Downregulation of nNOS and synthesis of PGs associated with endotoxin-induced delay in gastric emptying

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Calatayud, Sara, Eugenia García-Zaragozá, Carlos Hernández, Elsa Quintana, Vicente Felipo, Juan Vicente Esplugues, and María Dolores Barrachina. Downregulation of nNOS and synthesis of PGs associated with endotoxin-induced delay in gastric emptying. Am J Physiol Gastrointest Liver Physiol 283: G1360–G1367, 2002; 10.1152/ajpgi.00168.2002.—A single intraperitoneal injection of endotoxin (40 μg/kg) significantly delayed gastric emptying of a solid nutrient meal. Blockade of nitric oxide synthase (NOS) with 30 mg/kg ip Nω-nitro-L-arginine methyl ester or 20 mg/kg ip 7-nitroindazole [neuronal NOS (nNOS) inhibitor] significantly delayed gastric emptying in control animals but failed to modify gastric emptying in endotoxin-treated rats. Administration of 2.5, 5, and 10 mg/kg ip N5-iminoethyl-L-lysine [inducible NOS (iNOS) inhibitor] had no effect in either experimental group. Indomethacin (5 mg/kg sc), NS-398 (cyclooxygenase-2 inhibitor; 10 mg/kg ip), and dexamethasone (10 mg/kg sc) but not quinacrine (20 mg/kg ip) significantly prevented delay in gastric emptying induced by endotoxin but failed to modify gastric emptying in vehicletreated animals. Ca2+-dependent NOS activity in the antrum pylorus of the stomach was diminished by endotoxin, whereas Ca2+-independent NOS activity was not changed. In addition, decreased nNOS mRNA and protein were observed in the antrum pylorus of endotoxin-treated rats. Our results suggest that downregulation of nNOS in the antrum pylorus of the stomach and synthesis of prostaglandins mediate the delay in gastric emptying of a solid nutrient meal induced by endotoxin.

Nitric oxide (NO) has a leading role as an inhibitor neurotransmitter of peripheral nonadrenergic noncholinergic nerves (1, 8). In the gastrointestinal tract, neuronal NO plays a role in the physiology of gastric motor function. NO is involved in the reflex relaxation of the gastric fundus to accommodate food or fluid (7) and mediates pyloric relaxation and intestinal feedback regulation, thereby facilitating gastric emptying (2). A recent study (21) analyzing gastric emptying in neuronal NO synthase (nNOS) knockout mice points to a prominent role for this isoenzyme in the modulation of gastric motor function. However, little is known about the role of nNOS in changes in gastric emptying associated with pathophysiological circumstances such as endotoxemia. Taking into account that endotoxin has been widely shown to increase the expression of the inducible NOS (iNOS) in different tissues (5), the specific role of nNOS and iNOS in endotoxin-induced delay in gastric emptying has been evaluated.

Prostaglandins synthesized from arachidonic acid act as local regulatory agents that modulate gastric motor function (32). Endogenous prostaglandins have been involved in the inhibition of gastric emptying induced by IL-1β (35), and exogenous administration of these prostanoids delays gastric emptying (33). Synthesis of prostaglandins is carried out by cyclooxygenase (COX), which exists as two isoenzymes, COX-1 and -2, and the role of prostaglandins on gastric emptying has been established mainly through the use of nonselective COX inhibitors. Both isoenzymes synthesize prostanoids that mediate physiological functions (38). However, the formation of proinflammatory prostaglandins is mostly catalyzed by COX-2, and expression of this isofrom is induced by a variety of stimuli, including endotoxin (12). This study also aims to determine the role and the enzymatic source of prostaglandins in the delay of gastric emptying induced by endotoxin.

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ENDOTOXEMIA AFTER INFECTION with gram-negative bacteria is associated with clinical abnormalities of gastric motor function (such as vomiting) broadly characterized by a decrease in gastric emptying. Experimental administration of the lipopolysaccharide of Escherichia coli is known to delay gastric emptying (36, 37, 39), and capsaicin-sensitive afferent neurons and nonadrenergic noncholinergic mechanisms have recently been shown to be involved (6).

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats (250–300 g) (Harlan Laboratories, Barcelona, Spain) were maintained ad libitum on standard Purina laboratory chow and tap water and were housed under conditions of controlled temperature (21 ± 1°C), humidity (30–35%), and lighting (0700–1900 h). All experiments began between 0900 h and 1000 h and were performed in animals deprived of food for 16–18 h but with access to water before and during the experiments.

All protocols comply with the European Community guidelines for the use of experimental animals and were approved by the ethics committee of the Faculty of Medicine of Valencia.

Measurement of Gastric Emptying

Gastric emptying of solids was measured as previously described (6). Rats were placed in individual cages and were given access to preweighed food for 3 h. Food was then removed and 40 μg/kg ip endotoxin (E. coli lipopolysaccharide) or 1 ml/kg ip saline was administered to the animals. Four hours later, the rats were killed by cervical dislocation. The stomach was exposed by laparotomy, quickly ligated at both the pylorus and cardia, and removed, and its wet content was weighed. Gastric emptying (GE) was calculated according to the following formula: GE = (1 – wet wt of food recovered from stomach/weight of food intake) × 100.

Treatments

To analyze the role of NO in the rate of gastric emptying of a solid nutrient meal, animals received a single intraperitoneal injection of 30 mg/kg Nω-nitro-l-arginine methyl ester (l-NAME) (a NOS inhibitor), 20 mg/kg 7-nitroindazole (a selective nNOS inhibitor), or the respective vehicle (1 ml/kg ip saline) 2 h after the administration of endotoxin or saline. Some rats received 2.5, 5, or 10 mg/kg ip Nω-iminoethyl-l-lysine (l-NIL) (a selective iNOS inhibitor) or its vehicle (1 ml/kg ip saline) 15 min before endotoxin or saline, and gastric emptying was determined as described above.

To determine the role of prostaglandins in the rate of gastric emptying of a solid nutrient meal, rats were pre-treated with 5 mg/kg sc indomethacin (a dual inhibitor of COX-1 and -2), a COX-2-selective dose of 10 mg/kg ip NS-398 (20), or their respective vehicles (1 ml/kg sc NaHCO3 5%, or 0.5 ml/kg ip DMSO) 60 min before the administration of endotoxin or saline. Some animals received a single intraperitoneal injection of 20 mg/kg quinacrine (a phospholipase A2 inhibitor) or its vehicle (1 ml/kg ip saline) 15 min before endotoxin or saline.

In the last group, rats were treated with dexamethasone (5 mg/kg sc, 16 h and 1 h before endotoxin) or saline (1 ml/kg sc), and the rate of gastric emptying was analyzed as mentioned above.

Determination of NOS Activity

Rats were killed 4 h after the administration of endotoxin (40 μg/kg ip) or saline (1 ml/kg ip). In short, the antrum pylorus tissue was cut into small pieces, frozen in liquid nitrogen, and stored at −80°C. RNA extraction and cDNA synthesis. Total RNA from frozen gastric tissues was isolated with TriPure isolation reagent (Roche Diagnostics) following the manufacturer’s protocol. RNA was resuspended in diethylpyrocarbonate-treated H2O and stored at −80°C. Total RNA was treated with DNA-free (Ambion) to eliminate traces of contaminating genomic DNA. Resulting total RNA was quantified by ultraviolet spectrophotometry, and its integrity was evaluated by agarose gel electrophoresis. RT from 2 μg of total RNA was carried out with SuperScript RT RNase H− (Life Technologies), using 0.8 μg oligo(dT)16 (TIB Molbiol; Roche Diagnostics) and 40 units RNase inhibitor (Roche Diagnostics) in a reaction volume of 20 μl. Controls without RT for each sample and a negative control with water in place of RNA were performed. Synthesized cDNA was stored at −20°C.

Quantiﬁcation PCR. Quantitative PCR was carried out in a LightCycler instrument (Roche Diagnostics) with the use of LightCycler-FastStart DNA Master SYBR Green I kit. Samples of 1 μl of cDNA were amplified through speciﬁc primers for each gene, 0.5 μM cyclophilin A (CyPA), 0.5 μM nNOS, or...
1 μM iNOS, in a solution containing 2 mM MgCl₂ and 5% DMSO (final volume 10 μl). Reactions were performed in duplicate, and a negative control with water instead of cDNA was included in each run. Protocol reaction includes an initial period of 10 min at 95°C to activate the polymerase. Each PCR cycle involved denaturation at 95°C for 30 s, annealing at Tₘ (shown in Table 1) for 30 s and extension at 72°C for 30 s. Fluorescence was measured at the end of each cycle. Specificity of reactions was tested by analysis of the melting curve (Tₘ, shown in Table 1) and agarose gel electrophoresis. To quantify input amounts of templates, a standard curve was obtained with serial dilutions of total RNA of a positive control (Table 1) for each analyzed gene, also after RT-PCR. To normalize the results, interpolated values for each sample were divided by values for the housekeeping gene CyPA, and results are expressed as an NOS/CyPA ratio.

### Statistical Analysis

All data are expressed as means ± SE. Comparisons between two groups were performed using the Student’s t-test and among three or more groups by one-way ANOVA followed by the Newman-Keuls test. P values < 0.05 were considered significant.

### Drugs

Endotoxin (LPS from E. coli, serotype 0111:B4), l-NAME, l-NIL, 7-nitroindazole, NS-398, indomethacin, quinacrine, and all reagents used for determination of NOS activity were purchased from Sigma (St. Louis, MO). L-[U-14C]arginine was obtained from Amersham Life Science (London, UK). Dexamethasone (Fortecortin) was used as a clinically available preparation. Unless mentioned otherwise, all drugs were dissolved in saline.

### RESULTS

#### Effects of Endotoxin on Gastric Emptying of a Solid Nutrient Meal

The amount of rat chow eaten for 3 h by Sprague Dawley rats after a 20-h fast was 5.1 ± 0.2 g (n = 10). The 4-h rate of gastric emptying in animals treated with vehicle was significantly higher than that observed in endotoxin-treated rats (Fig. 1). This dose of endotoxin has previously been shown to lack effects on gastric emptying (9).

Role of NO. Nonisoform-selective blockade of NO synthesis by pretreatment with l-NAME (30 mg/kg ip) significantly decreased the rate of gastric emptying in saline-treated rats, whereas it did not significantly change the rate of gastric emptying in endotoxin-treated animals (Fig. 1). In a similar manner, selective blockade of the nNOS isoform by pretreatment with 7-nitroindazole (20 mg/kg ip) induced a pronounced reduction in the rate of gastric emptying in saline-treated animals, whereas it did not modify the rate of gastric emptying in animals receiving endotoxin (Fig. 1). Selective inhibition of the iNOS isoform by the administration of L-NIL (2.5, 5, and 10 mg/kg ip) did not significantly modify the rate of gastric emptying in vehicle- or endotoxin-treated animals (Fig. 2).

Role of prostaglandins. The delay in gastric emptying induced by endotoxin was significantly prevented by pretreatment with the nonselective COX-1 and -2 inhibitor indomethacin (5 mg/kg sc; Fig. 3). In a similar manner, selective inhibition of the COX-2 isoform by pretreatment with NS-398 (10 mg/kg ip) also significantly prevented the inhibitory effects of endotoxin on gastric emptying (Fig. 3). Both indomethacin and NS-398 when administered to saline-treated animals lacked any effect on the rate of gastric emptying (Fig. 3). Blockade of arachidonic acid supply by pretreatment with a phospholipase 2 inhibitor, quinacrine (20 mg/kg ip), did not alter the rate of gastric emptying in endotoxin- or saline-treated rats (Fig. 4).

#### Effects of dexamethasone

Pretreatment with dexamethasone (10 mg/kg sc) significantly prevented the inhibition of gastric emptying by endotoxin but did not significantly change the rate of gastric emptying in vehicle-treated animals (Fig. 4).

#### Effects of Endotoxin on NOS Activity

NOS activity analyzed by the rate of conversion of L-arginine to L-citrulline was present in the antrum pylorus of saline-treated animals. With the use of a calcium chelating agent, we observed that NOS activity in these conditions was mainly Ca²⁺-dependent, whereas Ca²⁺-independent NOS activity (iNOS) was almost nonapparent (Fig. 5). Pretreatment (4 h) with endotoxin induced a significant reduction of the Ca²⁺-dependent NOS activity (26.7%) in the antrum pylorus of the rats, whereas it did not significantly change the Ca²⁺-independent NOS activity (iNOS), which was similar to that observed in saline-treated rats (Fig. 5).

### Table 1. Primer sequences, reaction data, and characteristics of specific PCR products for each analyzed gene

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequences (5'-3')</th>
<th>Tₘ (°C)</th>
<th>PCR, cycles</th>
<th>Tₘ (°C)</th>
<th>Size, bp</th>
<th>Positive Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CyPA</td>
<td>CGTCTGCTTCCAGCTGTGGT</td>
<td>60</td>
<td>30</td>
<td>81.7</td>
<td>464</td>
<td>Cerebellum</td>
</tr>
<tr>
<td></td>
<td>GTAAAATGCCCGCAATGCAAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nNOS</td>
<td>ACTCTGAGCTCTGAGGAGG</td>
<td>55</td>
<td>35</td>
<td>85.1</td>
<td>513</td>
<td>Cerebellum</td>
</tr>
<tr>
<td></td>
<td>ACTGTTGAGGAGCTGACGACAG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>GCTACACTTCCAAAGCGACAAC</td>
<td>60</td>
<td>40</td>
<td>84.6</td>
<td>293</td>
<td>Lung, endotoxin, 1 mg/kg, 12 h</td>
</tr>
</tbody>
</table>
Effects of Endotoxin on nNOS and iNOS mRNA

Both nNOS and iNOS mRNA were present in the antrum pylorus of vehicle-treated animals as analyzed by real-time RT-PCR. A single intraperitoneal injection of endotoxin (40 µg/kg) induced 4 h later a significant diminution in the amount of nNOS mRNA and a significant increase in the amount of iNOS mRNA in the antrum pylorus of the stomach (Table 2).

DISCUSSION

The present study shows a role for NO synthesis in the physiological control of gastric emptying of a solid nutrient meal. Moreover, a downregulation of the nNOS protein in the antrum pylorus of the stomach seems to be involved in the pathophysiological delay of gastric emptying of nutrient meals associated with endotoxemia.

Gastric emptying of nutrient meals is a complex function of the gut mediated by the integrated response of the proximal and distal stomach and duodenum (22), and NO has been shown to play a role in the specific functions carried out by these areas (2, 7, 26). In the present study, blockade of NO synthesis by systemic administration of L-NAME significantly delayed the rate of gastric emptying of a solid nutrient meal, as previously reported for nonnutrient meals (25, 27). This study extends previous observations and shows that selective inhibition of the nNOS isoform by pretreatment with 7-nitroindazole (3) also delays gastric emptying.
emptying, whereas blockade of the iNOS isoform with 1-NIL fails to exert the same effect, reinforcing the physiological role of neuronal NO in the modulation of gastric emptying. The specificity of 7-nitroindazole on nNOS has been questioned, and intestinal motor depression through an action unrelated to NOS inhibition has been reported in vitro (17). Taking into account that, in this study, L-NAME also decreased gastric emptying, such a possibility seems unlikely. Additionally, inhibition of nNOS by 7-nitroindazole has recently been reported to increase iNOS expression in the rat small intestine (28). Considering that NO synthesized from iNOS (36) or exogenously administered (13) has been shown to delay gastric emptying, it is possible that increased synthesis of NO by iNOS rather than diminution of nNOS activity is responsible for this effect.

Fig. 3. Gastric emptying of a solid nutrient meal in rats receiving endotoxin or saline and treated with indomethacin (5 mg/kg sc), NS-398 (10 mg/kg ip), or the respective vehicles (NaHCO₃ 5% sc or DMSO 0.5 ml/kg ip). Bars represent means ± SE of the number of animals shown above columns. *P < 0.05 compared with all experimental groups in the same graph.

Fig. 4. Gastric emptying of a solid nutrient meal in rats receiving endotoxin or saline and treated with quinacrine (20 mg/kg ip), dexamethasone (5 mg/kg sc), or the respective vehicles (1 ml/kg saline). Bars represent means ± SE of the number of animals shown above columns. *P < 0.05 compared with the respective saline-treated group.
for the delay in gastric emptying observed with 7-nitroindazole. However, the fact that L-NAME, which inhibits both nNOS and iNOS, also significantly decreased gastric emptying, combined with the following experiments analyzing the relationship between NOS isoforms and gastric emptying, strongly suggests that specific diminution of nNOS activity delays gastric emptying of nutrient meals.

Endotoxemia is associated with delayed gastric emptying, and synthesis of NO has been involved in the modulation of gastric function by endotoxin (9). However, the present study in which both nonselective NOS inhibitors and selective blockade of the nNOS isoform have been used do not support a role for NO synthesis in the delay in gastric emptying of a nutrient meal induced by endotoxin. Gastric emptying is inversely controlled by tones of gastric body and pyloric sphincter, and some evidence supports an inhibitory role of NO on gastric emptying through an action related with relaxation of the gastric fundus (36). However, NO synthesis has been widely associated with an acceleration of gastric emptying mainly due to pyloric sphincter relaxation (2, 25, 27). Lack of effect of NOS inhibitors in the delayed gastric emptying of endotoxin-treated rats, considered in the light of a recent study (10) showing an attenuation of the nonadrenergic noncholinergic relaxation of the pyloric sphincter by endotoxin, led us to think that the antrum pylorus of the stomach is the main target of endotoxin to inhibit gastric emptying.

Analysis of NOS activity in the antrum pylorus of the stomach of endotoxin-treated animals exhibited a significant reduction of Ca\textsuperscript{2+}-dependent NOS activity compared with that observed in vehicle-treated animals. Although Ca\textsuperscript{2+}-dependent NOS activity involves both endothelial NOS and nNOS, the fact that analysis of the NOS activity has been performed in the crude homogenate rather than in the membranous fraction (18) and the predominance of nNOS isoform in the gut (29) led to an important diminution of the nNOS activity associated with endotoxin. More specific analysis of the nNOS isoform showed a marked reduction of the nNOS protein content and nNOS mRNA in the same gastric area 4 h after treatment with endotoxin. Considered as a whole, these results suggest that endotoxin induces a transcriptional downregulation of the nNOS protein that implies diminution of NO synthesis in the antrum pylorus of the stomach, thereby increasing the tone of the pyloric sphincter and impeding gastric emptying.

Downregulation of nNOS protein in the stomach has been described with different proinflammatory stimuli such as platelet activating factor (29) and interferon-γ (4), usually associated with an increased synthesis of NO from iNOS. In addition, iNOS-derived NO has been involved in changes in gastric function over a long period of time, generally related to more severe insults, such as ischemia-reperfusion (15) or higher doses of LPS (36). The present study shows, 4 h after the administration of low doses of endotoxin, an increase in iNOS mRNA in the antrum pylorus of the stomach. However, no changes in iNOS activity were observed in the same area, suggesting that no significant synthesis of NO from the iNOS isoform is taking place at that time. Synthesis of NO from the iNOS, which is an inducible enzyme, requires protein gene expression.

Table 2. nNOS and iNOS gene expression of saline- and endotoxin-treated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>nNOS/CyPA Ratio</th>
<th>n</th>
<th>iNOS/CyPA Ratio</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline, 1 ml/kg ip, 4 h</td>
<td>0.0416 ± 0.0045</td>
<td>6</td>
<td>0.2603 ± 0.0312</td>
<td>5</td>
</tr>
<tr>
<td>Endotoxin, 40 μg/kg ip, 4 h</td>
<td>0.0272 ± 0.0049*</td>
<td>8</td>
<td>0.9892 ± 0.1714*</td>
<td>8</td>
</tr>
</tbody>
</table>

Results are means ± SE; n, no. of animals. Relative amounts of nNOS, iNOS, or CyPA mRNA were obtained from their standard curves (cerebellum, lung of endotoxin-treated animals at 1 mg/kg ip 12 h, or cerebellum, respectively). *P < 0.05 compared with the respective saline-treated group.
synthesis of the protein, and functional activity of the protein including dimerization of the iNOS that has been shown to be a slow process (34). It seems that 4 h after the administration of low doses of endotoxin the iNOS gene expression in the antrum pylorus has already been started but synthesis of NO from the iNOS is not significant.

A time lag between iNOS gene expression and synthesis of NO has widely been reported (16, 18, 19). Lack of iNOS-derived NO synthesis 4 h after the administration of endotoxin is reinforced by functional studies showing that pretreatment with both the selective iNOS inhibitor L-NIL and the nonselective NOS inhibitor L-NAME at doses reported to block iNOS-derived NO synthesis (23, 40) did not modify the rate of gastric emptying in endotoxin-treated animals. It is believed that once iNOS is functionally active, it synthesizes high amounts of NO (5, 6), which at the level of the antrum pylorus would cause impaired antral contractions. However, such amounts of NO will necessarily decrease pyloric tone, allowing gastric emptying, an effect not observed in the present study.

The present results support a downregulation of the nNOS protein in the delay of gastric emptying induced by low doses of endotoxin. Mechanisms other than synthesis of NO from the iNOS previously reported (4) seem to be involved. Cross-talk interactions between the NOS and COX systems have been described, and specific modulation of nNOS by prostaglandins has been reported.

A role for endogenous prostaglandins in the inhibitory effects of endotoxin on gastric emptying is shown in the present study. However, these prostanooids do not seem to mediate gastric emptying in vehicle-treated animals. Synthesis of prostaglandins triggered by endotoxin seems to be mediated by the COX-2 isoenzyme, because pretreatment with a COX-2-selective dose of NS-398 (20) significantly prevented the effect of endotoxin in a similar manner to that observed in indomethacin-treated rats. Both induction of COX-2 protein and increase in the supply of arachidonic acid are required to enhance prostanoid production (14). In the present study, the administration of quinacrine, a phospholipase 2-inhibitor, did not significantly modify the rate of gastric emptying in endotoxin-treated animals, suggesting that the increased synthesis of prostanooids induced by endotoxin may be due to an increased expression of COX-2 rather than the release of arachidonic acid from the cellular membrane due to an increased activity of the phospholipase. This is reinforced by the fact that pretreatment with dexamethasone, which inhibits the expression of COX-2 without directly affecting its activity, significantly prevented the inhibition of gastric emptying by endotoxin. Dexamethasone has also been shown to inhibit the expression of iNOS (30). However, taking into account the lack of effect of the iNOS isoform on the delayed gastric emptying induced by endotoxin, the effects of dexamethasone on gastric emptying seem likely due to the inhibition of COX-2 expression.

The present study shows that the delay in gastric emptying of a nutrient meal induced by low doses of endotoxin is mediated by diminution of the nNOS activity in the antrum pylorus of the stomach and synthesis of prostaglandins. Although a possible cross-talk between prostaglandins and NO cannot be ruled out, the present results point to a transcriptional regulation of the nNOS and COX-2 carried out by endotoxin.

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