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Nitric Oxide Produced by Inducible Nitric Oxide Synthase Delays Gastric Emptying in Lipopolysaccharide-treated Rats

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Background: **Endotoxin induces nitric oxide synthase (NOS), resulting in relaxation of gastric smooth muscle. The authors examined the effect of NO produced in response to lipopolysaccharide (LPS) treatment on gastric emptying in rats, and they also examined the effects of a selective inhibitor of inducible NOS (iNOS), aminoguanidine, and a suppressor of iNOS gene expression, dexamethasone.**

Methods: **Male Wistar rats weighing 200–250 g were used. LPS-treated rats received LPS (0.2–10 mg/kg) diluted in physiologic saline intraperitoneally. Before and at different intervals up to 8 h after administration of LPS, measurements of gastric emptying were performed in groups of 3–5 rats, by determining the amount of phenol red remaining in the stomach 20 min after intragastric instillation. In additional group of LPS (2 mg/kg)-treated rats, the gastric fundus was isolated 6 h after administration, and the tension changes in response to L-arginine, a substrate for NOS, and electrical transmural stimulation (3 Hz, 5 s) were recorded isometrically.**

Results: **(1) Gastric emptying was delayed by pretreatment with LPS in a dose- and time-dependent fashion (reduction from 68 ± 12% to 22 ± 7% with a dose of 2 mg/kg for 6 h). Aminoguanidine (50 mg/kg) or dexamethasone (5 mg/kg) partially inhibited the delay (to 39 ± 4% or to 40 ± 10%, respectively). (2) L-arginine (0.1 mM) produced a relaxation (28 ± 2% reduction in active tension) in the gastric fundus strips isolated from LPS-treated rats but not from LPS-untreated rats. The relaxation was inhibited by aminoguanidine (1 mM). In contrast, the relaxation response to the electrical stimulation was not affected by aminoguanidine (0.1–1 mM).**

Conclusion: **The present study suggests that NO, probably produced by iNOS, is one of the factors involved in the delay of gastric emptying in the LPS-treated rats and probably in those with sepsis (Key words: Stomach, gastric emptying: phenol red. Pharmacology: aminoguanidine, dexamethasone, L-arginine.)**

Clinical symptoms of endotoxemia and sepsis are frequently those associated with an abnormal motility of the gastrointestinal tract such as nausea, vomiting, ileus, and diarrhea. It has now been reported that management with endotoxin inhibits gastric motility and delays gastric emptying.[1,2] Although a possible involvement of the autonomic nervous system,[1] eicosanoids,[2–4] platelet-activating factor,[5] and cholecystokinin[6] in delay has been suggested, the precise mechanisms have not been defined.

Nitric oxide (NO), produced from L-arginine by NO synthase (NOS), induces many physiologic and pathophysiologic effects.[7] Two major isoforms of NOS have been identified. One is a constitutive enzyme (cNOS) that depends on Ca^{2+} for activity, whereas the other is an inducible enzyme (iNOS) induced by bacterial endotoxin or cytokines in various types of cells and is Ca^{2+}-independent. The former are mainly localized to endothelium or central and peripheral nerves, whereas the latter is induced in immune cells such as macrophages and other cells such as vascular smooth muscle cells.[8] Especially, NO produced by iNOS in vascular smooth muscle cells relaxes the muscle and apparently plays a role in sepsis-associated hypotension.[9,10] In isolated gastric smooth muscle, iNOS also is induced by lipopolysaccharide (LPS)-treatment, and the resulting NO can relax the gastric muscle strips directly.[11,12] This relaxation (and perhaps the lack of peristalsis) may be related to delayed gastric emptying.

In the present study, to determine whether the NO produced by iNOS inhibits gastric motility, we investigated the effects of LPS, aminoguanidine (a selective inhibitor of iNOS[13]), and dexamethasone (a suppressor

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of iNOS gene expression[14]) on gastric emptying in LPS-treated rats. In addition, we confirmed the direct effects of aminoguanidine to gastric strips isolated from LPS-treated rats with functional experiments.

Methods

Animals

This investigation was approved by institutional animal care committee. Male Wistar rats weighing 200–250 g were used. They were housed under conditions of controlled temperature (23 ± 2°C) and illumination (light on 8 AM to 8 PM). Experiments were performed on animals deprived of food for 18 h but allowed free access to water up to the beginning of the experiments.

Drug treatments in vivo

Pyrogen-free physiologic saline or LPS (0.2–10 mg/kg) diluted in the saline was administered intraperitoneally. In preliminary experiments, intraperitoneal saline injection (control) was confirmed not to cause any change in gastric emptying. At various times after administration of LPS (2 mg/kg) or the saline, measurements of gastric emptying were performed. Aminoguanidine (5 or 50 mg/kg) was administered intraperitoneally 1 h before the measurement. Dexamethasone (5 mg/kg) was administered intraperitoneally 1 h before LPS or the saline administration.

Measurement of gastric emptying

Gastric emptying of non-nutrient solution was determined with a method using phenol red, essentially as described by Maeda and Tache.[15] Briefly, before and at different times after LPS administration, 1.5 ml of non-nutrient solution containing 1.5% methylcellulose and phenol red (50 mg/100 ml) was given orally into the stomach through a polyethylene tube, which was removed immediately after delivering the solution intragastrically. Twenty minutes later, the rats were killed by decapitation. The pylous and cardia of stomach were clamped, removed, and homogenized in 100 ml of 0.1 N NaOH. Protein in 5 ml of homogenate was precipitated with 0.5 ml of trichloroacetic acid (20% w/v). After centrifugation at 2,800 rpm for 20 min, 3 ml of the supernatant was mixed with 4 ml of 0.5 N NaOH, and then the absorbance was read at a wavelength of 560 nm. As standard, the absorbance of phenol red in the stomachs of rats immediately after administration of the non-nutrient solution was measured. The percentage of gastric emptying was calculated according to the following formula: (1 - amount of phenol red recovered from test stomach/amount of phenol red recovered from standard stomach immediately after administration) × 100.

Functional experiments with isolated fundus strips

Six hours after the intraperitoneal administration of LPS (2 mg/kg) or pyrogen-free saline, the gastric fundus was isolated from rats and placed in modified Krebs-Henseleit solution (mM; NaCl, 118; KCl, 4.7; $NaHCO_3$, 25; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $CaCl_2$, 2; glucose, 10; pH, 7.4). Circular fundus strips (15 × 3 mm) were carefully prepared under a dissecting microscope, and the mucosa was removed. The strips were suspended in organ chambers containing 20 ml of the modified Krebs-Henseleit solution, bubbled with 95% O_2–5% CO_2 at 37°C, and the tension changes were recorded isometrically.[12] A resting tension of 1 g was applied for 1 h, and then the strips were precontracted by 1 μM prostaglandin $F_{2\alpha}$, which produced approximately 80% of the maximal contraction. In the presence of atropine (1 μM) and guanethidine (5 μM) to block cholinergic and adrenergic nerve reactions, respectively, electrical transmural stimulation was induced *via* a pair of platinum-wire electrodes (15 mm long; 0.6 mm diameter; 3 mm distance between the electrodes) with an electronic stimulator (Nihon Koden, SEN-3201, Tokyo, Japan) and a current booster.[16] The stimulus parameters were 0.2 ms in duration at supermaximal voltage (12 V) for 5 s. The stimulus frequency used was 3 Hz. Stimulation was applied at a minimum of 5-min intervals.

Measurement of heart rate and blood pressure

Heart rate and blood pressure of conscious rats were measured sphygmomanometrically (Muromachi-Kikai, MK-1000, Osaka, Japan) 6 h after administration of LPS or sterile and pyrogen-free saline.[17]

Drugs

The following drugs were used: LPS (lipopolysaccharide from *Escherichia coli* 0111 extracted by the bovine method, Lot 14079, Difco Laboratories, Detroit, MI), atropine, guanethidine, tetrodotoxin, L-arginine hydrochloride, N^{ω}-nitro-L-arginine methyl ester, dexamethasone (Sigma, St. Louis, MO), aminoguanidine sulfate (Nakarai Tesque, Kyoto, Japan), and prostaglandin $F_{2\alpha}$ (Ono, Osaka, Japan).

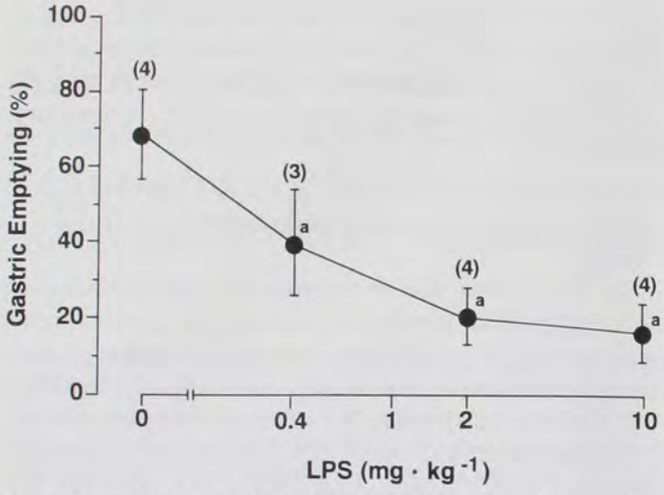

Fig. 1 Dose-dependent inhibition of LPS on gastric emptying in rats. Rats were treated intraperitoneally with LPS for 6 h, and then gastric emptying for 20 min was measured. Mean ± SD of 3–4 rats shown in parentheses. a; significantly different from control (LPS-untreated rats).

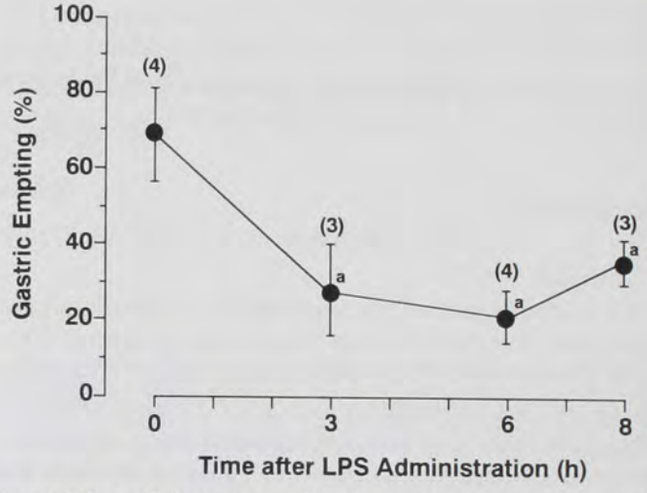

Fig. 2 Effect of lipopolysaccharide-treated period on gastric emptying in rats. LPS 2 mg/kg was administered intraperitoneally. Mean ± SD of 3–4 rats shown in parentheses. a; significantly different from at 0 h.

Statistical analysis

The results of experiments were expressed as mean ± SD. Statistical analyses were performed with analysis of variance - factorial (Scheffe F test), and a P value less than 0.05 was considered significant.

Results

Gastric emptying

At first, gastric emptying was measured in rats treated with various dose of LPS for 6 h. As shown in figure 1, LPS-treatment delayed gastric emptying dose-dependently. The maximal delay was produced in doses more than 2 mg/kg. The delay also depended on the treatment period. Figure 2 shows the relationship between the treatment period and gastric emptying, in which 2 mg/kg LPS was used. The delaying effect was gradually developed, and the maximal delay was observed 3 - 6 h after administration of LPS. Aminoguanidine (5 and 50 mg/kg) and dexamethasone (5 mg/kg) had no effect on the gastric emptying in LPS-untreated rats. However, treatment with aminoguanidine (50 mg/kg) or dexamethasone (5 mg/kg) significantly inhibited the delaying effect of LPS ($P < 0.05$; fig. 3).

Functional experiments with isolated fundus strips

In the strips isolated from LPS-untreated rats, the relaxation responses produced by electrical transmural stimulation were inhibited by subsequent addition of N^{ω}-nitro-L-arginine methyl ester (0.01 - 1 mM; n = 4; fig. 4A) and abolished by tetrodotoxin (1 μM; n = 3; data not shown). However, aminoguanidine (0.1 - 1 mM) had no effect on the relaxation responses to electrical stimulation (fig. 4B). In the strips isolated from the LPS-treated

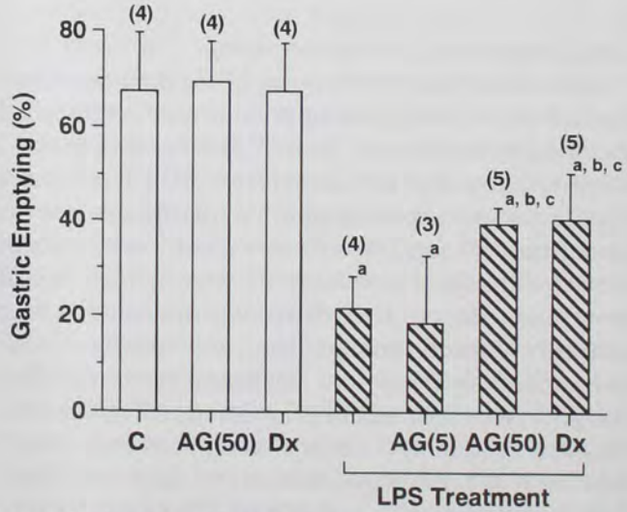

Fig. 3 Effects of LPS (2 mg/kg), aminoguanidine (AG; 5 or 50 mg/kg) and dexamethasone (Dx; 5 mg/kg) on gastric emptying. Mean ± SD of 3–5 rats shown in parentheses. a; significantly different from control (C; saline-treated rats). b; significantly different from rats treated with LPS alone. c; significantly different from rats treated with LPS and 5 mg/kg aminoguanidine.

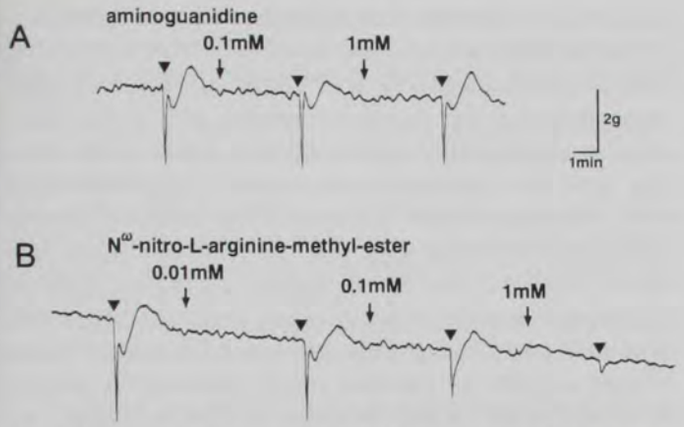

Fig. 4 Effects of aminoguanidine (0.1–1 mM) and N$^{\omega}$-nitro-L-arginine methyl ester (0.01–1 mM) on the relaxation responses to electrical transmural stimulation (▼; 3 Hz for 5 s) in gastric fundus strips isolated from rats. The rats were not treated with LPS. In the presence of atropine (1 μM) and guanethidine (5 μM), the strips were precontracted by prostaglandin $F_{2\alpha}$ (1 μM).

Table 1. Effects of Lipopolysaccharide (LPS), Aminoguanidine (AG), and Dexamethasone (Dex) on Heart Rate and Mean Arterial Blood Pressure (MABP) in Rats

Treatment	HR (bpm)	MABP (mmHg)
Control	445 ± 8	105 ± 7
AG (50 mg·kg^{-1})	423 ± 25	107 ± 5
Dex (5 mg·kg^{-1})	417 ± 22	128 ± 13*
LPS (2 mg·kg^{-1})	552 ± 35*	105 ± 9
LPS (2 mg·kg^{-1}) + AG (50 mg·kg^{-1})	484 ± 44†	112 ± 11
LPS (2 mg·kg^{-1}) + Dex (5 mg·kg^{-1})	475 ± 21†	116 ± 8

Values are mean ± SD of 4 or 5 animals. LPS was administered intraperitoneally and treated for 6 h. Aminoguanidine (AG) and dexamathasone (Dex) were administered intraperitoneally 1 h before the measurement and 1 h before LPS or saline administration, respectively.

* Significantly different from control (saline treatment).

† Significantly different from treatment with LPS alone.

(2 mg/kg, for 6 h) rats, L-arginine (0.1 mM) caused a relaxation (28 ± 4% decrease in the contractile level induced by prostaglandin $F_{2\alpha}$; n = 4), and the relaxation was reversed by aminoguanidine (1 mM; 105 ± 7% in the contractile level induced by prostaglandin $F_{2\alpha}$; n = 4; fig. 5). Such a relaxation response to L-arginine (0.1 mM) was not produced in the strips isolated from LPS-untreated rats or LPS-treated (2 mg/kg, for 6 h) rats preadministered dexamethasone (5 mg/kg).

Heart rate and blood pressure

Treatment with aminoguanidine (50 mg/kg) for 1 h did not affect either heart rate or mean arterial blood pressure in LPS-untreated rats (table 1). On the other hand, dexamethasone increased mean arterial blood pressure significantly. Mean blood pressure in the rats treated with LPS (2 mg/kg) for 6 h was not different from that of control rats. However, heart rate was significantly increased in LPS-treated rats. The increased heart rate was decreased by administration of aminoguanidine or dexamethasone without changing the mean blood pressure.

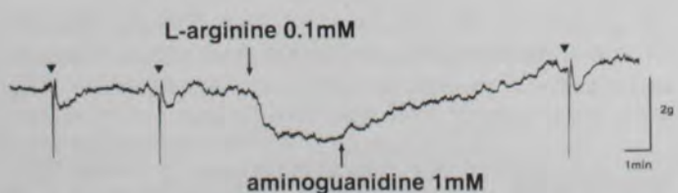

Fig. 5 Effects of L-arginine (0.1 mM), aminoguanidine (1 mM), and electrical transmural stimulation (▼; 3 Hz for 5 s) on gastric fundus strips isolated from rats treated with LPS (2 mg/kg) for 6 h. In the presence of atropine (1 μM) and guanethidine (5 μM), the strips were precontracted by prostaglandin $F_{2\alpha}$ (1 μM).

Discussion

It has been reported that treatment with LPS delays gastric emptying.[1,2] The present study confirmed such an inhibitory effect of LPS in rats. In addition, we found that the inhibitory effect of LPS remarkably depended on the LPS-treated period, suggesting a possibility that the delay is not directly caused by LPS.

Recently, it was shown that LPS treatment *in vivo* induces NOS and that iNOS produces much NO from L-arginine in many tissues, such as rat liver,[18] ileum,[19] myocardium,[20] and mesentery.[21] In such cases, it takes around 3–6 h to gain the maximal induction of the iNOS after administration of LPS. This time-course is consistent with that of the delaying effect on gastric emptying in the present study. Moreover, aminoguanidine (50 mg/kg), which can inhibit iNOS *in vivo*,[22] and dexamethasone (5 mg/kg), which can inhibit induction of iNOS *in vivo* but not inhibit activity of iNOS directly,[19,20] significantly reversed the delay of gastric emptying in LPS-treated rats, without affecting that in LPS-untreated rats. These results strongly suggest that induction of NOS is associated with the delay of gastric emptying.

More recently, the *in vitro* induction of iNOS after

LPS-treatment and the relaxation response to L-arginine, a substrate for NOS, were shown in isolated gastric smooth muscle.[11,12] In contrast to cNOS, iNOS always is activated without an increase in intracellular Ca^{2+} concentration and produces NO from L-arginine; thus iNOS activity is presumed to be roughly correlated to the amplitude of L-arginine-induced relaxation.[12,23,24] Although direct measurement of iNOS activity was not carried out in the present study, L-arginine produced aminoguanidine-sensitive relaxation in gastric fundus muscle isolated from LPS-treated rats but not from untreated rats, suggesting that *in vivo* treatment with LPS induces iNOS in stomach muscle and in other tissues reported.[18-21]

Lipopolysaccharide induces not only iNOS but also other enzymes such as arginase.[25,26] Thus, it may be possible that arginase depletes L-arginine, depriving iNOS of substrate even though iNOS is induced. However, it was reported that concentration of circulating L-arginine is enough to activate the iNOS in rats with sepsis.[27] There are also several studies that LPS increases N^G-hydroxy-L-arginine, which inhibits arginase activity, ensuring sufficient arginine availability for high-output production of NO.[28,29] In the present study, the fundus strips isolated from LPS-treated rats produced nitrergic relaxation even in L-arginine-free medium, indicating that depletion of L-arginine did not occur in the tissue.

As contractions of the proximal stomach play an important role in controlling gastric emptying of liquid meal,[30] it seems that the relaxation of the gastric fundus by NO produced by iNOS is one important factor delaying gastric emptying in LPS-treated rats. Aminoguanidine (50 mg/kg) or dexamethasone (5 mg/kg) used in present study partially inhibited the delay of gastric emptying induced by LPS. Because the dose of aminoguanidine was reported to inhibit iNOS activity submaximally,[22] the partial inhibition may reflect the insufficient inhibition of iNOS under the used conditions. However, the relaxant responses to L-arginine were not produced in the strips isolated from LPS-treated rats preadministered 5 mg/kg dexamethasone, suggesting that iNOS induction was inhibited completely. Therefore, we cannot disregard other factors affecting gastric emptying during treatment with LPS, such as the autonomic nervous system,[1] eicosanoids,[2-4] platelet-activating factor,[5] and cholecystokinin.[6]

In addition to the iNOS pathway, NO is released from nitrergic nerves (containing cNOS) and causes relaxation in stomach,[31] so the nitrergic NO also may affect the gastric emptying. Plourde *et al.*[32] and Orihata and Sarna[33] demonstrated that treatment with N^{ω}-nitro-L-arginine methyl ester (an inhibitor of cNOS and iNOS) delayed gastric emptying in LPS-untreated rats and dogs, suggesting that the delayed emptying may result from an increase in pyloric sphincter tone. Their results conflict with the present result because iNOS inhibition with aminoguanidine recovered the delayed gastric emptying. It is interesting that the NOS inhibitors produced opposite effects on gastric emptying. Gastric emptying is known to be inversely controlled by tones of gastric body and pyloric sphincter.[28] Under LPS-untreated conditions wherein iNOS induction is minor, NOS inhibitors block nitrergic (cNOS) relaxant responses, resulting in the delay of gastric emptying by increasing pyloric sphincter tone. On the other hand, a selective inhibitor of iNOS may inhibit the delayed gastric emptying in LPS-treated rats by increasing gastric body tone without affecting nitrergic relaxation in pyloric sphincter. Recently, it was reported that N^{ω}-nitro-L-arginine methyl ester and N^{ω}-nitro-L-arginine (an inhibitor of cNOS and iNOS) failed to reverse the delayed gastric emptying in sepsis rats.[34] This may suggest that an increase in pyloric sphincter tone by cNOS inhibition plays more dominant roles in gastric emptying even though gastric body tone is increased by iNOS inhibition.

Administration of 2 mg/kg LPS did not change arterial blood pressure, but it increased heart rate significantly. This change is obviously systemic response to LPS and may correspond to hyperdynamic state in sepsis. Inhibition of this change by aminoguanidine and dexamethasone also may suggest involvement of iNOS in the tachycardia. The present study further showed an increase in blood pressure in dexamethasone-treated rats. Although such an increase of blood pressure by dexamethasone often has been reported, the underlying mechanisms are unknown.[35]

In conclusion, the present study shows that the NO produced by inducible NO synthase is one of the factors in the delay of gastric emptying in the LPS-treated rats. The produced NO may be associated with nausea and anorexia, resulting in malnutrition during endotoxemia and sepsis.

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