min; time constant, 0.3 s; modulation amplitude, 0.32 mT; modulation frequency, 9.44 GHz; microwave power, 60 mW.

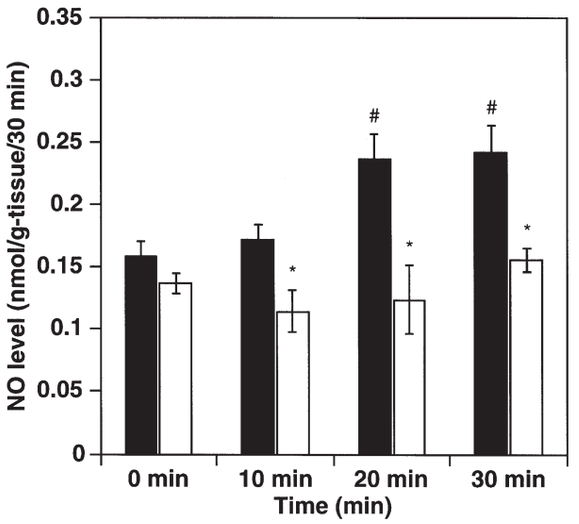

FIGURE 2 NO levels of the mucosal tissues resected from the rat stomach mounted on the lucite chamber before and during perfusion of ethanol (43%), which were evaluated from X-band EPR spectra as described in legend of Fig. 1. Open column, group with preadministration of L-NAME (10 mg/kg); closed column, group without it. The values are means ± SEM of five rats. $^{\#}p < 0.05$, vs. before perfusion of ethanol and 30 min after perfusion of ethanol (43%); $^{*}p < 0.05$, vs. with and without L-NAME (10 mg/kg).

of luminal NO_x levels on L-NAME treatment was eminent after perfusion of 43% ethanol (Fig. 3). The treatment with another NOS inhibitor, L-NNA treatment suppressed the NO_x levels in a similar manner as L-NAME (data not shown). Luminal NO_x levels gradually returned to the level before ethanol perfusions as saline reperfusion continued.

Changes in GMBF

GMBF was measured by laser-Doppler flowmetry. GMBF gradually increased during perfusion and 30 min after perfusion reached a level of 110–130% (Fig. 4). Preadministration of L-NAME (10 or 50 mg/kg) appreciably suppressed the increase in GMBF during and after perfusion of 43% ethanol, while L-NAME hardly affected GMBF prior to ethanol perfusion.

Hemorrhagic Gastric Mucosal Injury After Perfusion of Ethanol

The area of hemorrhagic injury caused by perfusion of ethanol was macroscopically assessed. While perfusion of 12.5% ethanol did not cause macroscopic hemorrhagic mucosal injury (data not shown), in specimens from rats treated with 43% ethanol the injured area encompassed 6.3 ± 0.6% of the total mass. Preadministration of L-NAME enhanced the area of hemorrhagic gastric mucosal injury from 6.3 ± 0.6% (L-NAME not given) to 7.3 ± 1.0% for 10 mg/kg L-NAME and to 12.5 ± 1.2% for 50 mg/kg (Fig. 5).

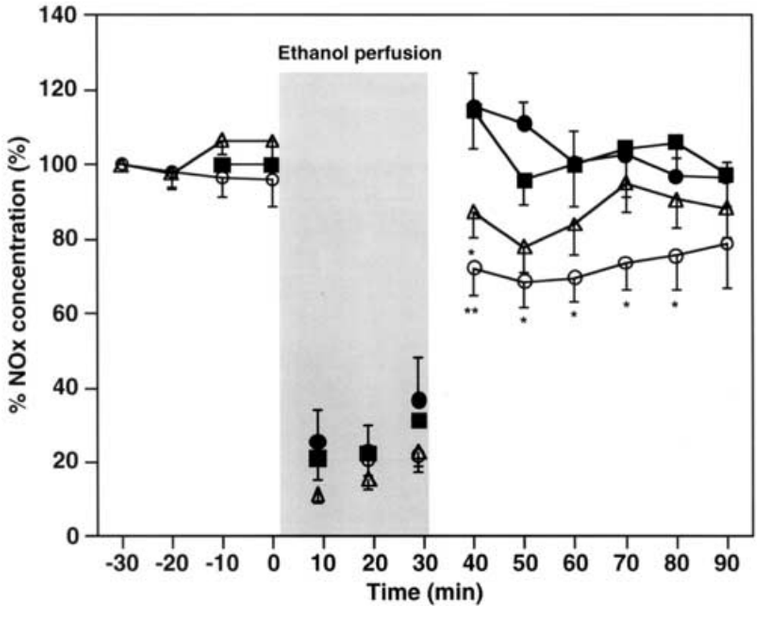

FIGURE 3 Sequential changes of percentage of the NO_x levels of luminal solutions perfused in chambered stomach; (■) before, during and after perfusion of ethanol (12.5%); (●), during and after perfusion of ethanol (43%); (△), before, during, and after perfusion of ethanol (43%) with preadministration of L-NAME (10 mg/kg); (○), before, during, and after perfusion of ethanol (43%) with preadministration of L-NAME (50 mg/kg). Nitrite plus nitrate (NO_x) of perfusate were assayed by high-performance liquid chromatography combined with Griess reagent-flow reaction system. The shaded area during ethanol perfusion exhibits the region where the NO_x levels cannot be exactly obtained in assay based on Griess reaction. The values are means ± SEM of five rats. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. with and without L-NAME (10 and 50 mg/kg) in the group of perfusion of ethanol (43%).

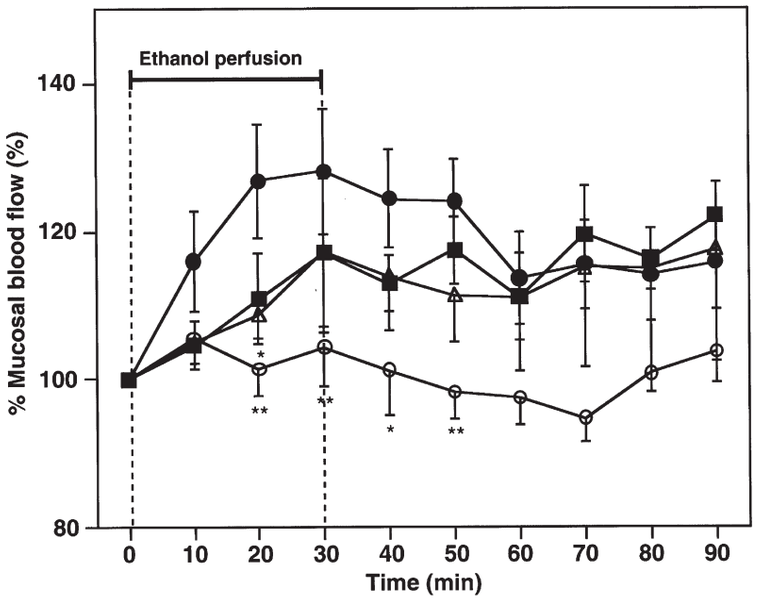

FIGURE 4 Sequential changes of GMBF during and after perfusion of ethanol (12.5 and 43%). (■), Perfusion of ethanol (12.5%); (●), perfusion of ethanol (43%); (△), perfusion of ethanol (43%) with preadministration of L-NAME (10 mg/kg); (○), perfusion of ethanol (43%) with preadministration of L-NAME (50 mg/kg). GMBF was measured by using the laser Doppler flow meter with optic probe putting gently on the gastric mucosa of the anterior wall in the corpus. The results are expressed as a percentage of the laser-Doppler flow measured before perfusion of ethanol (12.5 or 43%). The values are means ± SEM of five rats. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. with and without L-NAME (10 and 50 mg/kg) in the group of perfusion of ethanol (43%).

Changes in PD Before and After Perfusion of Ethanol

The basal PD level was about 30 mV. Since PD values can be interfered with by ethanol perfusion, the data during ethanol perfusion were presented as a shaded area in Fig. 6. The perfusion of 12.5% ethanol did not exhibit significant influence on the PD levels. On the other hand, perfusion of 43% ethanol caused a remarkable decrease in the PD. Furthermore, subsequent reperfusion of saline did not restore it to the basal level. L-NAME treatment (50 mg/kg) prior to 43% ethanol perfusion caused a further decrease in PD.

Changes in Luminal PH Before and After Perfusion of Ethanol

The pH of the perfused luminal fluid was measured. The basal pH level was approximately 3. Since the pH values can be affected by ethanol perfusion, the data during perfusion were presented as a shaded area in Fig. 7. Ethanol (12.5%) perfusion had little effect on pH. On the contrary, 43% ethanol perfusion caused a remarkable increase in the pH of the perfused luminal fluids; and even reperfusion of saline failed to restore it to the basal pH level within the period of measurement. L-NAME treatment (50 mg/kg) prior to 43% ethanol perfusion caused a further increase in the pH of the perfused luminal fluids. This tendency was also observed after the peritoneal injection of another NOS inhibitor, L-NNA (50 mg/kg) (data not shown).

DISCUSSION

The present study demonstrates that ethanol perfusion into the chambered stomach of rats causes gastric mucosal injury with macroscopic hemorrhage and simultaneously releases NO into the gastric lumen. Gastric NO production was directly measured by an NO trapping technique with EPR spectroscopy. Intragastric NO as well as luminal NO_x levels were discussed in relation to gastric pathophysiological parameters.

Vanin *et al.* first demonstrated NO measurement in the mouse stomach using an EPR NO trapping technique in which DETC-loaded yeast suspensions were injected perorally into the stomach.[40] However, no direct NO measurements by EPR spectroscopy have been performed using a rat model for acute gastric injury. In this study, an Fe–DETC complex as an NO trapping agent was administered via the systemic route. Fe–DETC is lipophilic and has a high specificity for NO;[31,36] therefore, it has strong affinity for gastric mucosal tissue and the ability to trap NO derived from the gastric mucosa. DETC and Fe–citrate injection did not interfere with the GMBF

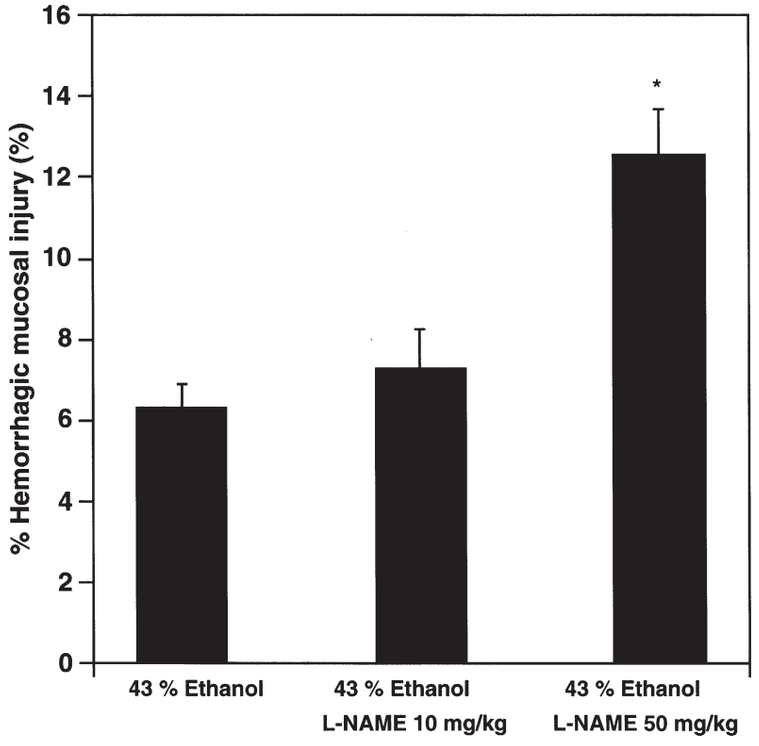

FIGURE 5 The area of the hemorrhagic mucosal damages after the perfusion of ethanol (43%) with or without the preadministration of L-NAME (10 or 50 mg/kg). Stomachs resected from treated rats were pinned out on black rubber board to take a photo with a digital camera. The area of macroscopic hemorrhagic gastric mucosal injury was analyzed by using the NIH Image Freeware (v. 1.58), The values are means ± SEM of five rats. *$p < 0.01$ vs. with and without L-NAME.

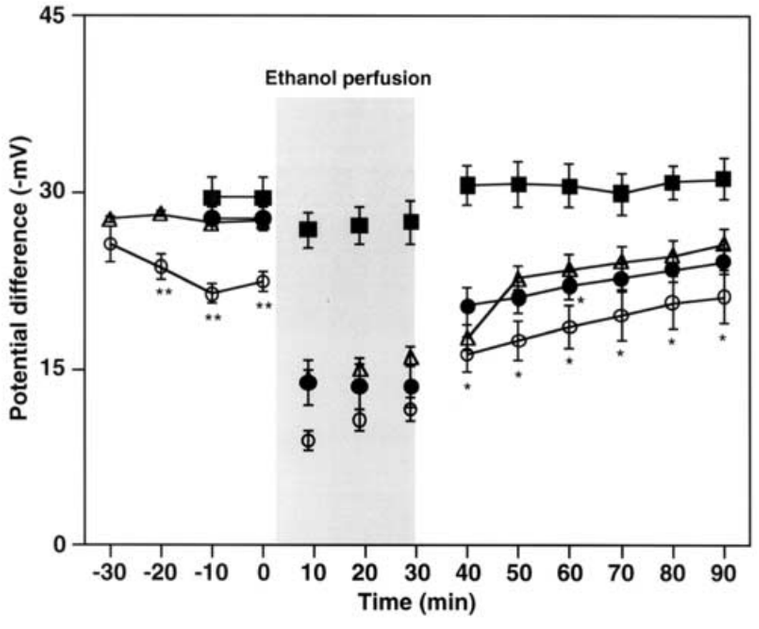

FIGURE 6 Sequential changes of PD in chambered stomach before, during, and after perfusion of ethanol (12.5 and 43%). (■), Perfusion of ethanol (12.5%); (●), perfusion of ethanol (43%); (△), perfusion of ethanol (43%) with preadministration of L-NAME (10 mg/kg); (○), perfusion of ethanol (43%) with preadministration of L-NAME (50 mg/kg). PD between lumen of the stomach and abdominal cavity was measured by electrometer using two agar bridges. Since PD values can be interfered by ethanol perfusion, the data during ethanol perfusion were presented as a shaded area. The values are means ± SEM of five rats. *$p < 0.05$, **$p < 0.01$ vs. with and without L-NAME (10 and 50 mg/kg) in the group of perfusion of ethanol (43%).

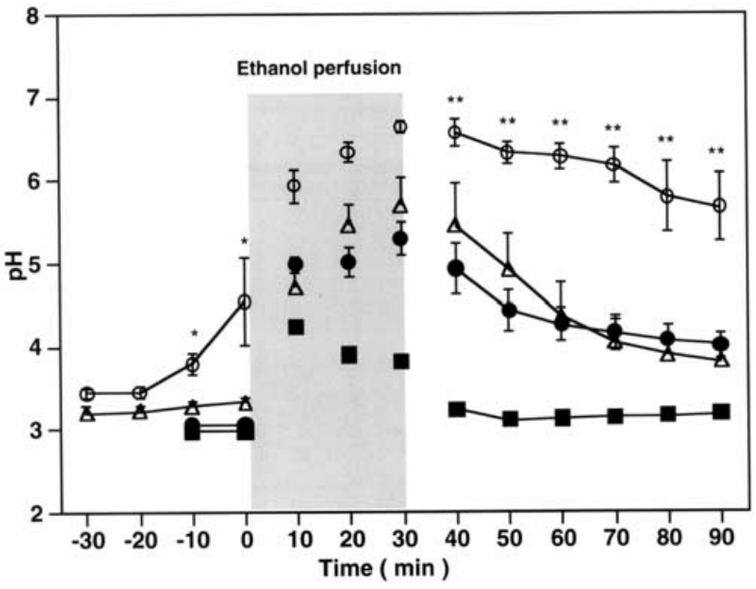

FIGURE 7 Sequential changes of pH of luminal fluid perfused in chambered stomach before, during, and after perfusion of ethanol (12.5 and 43%). (■), Perfusion of ethanol (12.5%); (●), perfusion of ethanol (43%); (△), perfusion of ethanol (43%) with preadministration of L-NAME (10 mg/kg); (○), perfusion of ethanol (43%) with preadministration of L-NAME (50 mg/kg). Since pH values can be interfered by ethanol perfusion, the data during ethanol perfusion were presented as a shaded area. The values are means ± SEM of five rats. *$p < 0.05$, **$p < 0.01$ vs. with and without L-NAME (10 and 50 mg/kg) in the group of perfusion of ethanol (43%).

or pH in the basal state. Accordingly, the EPR NO trapping technique is a valuable method to quantify NO produced in the gastric mucosa.

In this study, NO was measured within 60–120 min after the surgical procedures and 30 min after ethanol perfusion. iNOS has not been found in the gastric mucosa,[11] but two types of cNOS have been found in the normal rat stomach.[7,8] Reportedly, when endotoxin was administered in the rat, the expression of iNOS mRNA occurred 3 h later.[41,42] Therefore, the detected EPR signal was derived from NO that had been synthesized by cNOSs and not by iNOS.

Luminal NO_x concentrations in the perfusate were measured by a Griess reaction followed by HPLC. Luminal NO_x measured here can only be derived from the latest degradation products of NO (through the actions of NOSs in the gastric mucosa), because the shift of nitrite in the saliva into the stomach was inhibited by ligation of the lower esophagus. It appears likely that the luminal NO_x levels in the chambered stomach do not represent the total NO release in the gastric tissues, since NO in deeper tissues may be diffused only slightly to the lumen. Furthermore, nitrite, an oxidation product, may be reduced to gaseous NO under luminal acidic conditions and dissipate from the chamber. Finally, a small amount of NO_x is derived from normal saline. However, the luminal NO_x levels and their changes over time measured here can reflect (in real time) overall changes in gastric mucosa as well as the GMBF, PD between the gastric lumen and abdominal cavity, and luminal pH.

As shown in Fig. 3, the NO_x levels after ethanol perfusion were increased by approximately 0.5 μM from the basal level, though the difference was not significant. The NO_x levels were dose-dependently and significantly reduced by the preadministration of an NOS inhibitor. It has been shown that a drop in luminal NO_x levels during aqueous ethanol perfusion, as shown in Fig. 3, results from the removal of some compound formed through the reaction of ethanol with NO, and that this compound may be volatile ethylnitrite.[27] This suggests that ethanol may be an NO scavenger in gastric lumen and ethylnitrite thus formed may act as an NO or NO^+ donor in stomach or intestine.

These results for both the EPR NO measurements of the gastric mucosa and luminal NO_x assays of the perfusate in the ethanol perfusion model demonstrate that ethanol perfusion into the stomach enhances the endogenous intragastric NO levels. An NOS inhibitor markedly suppressed the increases; the NO levels are not readily restored to the basal level, and the reaction product of ethanol with NO may bring about some additional physiologic action.

Ethanol perfusion caused gastric injury with macroscopic hemorrhages, and this effect was enhanced dose-dependently by pre-administration of L-NAME (Fig. 5). This increase in the severity of hemorrhagic injury was accompanied by a decrease in the PD, which reflects the mucosal integrity.[43] These findings demonstrate that NO endogenously generated in the gastric tissues contributes to an

amelioration of hemorrhagic injury induced by ethanol treatments and to the maintenance of gastric mucosal integrity.

The perfusion also increased GMBF while preadministration of L-NAME suppressed the increase (Fig. 4), suggesting that NO is related to the increase in GMBF in ethanol induced acute mucosal injury. The gastric mucosal microcirculation is generally regarded as a critical component of gastric mucosal defense.[44,45] The hyperemic response that can be observed after exposure to damaging agents facilitates dilution, buffering, and removal of back-diffusing acid.[44–46] The endogenously produced NO regulates the GMBF and plays an important role in the mucosal defense.[47,48]

Luminal pH reveals the secretory response of the intact gastric mucosa,[49] but the response can be enhanced by acid back-diffusion when the gastric mucosa is injured.[50] Ethanol perfusion significantly increased the luminal pH, and preadministration of L-NAME enhanced this effect (Fig. 7). This rise in the luminal pH following gastric mucosal injury and intragastric NO production suggests the involvement of acid back-diffusion. In this study, basal levels of the luminal pH reflecting the gastric acid secretion increased after injection of L-NAME (50 mg/kg). It has been suggested that L-arginine analogues with alkyl ester modification, such as L-NAME, have specific muscarinic receptor blocking activity *in vivo* and *in vitro*.[51] However, in other studies, L-NAME did not have a very limited blocking activity.[52] In the stomach of rats, acid secretion is partly mediated by muscarinic receptors in gastric parietal cells in the epithelium.[53–55] We found in the present study that the effect of L-NAME on the basal acid secretion was similar to that of L-NNA, which is a non-alkyl ester of L-arginine. This suggests that the change of luminal pH after L-NAME treatment is not mediated through the muscarinic receptors of the epithelial cells.

The detailed mechanism of the enhancement of intragastric NO levels through ethanol perfusion into the stomach remains to be uncovered. When the gastric mucosa is damaged, acid back-diffusion can increase due to the mucosal disruption, and then acid can stimulate the afferent sensory neurons that release calcitonin gene-related peptide (CGRP).[50] The release of CGRP has been demonstrated to be one of the triggers of NO generation in the stomach.[44,56] Thus, the acid back-diffusion in ethanol-induced mucosal injury may be one critical factor in endogenous NO generation in the stomach.[57,58] On the other hand, there is increasing evidence indicating direct effects of ethanol on different NOS isoforms and cell types. Ethanol has been shown to enhance or inhibit the activity of NOS, or to have no effect, depending on the NOS isoform, cell type, or the conditions of administration and exposure.[59–62]

In this study, the *ex vivo* chambered stomach technique was applied to an ethanol-induced gastric injury model, and the levels of NO in the gastric mucosa and NO_x in lumen were directly evaluated to elucidate the pathophysiological role of NO in the gastric mucosal damage. The increase in intragastric NO levels, caused by perfusion of ethanol into the chambered stomach, was found to be related to changes in the area of hemorrhagic gastric mucosal injury, PD between the gastric lumen and abdominal cavity, blood flow in gastric mucosa, and luminal pH. On the other hand, ethanol has been shown to react with NO to form ethylnitrite during ethanol perfusion.[27] These findings corroborate the view that endogenously produced NO in the stomach maintains gastric mucosal integrity via regulation of the GMBF and suggest that oral intake of ethanol cannot only induce gastric mucosal injury but also affect physiologic roles of NO in stomach.

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