British Journal of Pharmacology (2001) 134, 325–332

www.nature.com/bjp

A cerebral nitrergic pathway modulates endotoxin-induced changes in gastric motility

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1 This study analyses the neural pathway involved in the modulation of gastric motor function by stress.

2 Systemic administration of low doses of endotoxin (40 μg kg^{-1}, i.v.) prevents the increase in gastric tone induced by 2-deoxy-D-glucose (200 mg kg^{-1}, i.v., 2-DG) in urethane-anaesthetized rats.

3 Functional inhibition of afferent neurones by systemic administration of capsaicin (20 + 30 + 50 mg kg^{-1}, i.m.) in adult rats prevented the inhibitory effects of endotoxin.

4 Pre-treatment with the nitric oxide synthase (NOS) inhibitor, N^{G}-nitro-L-arginine methyl ester (L-NAME), both i.v. (10 mg kg^{-1}) and i.c. (200 μg rat^{-1}), prevented the inhibitory effects of endotoxin on gastric tone induced by 2-DG.

5 Immunohistochemical studies show Fos expression in the dorsal vagal complex (DVC) of the brainstem of 2-DG-treated animals. Peripheral administration of endotoxin (40 μg kg^{-1}, i.p.) increased the number of Fos-immunoreactive cells induced by 2-DG, both in the nucleus tractus solitarii (NTS) and in the dorsal motor nucleus (DMN) of the DVC. Pre-treatment with L-NAME prevented the increase in Fos expression induced by endotoxin in both nuclei.

6 Endotoxin (40 μg kg^{-1}, i.p.) increased Ca^{2+}-dependent nitric oxide synthase (cNOS) activity in the brainstem. Addition of 7-nitroindazole (600 μM, 7-NI) to the assay significantly inhibited the increase in cNOS activity caused by endotoxin. No change in NOS activity of any isoform was observed in the stomach of animals treated with endotoxin.

7 The present study suggests that inhibition of gastric motor function by low doses of endotoxin involves activation of capsaicin-sensitive afferent neurones and neuronal NOS in the brainstem.

British Journal of Pharmacology (2001) **134,** 325–332

Keywords: Nitric oxide; gastric motor function; brain-gut; capsaicin; NTS; DMN; DVC; central nervous system; endotoxin

Abbreviations: ABC, avidin biotin complex; cNOS, Ca^{2+}-dependent nitric oxide synthase; 2-DG, 2-deoxy-D-glucose; D-arg, D-arginine; DVC, dorsal vagal complex; DMN, dorsal motor nucleus; ecNOS endothelial constitutive nitric oxide synthase; L-arg, L-arginine; L-NAME, N^{G}-nitro-L-arginine methyl ester; L-NNA, N^{G}-nitro-L-arginine; LPS, lipopolisaccharide; NANC, non-adrenergic non-cholinergic; ncNOS neuronal constitutive nitric oxide synthase; 7-NI, 7-nitroindazole; NTS, nucleus tractus solitarius

Introduction

Changes in gastric function are associated with stress. Acute administration of endotoxin, in doses that do not modify systemic arterial blood pressure or rectal temperature, selectively decreases gastric acid secretion and increases mucosal resistance to damage through a mechanism that involves the central nervous system (Barrachina *et al*., 1995a, b; Esplugues *et al*., 1996). Similar doses of endotoxin have recently been reported to delay gastric emptying of a solid nutrient meal through activation of afferent fibres (Calatayud *et al*., 2001), suggesting that the superior neuronal network is also involved in the gastric motor inhibitory mechanism triggered by endotoxin.

The DVC of the brainstem is comprised of two interacting nuclei: the NTS, which receives primary afferent fibres and the DMN, where preganglionic motor neurones innervating the gastrointestinal tract are located (Kalia & Mesulam, 1980). Functional active pathways in the brain have been characterized using Fos immunohistochemistry as a neural tracing technique. Systemic administration of endotoxin has been shown to activate the early-appearing gene product c-*fos* in visceral afferents, neuroendocrine and autonomic regions of the brain (Hermann *et al*., 2001; Lin *et al*., 1998b). However, relatively high doses of endotoxin have been used and no relationship between brainstem neuronal activation and modulation of gastric motor function by endotoxin has been reported.

Nitric oxide (NO) acts as an intercellular messenger in the central nervous system (Garthwaite *et al*., 1988). Three different NOS have been characterized: two Ca^{2+}-dependent NOS, the neuronal (ncNOS) and endothelial (ecNOS) isoform, and a third Ca^{2+}-independent isoform (Barrachina *et al*., 2001; Moncada *et al*., 1991). Immunohistochemical studies have reported the presence of NOS in the DVC of the brainstem. In particular, vagal afferents and intrinsic neurones of the NTS as well as neurones of the DMN express NOS immunoreactivity (Lin *et al*., 1998a; Zheng *et al*., 1999). In addition, a role for NO in central brainstem

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circuits which control gastric secretory and motor functions has been reported (Esplugues *et al*., 1996; Krowicki *et al*., 1997). Thus, synthesis of NO is involved in the inhibition of gastric acid secretion by stress (Beltrán *et al*., 1999; Esplugues *et al*., 1996) or modulates the inhibition of gastric motor function by substance P (Krowicki & Hornby, 1996).

In the present study, in order to characterize the neural pathway and endogenous mediators involved in the changes in gastric motor function associated with stress, we evaluated the effects of low doses of endotoxin on: (1) intragastric pressure induced by the antimetabolic glucose analogue, 2-DG; (2) the pattern of brainstem neuronal activation and (3) the NOS activity both in the brainstem and the stomach.

Methods

Male Sprague-Dawley rats (220–250 g) were fasted for 16–20 h before the experiments but were allowed access to drinking water.

Determination of intragastric pressure

Rats were anaesthetized with urethane (1.5 g kg^{-1}, i.p.), the trachea intubated and a jugular vein cannulated. After performing a laparotomy, an intraluminal latex balloon was inserted in the stomach through an incision in the forestomach and held in place with a ligature. The balloon and catheter system was connected to a pressure transducer and intragastric pressure was registered on line by a multi-channel recorder (Power Lab.). After reaching an intragastric pressure of between 4–5 cm H_2O, by filling the balloon with 2–3 ml H_2O at 37°C, rats were allowed to stabilize for 1 h. Rectal temperature was monitored during the experiment and maintained at 36–37°C. Unless mentioned otherwise, data is expressed as Δ intragastric pressure, calculated as the difference between stimulated (the average measurement 30 to 60 min after 2-DG administration) and basal (the average in the last 10 min before 2-DG administration) intragastric pressure.

Experimental protocol

In a first group of experiments, endotoxin (40 μg kg^{-1}, i.v.) was administered 30 min prior to a bolus injection of 2-DG (200 mg kg^{-1}, i.v.) and intragastric pressure was monitored for 60 min. In order to analyse the role of sensory fibres, rats were administered with capsaicin (a selective neurotoxin on C-fibres; 20 + 30 + 50 mg kg^{-1}, i.m.) or its vehicle (10% ethanol + 10% Tween-80 + 80% saline, 1 ml kg^{-1}, i.m.) for 3 consecutive days, 15 days prior to the experiments. The role of NO in the effects of endotoxin was analysed by pretreatment (15 min) with the NO synthesis inhibitor, L-NAME (10 mg kg^{-1}, i.v. or 200 μg rat^{-1}, i.c.) or its vehicle (1 ml kg^{-1} or 10 μl rat^{-1}, respectively).

In order to analyse further the direct role of NO synthesis in the brain on intragastric pressure, in a second group of experiments some animals received an i.c. injection of L-arginine (200 μg rat^{-1}, L-arg), D-arginine (200 μg rat^{-1}, D-arg) or saline (10 μl rat^{-1}) and gastric contractility was monitored for 60 min.

Fos immunohistochemistry

Procedures were carried out as previously described (Barrachina *et al*., 1997). Ten minutes prior to the administration of 2-DG (200 mg kg^{-1}, i.p.), rats received two consecutive i.p. injections of either L-NAME (10 mg kg^{-1}) + endotoxin (40 μg kg^{-1}), L-NAME + saline (1 ml kg^{-1}), vehicle (1 ml kg^{-1}) + endotoxin or vehicle + saline. Additional groups of rats received a single i.p. injection of saline, endotoxin or L-NAME and were included as control groups. Two hours later, animals were anaesthetized with pentobarbitone (280 mg kg^{-1}, i.p.) and transcardially perfused with 0.9% saline followed by paraformaldehyde solution (4%). Brains were postfixed in the same fixative and cryoprotected overnight by immersion in 30% sucrose. Immunohistochemistry for Fos expression was processed on frozen brain coronal sections (50 μm thick) using the avidin–biotin complex (ABC) method (Hsu *et al*., 1981). Sections were incubated for 24 h at 4°C with the primary antibody c-*fos* (sheep policlonal, Genosys) diluted at 1 : 2000. Then, sections were incubated with biotynylated anti-sheep IgG (Vector Labs.), diluted at 1 : 200, followed by ABC (Vectastain ABC Kit, Vector Labs.), in both cases for 1 h at 25°C. Sections were incubated for 8 min in a substrate for peroxidase kit (Vector VIP, Vector Labs.) and were mounted, air-dried, dehydrated, cleared and coverslipped. The sections were observed and photographed using a brightfield microscope (Zeiss). The counting of Fos-immunoreactive cells was performed bilaterally in six sections per animal, regardless of the intensity of staining, for the NTS and DMN. The L.W. Swanson rat brain atlas was used to determine the anatomical locations of the nuclei.

Determination of NOS activity

Rats were administered with endotoxin (40 μg kg^{-1}, i.p.) or saline (1 ml kg^{-1}, i.p.) and sacrified by cervical dislocation 30 min later. Both, a section of the brainstem containing the DVC (+1 mm to −1.4 mm to the obex) and the stomach, were quickly introduced in liquid nitrogen and stored at −80°C. NO synthase activity was measured as the rate of conversion of L-[U-^{14}C]-arginine to L-[U-^{14}C]-citrulline (Salter *et al*., 1990). Briefly, the samples were homogenized (Ultra-Turrax) in an ice-cold buffer (330 mg ml^{-1}; pH 7.2) containing 320 mM sucrose, 20 mM HEPES, 1 mM EDTA, 1 mM DL-dithiothreitol, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} soybean trypsin inhibitor and 2 μg ml^{-1} aprotinin followed by centrifugation at 10,000 × *g* for 20 min at 4°C. Afterwards centrifugation, 40 μl of supernatant was incubated at 37°C for 20 min in assay buffer (pH 7.4) containing (mM) KH_2PO_4 50, $MgCl_2$ 1, $CaCl_2$ 0.2, L-valine 50, L-citrulline 1, L-arginine 0.02, DL-dithiothreitol 1, and 100 μM NADPH, 3 μM FAD, 3 μM FMN, 3 μM BH_4 and 950 nM L-[U-^{14}C]-arginine (348 mCi $mmol^{-1}$). The specificity of L-arginine conversion by NOS to L-citrulline was further confirmed using the NO synthesis inhibitors, N^G-nitro-L-arginine (L-NNA, 1 mM). Additionally, 1 mM EGTA, a calcium chelating agent was used to differentiate between Ca^{2+}-dependent and Ca^{2+}-independent isoform of NOS. The specific inhibitor of neuronal constitutive NOS (ncNOS), 7-nitroindazole (600 μM, 7-NI; a dose chosen from a previous dose-response curve; Babbedge *et al*., 1993) was used to differentiate

between ncNOS and endothelial constitutive NOS (ecNOS) isoform activity. All activities are expressed as picomol of product generated per minute per gram of tissue.

Drugs

Urethane, 2-deoxy-D-glucose, *Escherichia coli* endotoxin (serotype 026:B6), L-NAME, L-arginine, D-arginine, Tween 80 and all reagents used for determination of NOS activity were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). L-[^{14}C]-arginine was obtained from Amersham Life Science and Capsaicin was purchased from Fluka Chemic AG. Sodium pentobarbitone was used as a commercially available preparation (Abbott, Madrid). Unless mentioned otherwise all drugs have been dissolved in saline.

Statistical analysis

Data are expressed as mean ± s.e.mean. Comparisons between groups were performed by one-way analysis of variance followed by a Newman-Keuls test. Data are considered statistically significant when $P<0.05$.

Results

Effects of endotoxin on intragastric pressure

Urethane-anaesthetized rats fixed with a gastric balloon filled with 2.8 ± 0.2 ml of water exhibited an intragastric pressure of 5.4 ± 0.5 cm H_2O ($n=10$). A single i.v. injection of endotoxin (40 μg kg^{-1}) did not significantly modify intragastric pressure (5.3 ± 0.5 cm H_2O, $n=10$) in the 30 min following injection (Figure 1).

Administration of 2-DG (200 mg kg^{-1} i.v., $n=10$) induced a peak increase in gastric tone at 5 min (9.4 ± 0.9 cm H_2O) that was maintained above basal level for more than 60 min (6.7 ± 0.6 cm H_2O). Pre-treatment with endotoxin (40 μg kg^{-1}, i.v., $n=10$) significantly ($P<0.05$) impeded both the contraction peak (6.2 ± 0.7 cm H_2O) and the increase in gastric tone (5.1 ± 0.4 cm H_2O) induced by 2-DG (Figure 1).

As shown in Figure 2, functional inhibition of capsaicin-sensitive afferent neurones significantly prevented the inhibitory effects of endotoxin on 2-DG-increased intragastric pressure while it did not significantly alter gastric contractility in animals treated with 2-DG alone.

Basal gastric tone (5.4 ± 0.5 cm H_2O, $n=14$) was not significantly changed by inhibition of NO synthesis through systemic administration of L-NAME (4.9 ± 0.3 cm H_2O, $n=14$, measured 15 min after administration of L-NAME). Pre-treatment with i.v. L-NAME did not significantly modify the increase in intragastric pressure induced by 2-DG (Figure 3a). However, systemic inhibition of NO synthesis completely reversed the reduction by endotoxin of intragastric pressure

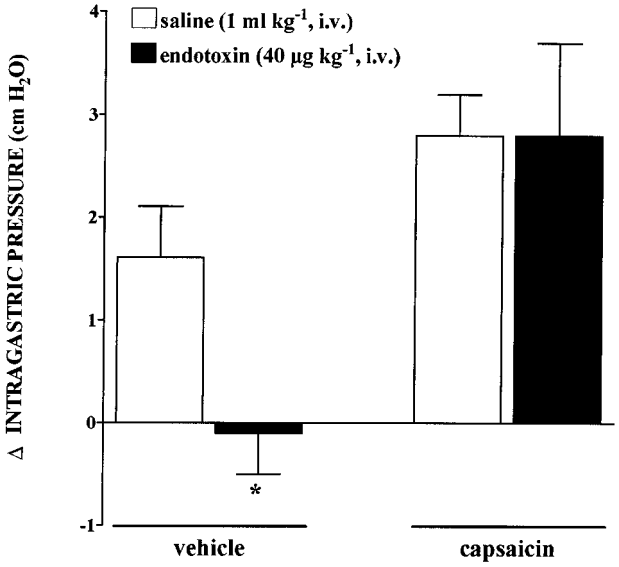

Figure 2 Effects of endotoxin or saline on intragastric pressure induced by 2-DG (200 mg kg^{-1}, i.v.) after pre-treatment (15 days) with capsaicin (125 mg kg^{-1}, i.m.) or its vehicle (ethanol, Tween 80 and saline, 10 : 10 : 80, 1 ml kg^{-1}, i.m.). Each bar represents mean ± s.e.mean of at least four animals. Significant difference from all other groups is shown by $*P<0.05$.

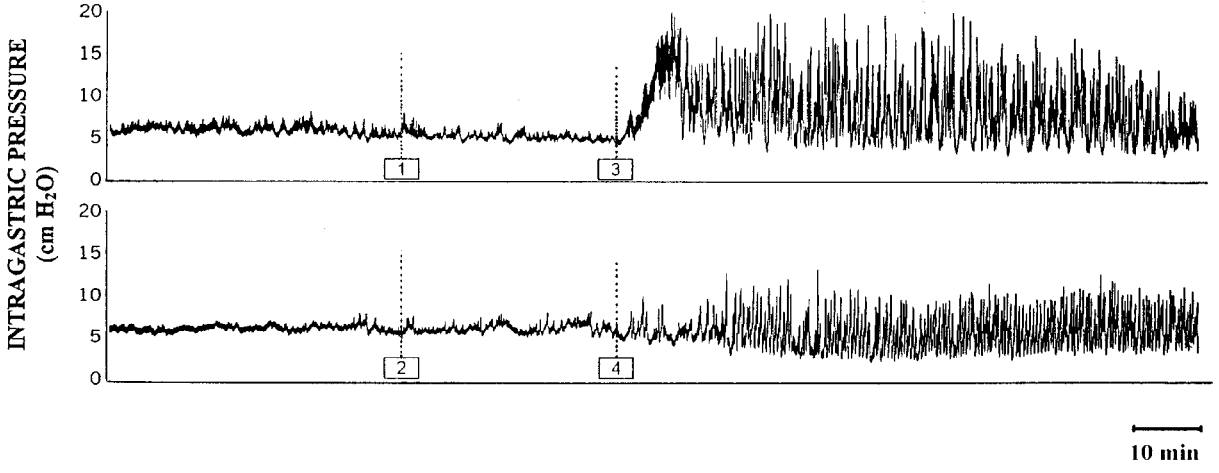

Figure 1 Original recording showing the effects of i.v. administration of vehicle (1 ml kg^{-1}, **1**) or endotoxin (40 μg kg^{-1}, **2**) on intragastric pressure induced by 2-Deoxy-D-Glucose (200 mg kg^{-1}, i.v.; **3, 4**).

induced by 2-DG (Figure 3a). Similar results were obtained when lower doses of L-NAME (200 μg rat^{-1}) were administered i.c. Central inhibition of NO synthesis did not significantly change basal or 2-DG stimulated intragastric pressure while it did prevent the inhibitory effects of endotoxin (Figure 3b).

Gastric tone in basal conditions (5.6$\pm$0.6 cm H_2O, $n=5$) was significantly ($P<0.05$) diminished by a single i.c. injection of L-arg (2.4$\pm$0.8 cm H_2O, $n=5$; 200 μg rat^{-1}). Neither an i.c. injection of vehicle (4.6$\pm$0.6 cm H_2O, $n=3$; 10 μl rat^{-1}), nor administration of D-arg (4.0$\pm$1.1 cm H_2O, $n=3$; 200 μg rat^{-1}) significantly modified basal intragastric pressure.

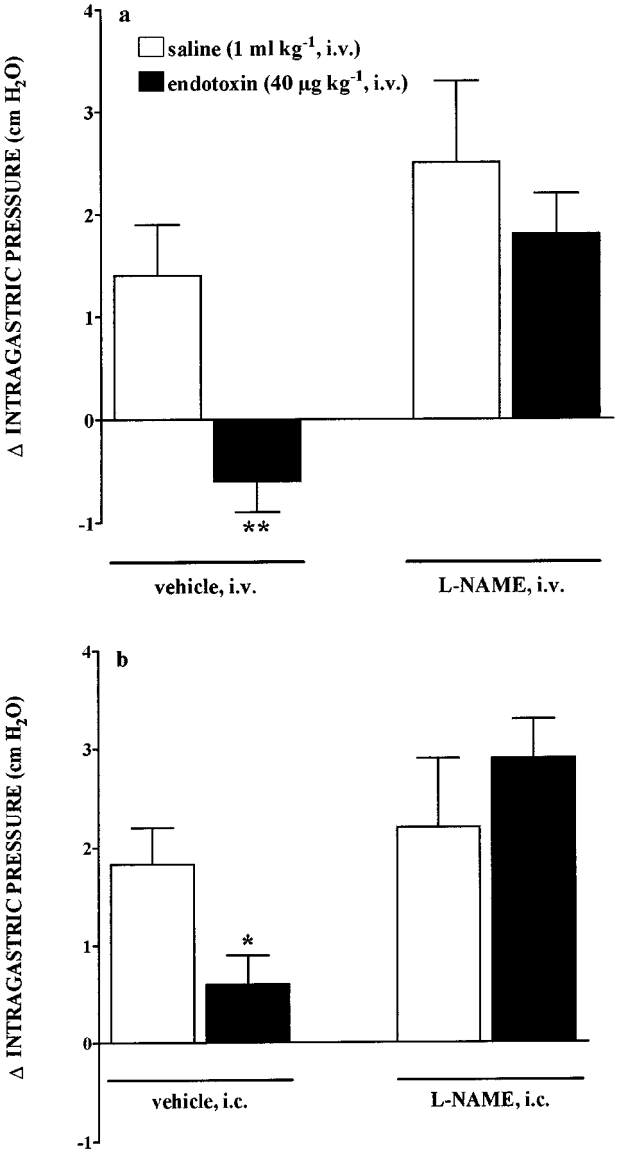

Figure 3 Effects of endotoxin or saline on intragastric pressure induced by 2-DG (200 mg kg^{-1}, i.v.) in animals pretreated: (a) with i.v. administration of L-NAME (10 mg kg^{-1}) or vehicle (1 ml kg^{-1}); (b) with i.c administration of L-NAME (200 μg rat^{-1}) or vehicle (10 μl rat^{-1}). Each bar represents mean$\pm$s.e.mean of at least six animals. Significant difference from all respective groups included in the same graph is shown by $^{**}P<0.01$ or $^{*}P<0.05$.

Effects of endotoxin on Fos expression

Fos expression was not observed either in the NTS or in the DMN of fasted rats injected i.p. with saline ($n=3$). Administration of 2-DG (200 mg kg^{-1}, i.p.) induced a pronounced staining for Fos immunoreactivity in the NTS and a slight Fos expression in the DMN (Figure 4a,d). Pre-treatment with endotoxin (40 μg kg^{-1}, i.p.) significantly increased the number of Fos-positive cells both in the NTS and the DMN of 2-DG-treated animals (Figure 4b,d). If administered alone, endotoxin induced a moderate increase in the number of Fos-immunoreactive cells in the NTS (9.3$\pm$0.1 nb/section, $n=3$) while having no effect on Fos expression in the DMN.

Pre-treatment with L-NAME (10 mg kg^{-1}, i.p.) significantly increased the number of Fos-positive neurones in both the NTS and the DMN of 2-DG-treated rats. Endotoxin did not augment the number of Fos-immunoreactive cells in animals treated with L-NAME + 2-DG (Figure 4d). L-NAME administered alone ($n=3$) increased Fos expression in the NTS (54.2$\pm$10.2 nb/section) and the DMN (1.8$\pm$1.2 nb/section), while vehicle-treated animals showed no Fos staining in both nuclei.

Effects of endotoxin on NOS activity

Brainstem NOS activity Ca^{2+}-independent NOS activity in the brainstem of vehicle-treated animals was almost non-apparent (4.5$\pm$0.9 pmol citrulline min^{-1} g tissue^{-1}), compared with cNOS activity (Figure 5a). Pre-treatment (30 min) with endotoxin (40 μg kg^{-1}, i.p., $n=4$) had no effect on Ca^{2+}-independent NOS activity (3.7$\pm$0.7 pmol citrulline min^{-1}g tissue^{-1}) while significantly ($P<0.05$) increased the activity of cNOS (Figure 5a).

cNOS activity in the brainstem of vehicle- or endotoxin-treated animals seems to be principally due to the ncNOS isoform, since addition of 7-NI (600 μM) to the assay significantly inhibited (77.8$\pm$1.6 or 82.9$\pm$2.8%, respectively) cNOS activity (Figure 5a).

Stomach NOS activity cNOS activity in the stomach of vehicle-treated animals was one-tenth than that exhibited in the brainstem of the same animals (Figure 5a,b). In a similar manner to that observed in the brainstem, Ca^{2+}-independent NOS exhibited low levels of activity (28.2$\pm$4.6 pmol citrulline min^{-1} g tissue^{-1}) in the stomach. Pre-treatment with endotoxin (30 min) did not significantly modify the Ca^{2+}-independent NOS activity (23.5$\pm$2.6 pmol citrulline min^{-1} g tissue^{-1}) or cNOS in the stomach (Figure 5b).

cNOS activity in the stomach of vehicle- or endotoxin-treated animals seems to be attributted to the ncNOS isoform, since addition of 7-NI (600 μM) to the assay significantly inhibited (66.6$\pm$6.5 or 74.0$\pm$2.1%, respectively) the cNOS activity (Figure 5b).

Discussion

Intravenous administration of 2-DG induced an increase in intragastric pressure that was rapid in onset, maintained itself for more than 60 min and was independent of both capsaicin-sensitive afferent neurones and NO synthesis in the brain-

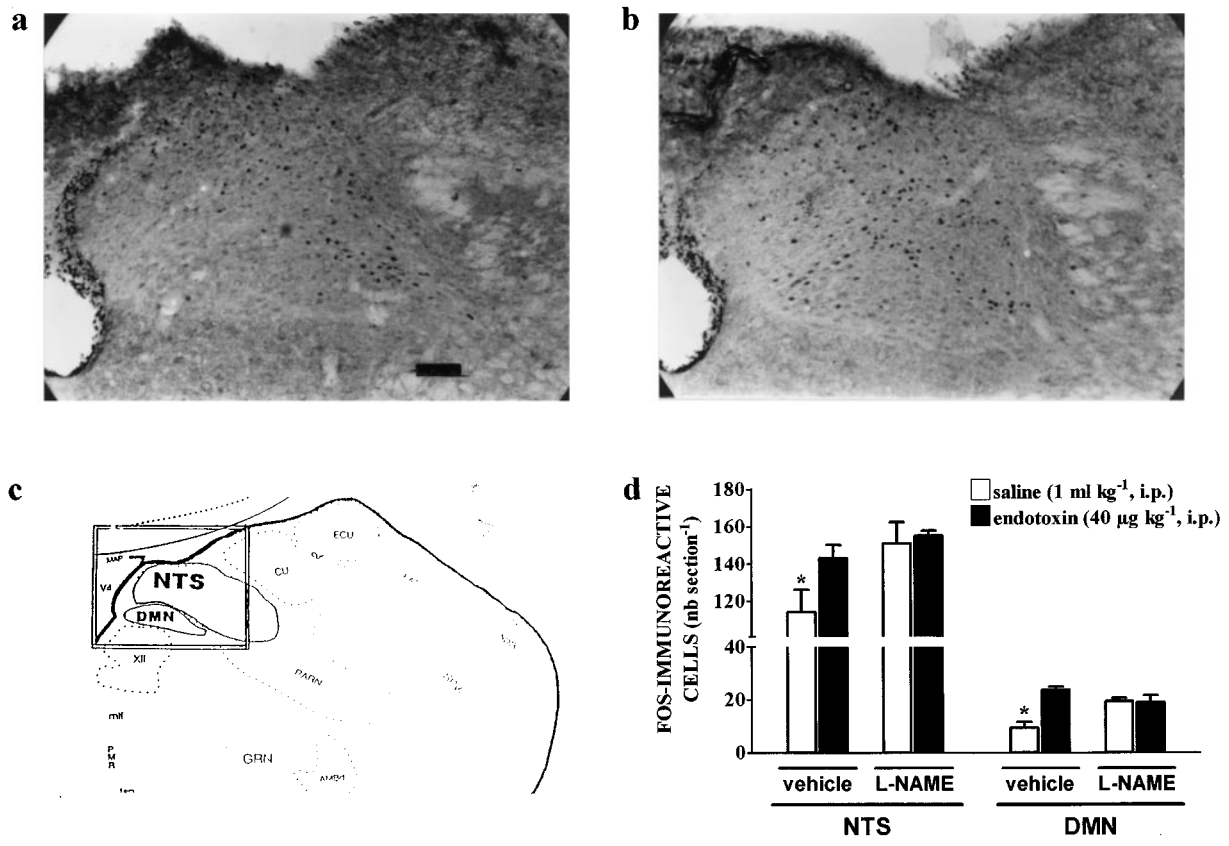

Figure 4 Fos immunoreactivity in the DVC of the brainstem after administration of endotoxin or saline in 2-DG-treated animals (200 mg kg^{-1}, i.p.). Representative microphotographs show Fos immunoreactivity in the NTS and DMN of the DVC, 2 h after injection of (a) vehicle + 2-DG, (b) endotoxin + 2-DG; Scale bar = 100 μm. (c) Coronal drawing section from brainstem adapted from Swanson rat brain atlas at the level of −13.6 mm from bregma, showing the localization of the NTS and DMN. (d) Graph showing the effects of endotoxin or saline on the number of Fos immunoreactive cells (nb/section, bilateral) induced by 2-DG in vehicle- (1 ml kg^{-1}, i.p.) or L-NAME- (10 mg kg^{-1}, i.p.) treated animals. Each bar represents mean ± s.e.mean of at least three animals. Significant difference from the rest of groups in the same nucleus is shown by $*P<0.05$.

stem. Pretreatment with doses of *E. coli* lipopolisaccharide (LPS) which do not modify arterial blood pressure or rectal temperature (Barrachina *et al*., 1995b; García-Zaragozá *et al*., 2000), both of which are associated with severe septicaemia, significantly prevented the increase in intragastric pressure induced by 2-DG. We have recently demonstrated that a similar dose of endotoxin significantly delays the 4 h rate of gastric emptying of a solid nutrient meal in conscious animals (Calatayud *et al*., 2001). The present results thus suggest that the rapid diminution of gastric tone induced by endotoxin is involved in the mechanism by which peripheral endotoxin delays gastric emptying. In accordance with this observation, in the present study, activation of capsaicin sensitive afferent neurones is shown to mediate the diminution of intragastric pressure by endotoxin, in a similar manner to that previously reported for the delay in gastric emptying by LPS (Calatayud *et al*., 2001). These sensory fibres are involved in the modulation of gastric acid secretion (Martínez-Cuesta *et al*., 1994) and resistance of the gastric mucosa to damage (Barrachina *et al*., 1995b) by peripheral endotoxin. However, the precise role of this activation is still unknown. Capsaicin-sensitive afferent neurones in the gastrointestinal tract appear to act through two mechanisms. One is related to the local effector function of these fibres, whereby several neuropeptides are released (Holzer, 1988); in this way, we have recently shown a role for endogenous release of CGRP in endotoxin-induced delay in gastric emptying (Calatayud *et al*., 2001). The second mechanism is related to sending information to the superior neuronal network in the brain and spinal cord, which initiates the efferent reflex loop (Holzer, 1998). In the present study, pretreatment with endotoxin, at doses that prevented the increase in intragastric pressure induced by 2-DG, significantly increased the number of Fos-immunoreactive neurones in the DVC of the brainstem of 2-DG-treated animals. This complex is comprised of the NTS and the DMN, and both nuclei exhibited Fos immunoreactivity after systemic administration of 2-DG. The increase in Fos expression observed reflects an interaction between 2-DG and endotoxin, since LPS injected alone had no influence on the number of Fos-positive neurones in the DVC of the brainstem. These observations may be significant in attempting to understand the diminution in intragastric pressure induced by peripheral endotoxin. The higher activity observed in neurones of the NTS in presence of endotoxin suggest that vagal primary afferents are excited by peripheral endotoxin, which would be in accordance with the functional part of the study. However, according to previous studies, activation of the NTS implies inhibition of the DMN, since many of the NTS neurones that project into the DMN are inhibitory (Zhang *et al*., 1998). In

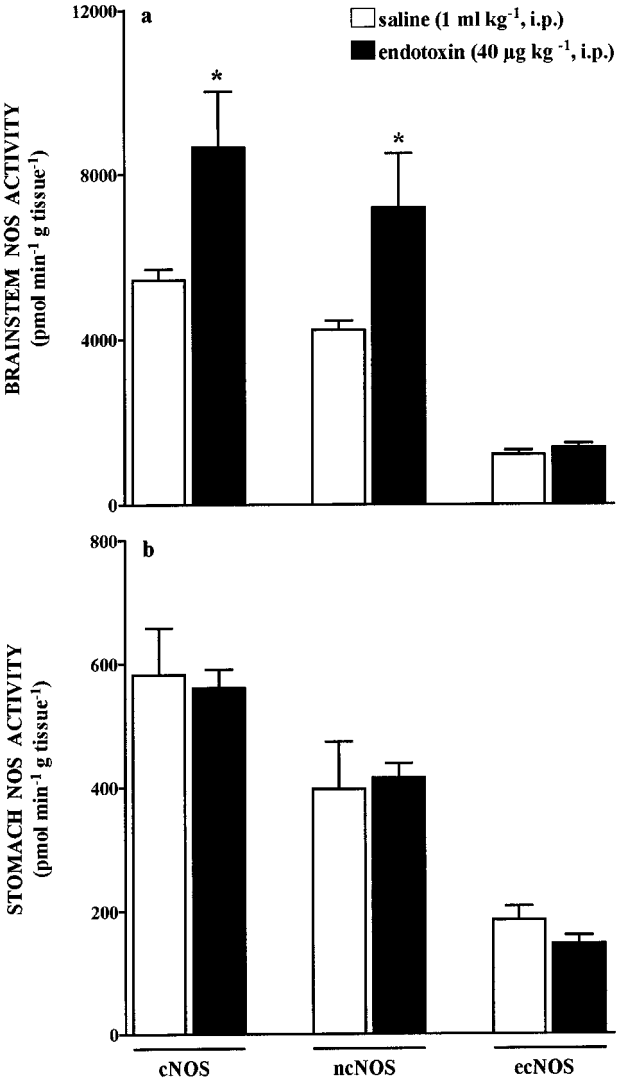

Figure 5 Ca^{2+}-dependent NOS (cNOS) activity in: (a) a section of the brainstem (+1 to −1.4 mm to the obex) and (b) the stomach of endotoxin or saline treated rats. Addition of 7-nitroindazole (600 μM) distinguishes the cNOS between neuronal NOS (ncNOS) and endothelial NOS (ecNOS) activity. Each bar represents mean ± s.e.mean of four animals. Significant difference from the respective saline treated group is shown by $^*P<0.05$.

this case we might expect that the DMN neurones would respond to peripheral endotoxin with a decrease in activity that would, thereby, mediate a decrease in gastric contractility. In contrast, the present results show an increase in Fos immunoreactivity of DMN neurones in response to peripheral endotoxin. Excitation of DMN neurones by different stimuli that inhibit gastric motor function has already been reported (Blackshaw *et al*., 1987; Miolan & Roman, 1984). Characterizing the nature of the vagal efferent fibres involved in the gastric effects of endotoxin would help us to better address this question.

It has been proposed that NO acts as an intercellular messenger in central brainstem circuits controlling gastric function (Esplugues *et al*., 1996; Krowicki *et al*., 1997). Pretreatment with L-NAME prevented the increase in Fos expression in the DVC induced by peripheral endotoxin, suggesting that Fos-immunoreactive cells are dependent on NO. However, results are not conclusive and the understanding of these observations is complicated since L-NAME alone significantly increased Fos expression in the DVC. Two basic assumptions can be made, each resulting in a different interpretation of our findings. One is that endotoxin and blockade of NO synthesis both activate the same population of neurones. If this were the case, results suggest that neurones activated by endotoxin are sensitive to the tonic inhibitor role of NO. The second possibility is that endotoxin and L-NAME activate a separate population of neurones. In this case, the inability of endotoxin to increase Fos expression in L-NAME-treated animals would suggest that NO, acting as a neurotransmitter or as second messenger, is responsible for the activation of these neurones. Previous functional studies support the second hypothesis (Barrachina *et al*., 1995b; Esplugues *et al*., 1996), although double immunostaining would be necessary to provide more conclusive results.

The present study demonstrates a role for cerebral NO synthesis in the diminution of intragastric pressure by peripheral endotoxin, since pre-treatment with i.c. L-NAME completely abolished the inhibitory effects of endotoxin. A role for NO in the brainstem in the modulation of gastric function has already been reported with exogenous administration of an NO-donor (Beltrán *et al*., 1999). This study extends previous observations and show a rapid decrease in intragastric pressure after i.c. administration of L-arg, an effect that seems specifically due to NO synthesis, since i.c. injection of the inactive enantiomer D-arg lacked a significant effect. Taken together results underline an important role for NO synthesis in the brainstem in the modulation of gastric function. In addition, the analysis of NOS activity in the brainstem shows that peripheral endotoxin significantly increases the activity of the enzyme, 30 min after administration. The NOS activity modulated by endotoxin was Ca^{2+}-dependent, which is in accordance with the present and previous studies (Barrachina *et al*., 1995a, b; Esplugues *et al*., 1996) showing that the effects of low doses of endotoxin on gastric function become apparent in less than 30 min. Furthermore, the use of an specific ncNOS inhibitor, 7-nitroindazole (Babbedge *et al*., 1993) significantly inhibited NOS activity in the brainstem, suggesting that the ncNOS isoform, rather than ecNOS, is responsible for the synthesis of NO induced by endotoxin. Taking into account that the ncNOS activity in the brainstem of control animals is responsible for most of the NOS activity, it would be reasonable to assume that this isoform is more sensitive to change. LPS has also been shown to stimulate the production of Ca^{2+}-independent NOS in the brain (Wong *et al*., 1996) but this change normally takes place several hours after the induction of endotoxaemia and, therefore, probably has little functional importance for the inhibition of gastric motor function shown in the present study.

The precise mechanism by which peripheral endotoxin increases the activity of ncNOS has not been evaluated in this study. Considering that the presence of NOS in vagal afferents which project into the NTS has been reported (Lin *et al*., 1998a), one possibility is the direct activation of ncNOS by stimulation of the afferent neurones. However, based mainly on the literature, we hypothesise a role for an intermediate neurotransmitter: (a) NO synthesis in the brain is predominantly regulated by Ca^{2+} influx through receptor operated channels, in particular following postsynaptic

stimulation by the excitatory neurotransmitter glutamate (Bredt & Snyder, 1989; Garthwaite *et al*., 1989). (b) Recent studies report that glutamate is the primary afferent neurotransmitter released in the NTS in the control of gastric function (Zheng *et al*., 1999). (c) The release of glutamate in the brainstem by peripheral endotoxin has been reported (Mascarucci *et al*., 1998) and we have recently shown that endogenous glutamate activates NO synthesis in the brain, which is in turn responsible for the inhibition of gastric acid secretion by peripheral endotoxin (García-Zaragozá *et al*., 2000). These observations, considered with a previous study reporting an inhibitory role on gastric motor function for glutamate acting in the NTS (Raybould *et al*., 1989) lead us to believe that endogenous release of this neurotransmitter is involved in the activation of ncNOS in the brainstem by peripheral endotoxin.

Irrespective of the afferent pathways involved, once synthesized in the brainstem, NO is involved in the diminution of intragastric pressure by endotoxin. NO in the DVC has been shown to modulate the firing rate of DMN neurons (Travagli & Gillis, 1994). Such a modulation implies activation of efferent vagal preganglionic fibres that would synapse with NANC inhibitory postganglionic neurones in the myenteric plexus. NO has also been consolidated as one of the non-adrenergic non-cholinergic (NANC) inhibitory neurotransmitters of the gastrointestinal tract. In the present study, systemic administration of L-NAME significantly prevented the reduction in intragastric pressure induced by endotoxin, suggesting a peripheral role for NO. However, taking into account that i.v. administration of L-NAME induces Fos expression in the DVC of the brainstem, and that similar doses of systemic L-NAME have been shown to affect the spontaneous discharge rate of NTS neurones (Ma *et al*., 1995), we cannot deduce the release of NO in the periphery. In addition, this role seems improbable since analysis of NOS activity in the stomach shows no change in the activity of any NOS isoform when evaluated 30 min after injection of endotoxin, a time interval in which changes in gastric function are already apparent. Regardless of whether or not activation of an inhibitory efferent neural pathway takes place in the inhibition of gastric motor function by endotoxin, the peripheral release of NO does not seem to be involved. Endogenous mediators such as VIP have also been reported as inhibitory neurotransmitters of the NANC innervation of the gastrointestinal tract. Further experiments are required to address this question.

In summary, the present results suggest that low doses of endotoxin reduce gastric contractility through activation of both capsaicin-sensitive afferent neurones and ncNOS in the brainstem. This inhibition may be part of the mechanism involved in the delay in gastric emptying associated with moderate endotoxaemia.

We would like to thank Prof C. Estrada and F. Portillo for their valuable help in the Fos immunohistochemistry. The present study has been supported by grants SAF 99-0114, SAF 98-0118 and 1FD97-1029 from Ministerio de Educación y Cultura. E. Quintana and E. García-Zaragozá are the recipients of a fellowship from Ministerio de Sanidad y Consumo and Ministerio de Educación y Cultura, respectively. Part of this study has been supported by the Royal Academy of Medicine of University of Valencia.

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(Received April 10, 2001
Revised June 19, 2001
Accepted June 26, 2001)