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# Low endotoxemia prevents the reduction of gastric blood flow induced by NSAIDs: role of nitric oxide

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**1** The role of nitric oxide (NO) in the effects of low endotoxemia on gastric damage and blood flow has been evaluated in indomethacin-treated rats.

**2** Pretreatment (−1 h) with endotoxin (40 $\mu$g kg$^{-1}$) reduced gastric damage induced by indomethacin (20 mg kg$^{-1}$) in conscious rats.

**3** Endotoxin prevented the reduction in gastric blood flow (laser Doppler flowmetry) induced by indomethacin in pentobarbital-anaesthetised rats.

**4** Pretreatment with an NO-synthase (NOS) inhibitor (L-NAME, 1 mg kg$^{-1}$) reversed the protective effect of endotoxin on gastric blood perfusion.

**5** Endotoxin did not modify the expression of mRNA for endothelial NOS or inducible NOS in the gastric corpus when evaluated 1 h postinjection. However, a 3.8-fold increase in inducible NOS mRNA and a 61% reduction in endothelial NOS mRNA were observed in the gastric corpus 4 h after endotoxin administration.

**6** Evaluation of both total and $Ca^{2+}$-dependent NOS activity by analysing the rate of conversion of L-arginine to L-citrulline in gastric corpus homogenates showed no differences between animals treated with endotoxin and those treated with saline 1 or 4 h beforehand. $Ca^{2+}$-independent NOS activity was almost non-apparent in control as well as in endotoxin-treated rats at all the time points analysed.

**7** Low endotoxemia preserves blood perfusion and protects the gastric mucosa against the deleterious effects of indomethacin through the endogenous NO release. NO synthesis in response to endotoxin does not involve the inducible NOS, but probably depends on the post-translational/biochemical regulation *in vivo* of a $Ca^{2+}$-dependent NOS, most probably endothelial NOS.
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**Keywords:** Endotoxin; nitric oxide; NSAIDs; gastric damage; gastric blood flow

**Abbreviations:** GBF, gastric blood flow; NO, nitric oxide; NOS, NO-synthase; eNOS, endothelial NOS; iNOS, inducible NOS; NSAIDs, nonsteroidal anti-inflammatory drugs

## Introduction

The gastric mucosa has the ability to respond to mild aggressions improving its resistance against damage. Thus, while endotoxic shock causes gastric damage, low endotoxemia reduces the susceptibility of the gastric mucosa to ulcerogen agents (Tsuji *et al*., 1993; Barrachina *et al*., 1995a). Endotoxin is one of the most common stimulus to induce the expression of the inducible nitric oxide (NO) synthase (iNOS) and, although this has been traditionally associated with the deleterious effects of endotoxin and NO, recent evidences suggest that iNOS-derived NO may be responsible for endotoxin's protective actions (Yu *et al*., 1997; Konturek *et al*., 1998). We have observed that low endotoxemia elicits acute neural responses involving capsaicin-sensitive afferent neurons and NO synthesis to protect the mucosa against damage, inhibit gastric acid secretion or modify gastrointestinal motility (Barrachina *et al*., 1995a; Esplugues *et al*., 1996; Calatayud *et al*., 2001b; Quintana *et al*., 2001), but a role for iNOS in these effects seems unlikely considering their quick appearance.

A reduced mucosal blood perfusion has consistently been proposed as a causative factor in the development of gastric lesions by nonsteroidal anti-inflammatory drugs (NSAIDs) (Tepperman & Jacobson, 1994), which are the drugs that most frequently challenge gastric mucosal integrity (Hawkey, 1999; Mitchell & Warner, 1999). The endogenous release of NO modulates resting mucosal blood flow (Piqué *et al*., 1989) and the exogenous administration of NO donors prevents both mucosal hypoperfusion and gastric damage induced by NSAIDs (Calatayud *et al*., 1999). The aim of the present study was to analyse the effects of NSAIDs on gastric blood flow in conditions of low endotoxemia and the role of NO and its enzymatic source in such a circumstance. Furthermore, and considering that NO interacts with vasodilator neuropeptides released from capsaicin-sensitive afferent neurons and prostaglandins to modulate microvascular tone in the gastric mucosa (Whittle, 1993), the role played by these substances in endotoxemia was also evaluated.

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## Methods

Male Sprague – Dawley rats (Harlan, 200 – 250 g) were deprived of food, but not water, for 18 – 20 h prior to the experiment.

### *Gastric damage studies*

Animals received an intraperitoneal injection of saline (1 ml kg$^{-1}$) or endotoxin (*Escherichia coli* lipopolysaccharide, serotype 0111:B4, 40 $\mu$g kg$^{-1}$) 1 h before administration of a gastrolesive dose of indomethacin (20 mg kg$^{-1}$ s.c.). Rats were killed by cervical dislocation 3 h after indomethacin injection. The stomachs were then removed, opened along the greater curvature, pinned to a wax block, coded to avoid observer bias and photographed on colour transparency film. The lengths of all macroscopic individual lesions were measured on the photographs and added together to provide a total lesion length (mm) for each rat.

### *Haemodynamic studies*

Animals were anaesthetised with sodium pentobarbitone (50 mg kg$^{-1}$ i.p. ) and the trachea, right carotid artery and jugular vein were cannulated with polyethylene tubing to facilitate spontaneous breathing, to measure systemic arterial blood pressure through a pressure transducer (Spectramed Sathan P- 23XL) connected to a channel recorder (GRASS RPS7C8B, Quincy, MA, U.S.A.) and for drug and additional anaesthetic administration, respectively.

A midline incision was performed and the pylorus was ligated with 4 – 0 silk. Thereafter, the stomach was opened along the greater curvature, pinned over a Plexiglas platform and clamped with a Plexiglas cylinder to form an *ex vivo* gastric chamber. The mucosa was then bathed with 5 ml of an isotonic solution of mannitol (200 mM + 50 mM HCl) that was continuously renewed (0.5 ml min$^{-1}$). Changes in gastric blood flow (GBF) were determined by laser Doppler flowmetry (Oxford Array, Oxford Optronix, Oxford, U.K.) as described before (Calatayud *et al*., 1999). In brief, a pencil probe (Array 1 OOA 036, Oxford Optronix, Oxford, U.K.) was placed perpendicularly 5 mm above the glandular mucosa surface so as to measure GBF. After a 30-min stabilisation period, animals received an i.v. injection of endotoxin (40 $\mu$g kg$^{-1}$) followed 1 h later by indomethacin (20 mg kg$^{-1}$ i.v.). GBF and mean arterial pressure were recorded continuously and the results presented correspond with values of these parameters observed 60 min after endotoxin/saline administration (time point = 60 min) and 60 min after indomethacin injection (time point = 120 min). Results are expressed as percentages of basal values.

In some experiments, animals were pretreated 10 min before endotoxin with an NO-synthase (NOS) inhibitor (L-NAME, 1 or 5 mg kg$^{-1}$ i.v.). Other rats received increasing doses of capsaicin for three consecutive days (20, 30 and 50 mg kg$^{-1}$ s.c.) with the aim of depleting sensory neuropeptides from primary afferent neurons. All capsaicin injections were performed under brief halothane anaesthesia and the rats were used 12 days after the last dose of capsaicin. Finally, one group of animals was treated with a dose of indomethacin capable of reducing gastric prostaglandin synthesis by more than 90% (5 mg kg$^{-1}$ i.v.) 1 h before endotoxin.

All experimental protocols were performed according to the guidelines approved by the Ethical Committee for Experimental Research of the Faculty of Medicine of the University of Valencia.

### *RNA extraction and cDNA synthesis*

Total RNA from frozen gastric tissues was isolated with TriPure Isolation Reagent (Roche Diagnostics, Barcelona, Spain), following the manufacturer's protocol. RNA was finally resuspended in DEPC-treated $H_2O$ and stored at −80°C. Total RNA was treated with DNA-free (Ambion, Huntington, U.K.) to eliminate traces of contaminating genomic DNA. Resulting total RNA was quantified by UV spectrophotometry and its integrity evaluated by agarose gel electrophoresis.

Reverse transcription (RT) from 2 $\mu$g of total RNA was carried out with SuperScript RT RNase H- (Life Technologies, Barcelona, Spain), using 0.8 $\mu$g of oligodT16 (TIB Molbiol, Roche Diagnostics) and 40 U of RNase inhibitor (Roche Diagnostics) in a reaction volume of 20 $\mu$l. To check for absence of genomic DNA, controls without reverse transcriptase (−RT controls) for each sample were performed. To check for contamination in RT reagents, negative controls with water instead of RNA were also performed. Synthesized cDNA was stored at −20°C until used for real-time PCR.

### *Real time PCR*

Quantitative real-time PCR was carried out in a LightCycler instrument (Roche Diagnostics) by using the DNA Master SYBR Green I kit. Samples (1 $\mu$l of cDNA) were amplified by means of specific primers for each gene in a final volume of 10 $\mu$l, with 2 mM $MgCl_2$, 5% DMSO and 0.5 $\mu$M of primers for cyclophilin A and eNOS or 1 $\mu$M for the iNOS primer. 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 for denaturation and polymerase activation, a cycling programme (30 s of denaturation at 95°C, 30 s of primer annealing and 30 s of extension at 72°C) and the measurement of fluorescence at the end of each cycle. The specificity of reactions was tested through analysis of the melting curve and agarose gel electrophoresis. The experimental conditions are specified in more detail in Table 1.

To quantify input amounts of templates, a standard curve was constructed with serial dilutions of total RNA of a positive control (Table 1) for each analysed gene. In order to standardise the results, interpolated values for each sample were divided by values for the housekeeping gene cyclophilin A (Table 2).

### *Determination of NOS activity*

Rats were administered with endotoxin (40 $\mu$g kg$^{-1}$, i.p.) or saline (1 ml kg$^{-1}$, i.p.) and killed by cervical dislocation 1 or 4 h later. To summarise, the gastric corpus was cut into small pieces and quickly introduced into liquid nitrogen and stored at −80°C. NOS activity was measured as the rate of conversion of L-[U-$^{14}$C]-arginine to L-[U-$^{14}$C]-citrulline (Salter *et al*., 1991). In brief, the samples were homogenised (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-dithiothrei-

**Table 1** Primer sequences, reaction data and characteristics of specific PCR products for each analysed gene

| *Target gene* | *Primer sequences(5′–3′)* | *Tann (°C)* | *PCR cycles* | *$T_m$ (°C)* | *Size (bp)* | *Positive control* |
|---|---|---|---|---|---|---|
| CyPA | CGTCTGCTTCGAGCTGTTTG (s)<br>GTAAAATGCCCGCAAGTCAA (as) | 60 | 30 | 81.7 | 464 | Cerebellum |
| eNOS | GCCACAATCCTGGTGCGTCT (s)<br>CCACCAGGGCTGCCTTTTTC (as) | 58 | 40 | 86.6 | 188 | Aorta |
| iNOS | GCTACACTTCCAACGCAACA (s)<br>ACAATCCACAACTCGCTCCA (as) | 60 | 40 | 84.6 | 293 | Lung (LPS treated) |

Primers were designed according to the sequences with the GenBank accession no. NM_017101 (cyclophilin), U02534 (eNOS) and D12520 (iNOS).

**Table 2** Expression of the mRNA for cyclophilin A as analysed by real-time RT–PCR in gastric corpus from rats receiving the specified treatments

| *60 min* | | *240 min* | |
|---|---|---|---|
| *Saline* | *Endotoxin* | *Saline* | *Endotoxin* |
| 0.26±0.11 (5) | 0.25±0.07 (7) | 0.16±0.02 (7) | 0.18±0.04 (6) |

Rats were administered with saline (1 mg kg$^{-1}$, i.v.) or endotoxin (40 μg kg$^{-1}$, i.v.) 60 or 240 min beforehand. Results are expressed as μg of total RNA of positive control and correspond with mean±s.e.m. of (*n*) experiments ($P=0.73$, one-way ANOVA).

tol, 10 μg ml$^{-1}$ leupeptin, 10 μg ml$^{-1}$ soybean trypsin inhibitor and 2 μg ml$^{-1}$ aprotinin. Samples were then centrifuged (10,000 × *g*, 20 min, 4°C) and the supernatant (40 μl) was incubated in assay buffer (pH 7.4, 37°C, 20 min) containing 50 mM $KH_2PO_4$, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 mM L-valine, 1 mM L-citrulline, 0.02 mM L-arginine, 1 mM DL-dithiothreitol, 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 inhibitor $N^G$-nitro-L-arginine (L-NNA, 1 mM). Additionally, 1.5 mM EGTA, a calcium-chelating agent was used to differentiate between the $Ca^{2+}$-dependent and $Ca^{2+}$-independent isoforms of NOS. All activities are expressed as picomole of product generated per minute and per gram of tissue.

### *Drugs*

*E. coli* endotoxin (serotype 0111:B4), L-NAME and indomethacin were purchased from Sigma Chemical Co. Sodium pentobarbitone (Pentothal®, Abbot, Barcelona, Spain) and mannitol (Apiroserum Manitol 20%®, Pharmacia, Barcelona, Spain) were used as clinically available preparations. Capsaicin was obtained from Fluka Chemic.

Indomethacin was dissolved in 5% sodium bicarbonate and capsaicin was prepared with ethanol : Tween80 : saline (10 : 10 : 80, v:v:v). All other drugs were dissolved in saline immediately before use and administered in a volume of 1 ml kg$^{-1}$.

## Results

Indomethacin (20 mg kg$^{-1}$, s.c.) induced macroscopic gastric damage as analysed 3 h after administration. Pretreatment with endotoxin (40 μg kg$^{-1}$, i.p., −1 h) significantly protected the gastric mucosa against the damaging actions of indomethacin (Figure 1). Endotoxin did not induce any macroscopically appreciable change in the gastric mucosa of control animals. Intravenous administration of this dose of indomethacin to pentobarbital-anaesthetised rats induced a significant reduction in gastric blood flow that reached its maximum 1 h after drug injection. Pretreatment with endotoxin (40 μg kg$^{-1}$, i.v., −1 h) significantly prevented the fall in gastric blood flow induced by indomethacin (Figure 2).

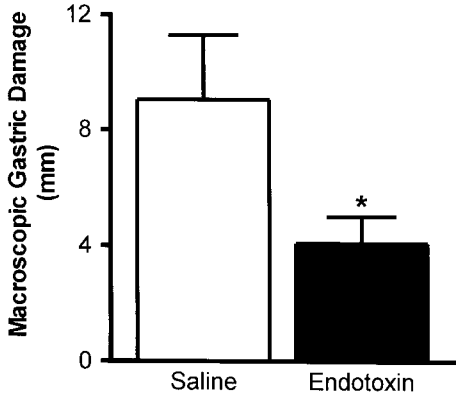


**Figure 1** Effect of pretreatment with endotoxin (40 μg kg$^{-1}$, i.p., −60 min) on the gastrolesive effect induced by indomethacin (20 mg kg$^{-1}$, s.c., 3 h). Results are expressed as mean±s.e.m. ($n=12$ per group) *$P<0.05$ vs saline (Student's *t*-test).

Pretreatment with a dose of L-NAME which did not modify basal blood flow (1 mg kg$^{-1}$) prevented the protective action of endotoxin against the noxious effect of indomethacin on gastric mucosal blood perfusion (Figure 3b). Administration of a higher dose of L-NAME (5 mg kg$^{-1}$, i.p.) also abolished the protective effects of endotoxin on the fall of gastric blood flow induced by indomethacin (56±4% reduction in saline-treated rats, 46±8% reduction in endotoxin-treated animals). However, these results were not conclusive as this dose of L-NAME itself induced a significant reduction in gastric blood flow in the first hour postinjection (38±2% reduction in saline-treated rats, 35±2% reduction in endotoxin-treated animals).

In capsaicin-treated animals, indomethacin induced a significant reduction of gastric blood flow similar to that observed in control rats. However, in these rats, administration of endotoxin itself induced a significant fall in resting blood flow that was not further reduced after indomethacin injection (Figure 3c). The effects of capsaicin pretreatment on resting mucosal blood flow could not be evaluated in the

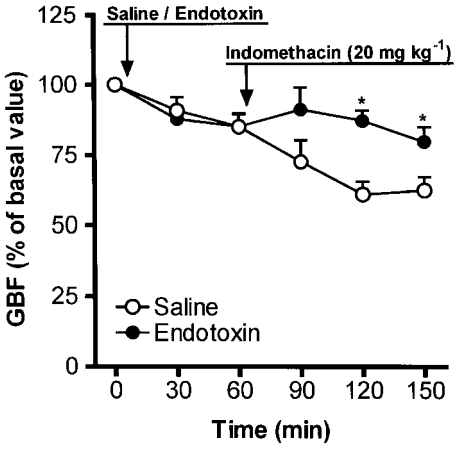


**Figure 2** Gastric blood flow (GBF) in rats receiving saline (1 ml kg$^{-1}$, i.v.) or endotoxin (40 $\mu$g kg$^{-1}$, i.v.). Indomethacin (20 mg kg$^{-1}$, i.v.) was administered 60 min after endotoxin/saline injection. GBF is expressed as a percentage of the respective basal value. Results correspond with mean $\pm$ s.e.m. ($n=4$ per group). *$P<0.05$ vs same time point in saline-treated rats (Student's $t$-test).

present investigation because of the relative, rather than absolute, values of blood flow determined by laser Doppler flowmetry.

Rats receiving a lower dose of indomethacin (5 mg kg$^{-1}$, i.v.) showed a reduced gastric blood flow 2 h later (40 $\pm$ 5% reduction in saline-treated rats). Administration of endotoxin 1 h after this dose of indomethacin partially prevented this reduction (25 $\pm$ 9% reduction, $P<0.05$) and attenuated the action of the ulterior treatment with 20 mg kg$^{-1}$ of indomethacin (46 $\pm$ 3% reduction in saline-treated rats, 34 $\pm$ 4% reduction in endotoxin-treated animals).

Endotoxin (40 $\mu$g kg$^{-1}$, i.v.) did not significantly modify systemic blood pressure in pentobarbital-anaesthetised rats throughout the experimental period. However, a significant reduction in blood pressure was observed 60 min after administration of this dose of endotoxin to capsaicin-treated animals. Pretreatment with 1 or 5 mg kg$^{-1}$ of L-NAME did not affect blood pressure in either control or endotoxin-treated rats (Table 3).

Analysis by real time RT – PCR of samples from the gastric corpus revealed no differences in the expression of the mRNA for eNOS and iNOS between saline- and endotoxin-treated animals 1 h after treatment. However, a 3.8-fold increase in iNOS-mRNA and a 61% reduction in eNOS-mRNA were observed in the gastric corpus 4 h after endotoxin administration (Figure 4). Evaluation of total NOS activity by the rate of conversion of L-arginine to L-citrulline in gastric corpus homogenates showed no differences between samples from animals treated with endotoxin and those receiving saline 1 or 4 h beforehand. Equivalent results were observed when $Ca^{2+}$-dependent NOS activity was analysed. $Ca^{2+}$-independent NOS activity was almost non-apparent in control as well as in endotoxin treated rats at all the time points analysed (Figure 5).

## Discussion

The gastrointestinal system is highly sensitive to stress, and moderate levels of somatic strain such as low endotoxemia

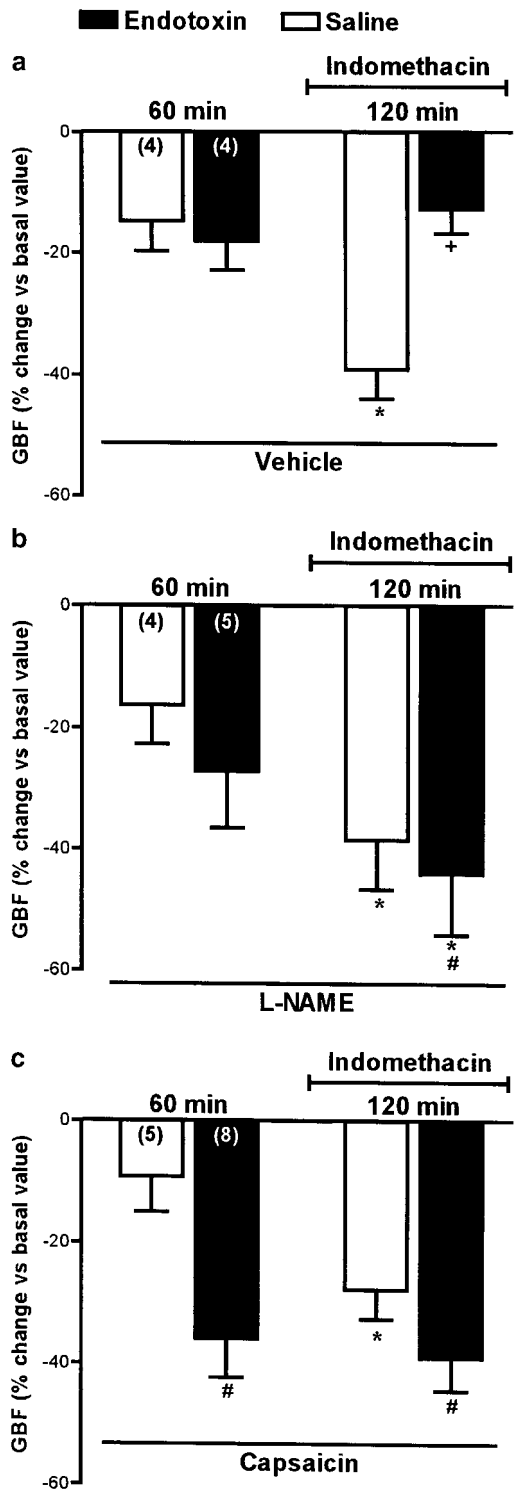


**Figure 3** Gastric blood flow (GBF) 60 min after saline (1 ml kg$^{-1}$, i.v.) or endotoxin (40 $\mu$g kg$^{-1}$, i.v.) administration in rats pretreated with (a) vehicle, (b) L-NAME (1 mg kg$^{-1}$, i.v., $-10$ min) or (c) capsaicin (100 mg kg$^{-1}$, s.c., $-12$ days) (time point = 60 min). Indomethacin (20 mg kg$^{-1}$, i.v.) was administered in all cases 60 min after endotoxin/saline injection and results correspond with GBF values observed 60 min later (120 min after endotoxin/saline administration, time point = 120 min). GBF is expressed as a percentage of the respective basal value. Results correspond with mean $\pm$ s.e.m. of ($n$) experiments. *$P<0.05$ vs respective value before indomethacin treatment, $^{+}P<0.05$ vs same time point in vehicle + saline-treated rats and $^{\#}P<0.05$ vs same time point in vehicle + endotoxin-treated rats (one-way ANOVA + Newman – Keuls test).

**Table 3** Values of mean arterial blood pressure (mm Hg) in pentobarbital anaesthetised animals receiving the specified treatments

| | *Basal* | | *60 min* | | *120 min* | |
|---|---|---|---|---|---|---|
| | *A* | *B* | *A: Saline* | *B:Endotoxin* | *A:Sal+Indo* | *B:Endo+Indo* |
| Vehicle | 127±9 | 124±11 | 114±9 | 101±11 | 110±14 | 96±19 |
| L-NAME | | | | | | |
| 1 mg kg$^{-1}$ | 132±3 | 124±3 | 122±7 | 125±7 | 97±13 | 92±17 |
| 5 mg kg$^{-1}$ | 130±5 | 134±4 | 150±4 | 129±12 | 135±5 | 139±8 |
| Capsaicin | 136±5 | 122±4 | 131±4 | 86±4* | 123±6 | 94±8* |

Mean arterial blood pressure was measured in basal conditions and 60 min after saline (1 ml kg$^{-1}$, i.v.) or endotoxin (40 $\mu$g kg$^{-1}$, i.v.) administration in animals pretreated with vehicle, L-NAME (1 or 5 mg kg$^{-1}$, i.v., −10 min) or capsaicin (100 mg kg$^{-1}$, s.c., −12 days) (time point = 60 min). Indomethacin (20 mg kg$^{-1}$, i.v.) was administered in all cases 60 min after endotoxin/saline injection and results correspond with values observed 60 min later (120 min after endotoxin/saline administration, time point = 120 min). Results are expressed as mean±s.e.m. *$P<0.05$ vs respective basal value (one-way ANOVA + Newman–Keuls test).

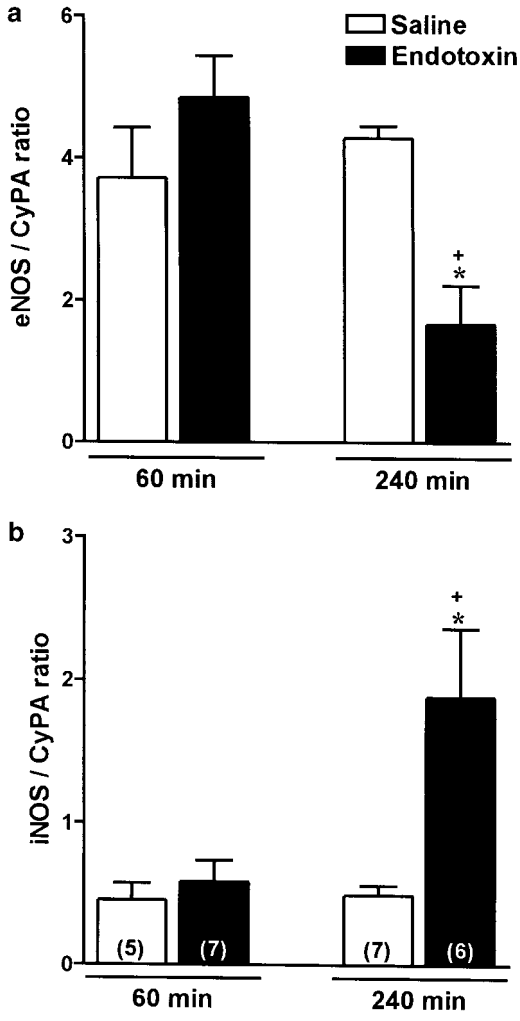


**Figure 4** Expression of the mRNA for eNOS (a) and iNOS (b) as analysed by real-time RT – PCR in gastric corpus from rats administered with saline (1 ml kg$^{-1}$, i.v.) or endotoxin (40 $\mu$g kg$^{-1}$, i.v.) 60 or 240 min beforehand. Results correspond with mean± s.e.m. of ($n$) experiments. *$P<0.05$ vs same time point in saline-treated rats and $^{+}P<0.05$ vs value obtained 60 min after treatment with endotoxin (one-way ANOVA + Newman – Keuls test).

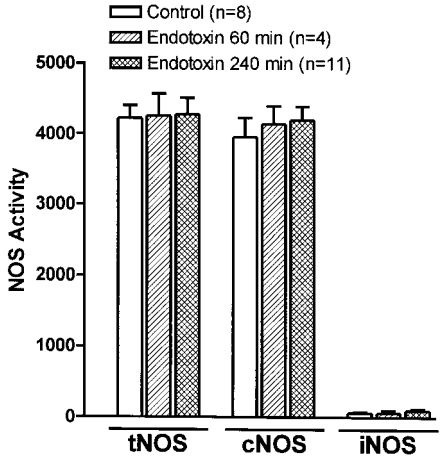


**Figure 5** NOS activity as evaluated by the rate of conversion of L-arginine to L-citrulline in gastric corpus homogenates from rats administered with saline (1 ml kg$^{-1}$, i.v.) or endotoxin (40 $\mu$g kg$^{-1}$, i.v.) 60 or 240 min beforehand. Results correspond with picomole of product generated per minute and per gram of tissue (mean±s.e.m. of $n$ experiments.). The activity of the $Ca^{2+}$-dependent and $Ca^{2+}$-independent NOS isoforms was differentiated by addition of a calcium-chelating agent (EGTA 1.5 mM). (tNOS: $P=0.98$, cNOS: $P=0.74$, iNOS: $P=0.55$, one-way ANOVA).

greatly modify its function. Together with secretory (Barrachina *et al*., 1995c) and motor changes all along the gastrointestinal tract (Martinez-Cuesta *et al*., 1997; Calatayud *et al*., 2001b; 2002), the gastric mucosa responds to the adverse environment and improves its ability to withstand posterior aggressions. Small doses of endotoxin have previously been reported to increase gastric mucosal resistance against the deleterious effects of agents such as severe stress, ethanol or NSAIDs (Tsuji *et al*., 1993, this paper; Barrachina *et al*., 1995a).

Reduction of gastric mucosal blood flow seems to be a leading factor in the ability of most ulcerogen agents to cause gastric lesions (Tepperman & Jacobson, 1994). In the present study, pretreatment with endotoxin prevented the reduction in gastric mucosal blood perfusion induced by a gastrolesive dose of indomethacin. Thus, endotoxin probably protects the mucosa against the ulcerative action of indomethacin by maintaining an adequate blood supply. This would guarantee tissue oxygenation and nutrient delivery, thereby maintaining the gastric mucosal barrier and preventing the accumulation of damaging substances such as acid or reactive oxygen species. The effects on mucosal blood flow may add to the inhibition of gastric acid secretion previously reported with these doses

of endotoxin (Esplugues *et al*., 1996) and, thereby, strengthen the ability of the gastric mucosa to withstand aggression.

Previous studies indicate that endotoxin protects the mucosa by enhancing the function of physiological regulators of mucosal integrity such as capsaicin-sensitive afferent neurons and NO, but not that of prostaglandins (Barrachina *et al*., 1995a). In the present study, we observed that administration of indomethacin ($5\,mg\,kg^{-1}$) 1 h before endotoxin did not prevent the protective effect of endotoxin on gastric microcirculation. This dose of indomethacin, which inhibits gastric prostaglandin synthesis by 90% but is devoid of other toxic actions of NSAIDs unrelated to COX inhibition and which take place at higher drug concentrations (Somasundaram *et al*., 1997; Calatayud *et al*., 2001c), also caused mucosal hypoperfusion. In this protocol, administration of endotoxin stopped the fall in gastric blood flow initiated 1 h beforehand by this dose of indomethacin. Thus, endotoxin protects gastric microcirculation in animals in which prostaglandin synthesis has previously been inhibited, thereby confirming, as expected, that the protective effect of endotoxin in the gastric mucosa is independent of endogenous prostaglandin synthesis. Moreover, these results indicate that the effect of endotoxin takes place immediately after its injection and points to a nervous reflex as being responsible for the endotoxin action. In capsaicin-treated rats, endotoxin lacked of any protective effect on the gastric microcirculation, which suggests the implication of these neurons in the prevention by endotoxin of the indomethacin action. However, endotoxin induced itself a significant fall in gastric blood flow when administered to capsaicin-treated rats. Thus, in contrast to what occurs in physiological circumstances (Holzer, 1998), resting mucosal blood flow depends on capsaicin-sensitive afferent neurons in conditions of low endotoxemia. These results corroborate the relevance of these sensory neurons as an emergency system which allows the organism to adapt to slight alterations of the physiology and enhances the body ability to withstand ulterior aggressions (Holzer, 1998). The role of this emergency nervous system is not limited to the gastrointestinal tract but seems to be responsible for systemic homeostasis, since the present results show a reduction in systemic arterial blood pressure in rats pretreated with capsaicin and receiving this dose of endotoxin, which does not have any hypotensive effect in control animals.

Inhibition of NO synthesis with L-NAME ($1\,mg\,kg^{-1}$) abolished the protective action of endotoxin against the detrimental effect of indomethacin on mucosal blood flow, which indicates that this effect of endotoxin is mediated by the endogenous synthesis of NO. A higher dose of the NOS inhibitor ($5\,mg\,kg^{-1}$) significantly reduced resting mucosal blood flow in control animals thus confirming the well reported role of NO as a physiological regulator of mucosal blood perfusion (Whittle, 1993). Thus, endotoxin, through the NO synthesis maintains an adequate blood supply to the gastric mucosa and counteracts the detrimental effects of NSAIDs. A similar effect had been obtained with a gastroprotective dose of the nitric oxide donor nitroglycerin (Barrachina *et al*., 1995b; Calatayud *et al*., 1999). However, neither endotoxin nor nitroglycerin modified resting blood flow in control animals. It seems therefore that mucosal microvasculature is an important target for the gastroprotective effect of NO against NSAIDs and that endogenous and exogenous NO counteracts some action of indomethacin that results in a reduced blood supply to the mucosa rather than induce an unspecific vasodilatation.

Synthesis of NO may be carried out by three NOS isoforms: two constitutive $Ca^{2+}$-dependent enzymes (endothelial and neuronal NOS) and the inducible $Ca^{2+}$-independent iNOS (Esplugues, 2002). The latter one is not usually present in the organism but is synthesised in response to inflammatory or immunologic stimuli. Cytokines and lipopolysaccharides from Gram-negative bacteria are considered to be the most common stimulus of iNOS induction. However, we did not observe any increase in iNOS mRNA expression nor detect iNOS activity in the gastric mucosa 1 h after endotoxin administration, when the protective effect on mucosal blood flow takes place. Increased iNOS gene expression can be observed 4 h after endotoxin treatment but still no iNOS activity is registered in gastric corpus. In accordance with this, the NO-dependent gastroprotection elicited by this dose of endotoxin against ethanol was not modified by dexamethasone, which prevents iNOS expression (Barrachina *et al*., 1995a). These results suggest that the origin of the NO mediating the gastroprotection observed in endotoxemia is because of an increased activity of the constitutive NOSs, most probably eNOS. However, no changes in the activity of the constitutive NOS, as measured by the rate of conversion of L-arginine to L-citrulline, were observed in tissue homogenates from endotoxin-treated rats. Thus, it is possible that factors increasing the activity of NOS that are only present *in vivo* were responsible for the enhanced NO synthesis after endotoxin administration and, for this reason, no changes would be observed in tissue homogenates. Biosynthesis of NO by constitutive enzymes is regulated by calcium entry/mobilisation induced by receptor-dependent mechanisms or physical stimuli (Esplugues, 2002). Endotoxin may stimulate constitutive NOSs by increasing the release of vasoactive neurotransmitters such as acetylcholine or CGRP. In fact, both substances have been shown to mediate other gastrointestinal effects of endotoxin (Calatayud *et al*., 2001b; 2002). Furthermore, recent studies demonstrate that application of physiological levels of shear stress significantly modifies the endothelial synthesis of vasoactive mediators *in vitro*, reflecting the endothelial function when *in vivo* (Topper *et al*., 1996; McAdam *et al*., 1999; Wiest *et al*., 1999).

Our results contrast with previous observations suggesting that endotoxin protects against stress or ethanol-induced damage by preserving mucosal blood flow through the expression of iNOS and the consequent increase in NO release (Yu *et al*., 1997; Konturek *et al*., 1998). iNOS has traditionally been associated with pathological conditions and its deleterious effect on mucosal integrity has been widely proven (Calatayud *et al*., 2001a). These recent studies thus challenge the initial view of low levels of NO synthesised by constitutive NOS being protective while exaggerated NO levels after iNOS induction leading irremediably to cytotoxicity. Considered as a whole, these observations and the present results suggest that, in order to preserve homeostasis, the organism responds to challenge in multiple ways and that nitric oxide is a key factor in this emergency system. There is no doubt about the protective role of NO in physiological conditions and also, according to the present results, in challenging circumstances where NO synthesis depends on constitutive NOSs. However, when the mucosa is threatened and the organism synthesises iNOS, the role of NO becomes multiple and the final,

protective or deleterious, effect will probably depend on the nature of the insult, the environment involved and the interaction with other mediators. The different responses elicited by endotoxin in our study and in the above-mentioned reports may well be due to differences with respect to the dose of endotoxin used or the time course of the experiments. Indeed, we observed increased iNOS mRNA in the gastric corpus 4 h after endotoxin injection, which suggests that this isoenzyme may play the leading role at later time points. Together with the induction of iNOS mRNA, there was a reduction in the amount of eNOS mRNA in gastric corpus. Although we have not analysed the mechanisms leading to these changes, bibliographic data suggest that endotoxin reduces eNOS mRNA by shortening its half-life (Lu *et al*., 1996; Arriero *et al*., 2002). Thus, endotoxin seems to affect the L-arginine/NO system in multiple ways, which may include transcriptional, post-transcriptional and post-translational mechanisms, and hence modifies gastric function. Furthermore, the role of NO and its enzymatic source changes with time.

In summary, the present study indicates that low endotoxemia protects the gastric mucosa against the damaging effects of NSAIDs probably by maintaining an adequate blood supply to the mucosa. This protective effect requires the integrity of the capsaicin-sensitive afferent neurons and the NO synthesis, which supports the proposed interaction between endogenous NO and sensory neuropeptides (Whittle *et al*., 1990; Tepperman & Whittle, 1992) and reinforces its role on the maintenance of mucosal integrity.

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