Br. J. Pharmacol. (1993), **110**, 1189–1195

The induction of nitric oxide synthase and intestinal vascular permeability by endotoxin in the rat

[1]Nigel K. Boughton-Smith, Steven M. Evans, Ferenc Laszlo, [2]Brendan J.R. Whittle & Salvador Moncada

Department of Pharmacology, Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS

1 The effect of endotoxin (*E. coli* lipopolysaccharide) on the induction of nitric oxide synthase (NOS) and the changes in vascular permeability in the colon and jejunum over a 5 h period have been investigated in the rat.

2 Under resting conditions, a calcium-dependent constitutive NOS, determined by the conversion of radiolabelled L-arginine to citrulline, was detected in homogenates of both colonic and jejunal tissue.

3 Administration of endotoxin (3 mg kg^{-1}, i.v.) led, after a 2 h lag period, to the appearance of calcium-independent NOS activity in the colon and jejunum *ex vivo*, characteristic of the inducible NOS enzyme.

4 Administration of endotoxin led to an increase in colonic and jejunal vascular permeability after a lag period of 3 h, determined by the leakage of radiolabelled albumin.

5 Pretreatment with dexamethasone (1 mg kg^{-1} s.c., 2 h prior to challenge) inhibited both the induction of NOS and the vascular leakage induced by endotoxin.

6 Administration of the NO synthase inhibitor N^{G}-monomethyl-L-arginine (12.5–50 mg kg^{-1}, s.c.) 3 h after endotoxin injection, dose-dependently reduced the subsequent increase in vascular permeability in jejunum and colon, an effect reversed by L-arginine (300 mg kg^{-1}, s.c.).

7 These findings suggest that induction of NOS is associated with the vascular injury induced by endotoxin in the rat colon and jejunum.

Keywords: Nitric oxide; inducible nitric oxide synthase; endotoxin; vascular permeability; intestinal inflammation; corticosteriods; NO synthase inhibitor; N^{G}-monomethyl-L-arginine

Introduction

The formation of the endothelium-derived vasodilator mediator, nitric oxide (NO) from L-arginine, by the calcium-dependent constitutive enzyme NO synthase (NOS) is involved in the regulation of the cardiovascular system (Palmer *et al.*, 1987; 1988; Palmer & Moncada, 1989; Moncada *et al.*, 1991). NO thus plays a physiological role in the regulation of gastro-intestinal blood flow (Pique *et al.*, 1989; 1992a,b; Walder *et al.*, 1990). NO is also involved in the regulation of gastric mucosal integrity, interacting with other local protective mediators (Whittle *et al.*, 1990). In addition, NO is involved in the modulation of intestinal vascular integrity under physiological conditions (Kubes & Granger, 1992) as well as pathological situations following acute endotoxin challenge (Hutcheson *et al.*, 1990). Thus, the inhibitor of NOS, N^{G}-monomethyl-L-arginine (L-NMMA), markedly enhances the vascular permeability and haemorrhage produced after 15 min in the jejunum by high doses of endotoxin (Hutcheson *et al.*, 1990), effects reversed by the nitrosothiol NO donor, S-nitroso-*N*-acetyl penicillamine (Boughton-Smith *et al.*, 1990; 1992b).

Endotoxin and some cytokines can induce a calcium-independent NOS in vascular tissue (Busse & Mulsch, 1990; Radomski *et al.*, 1990; Szabo *et al.*, 1993). The induction of NOS, leading to the excessive production of NO has also been implicated in the endothelial cell damage *in vitro* brought about by prolonged incubation with endotoxin and cytokines (Palmer *et al.*, 1992). Experimental and clinical studies suggest that excessive NO production has an important pathological role in the hypotension, hyporesponsiveness to vasoconstrictors and the cardiovascular collapse associated with septic shock (Kilbourn *et al.*, 1990; Thiemermann & Vane, 1990; Fleming *et al.*, 1991; Nava *et al.*, 1991; Petros *et al.*, 1991; Wright *et al.*, 1992; Szabo *et al.*, 1993). The anti-inflammatory corticosteroid, dexamethasone inhibits the induction of NOS by endotoxin and cytokines both *in vitro* and *in vivo* in vascular tissue (Radomski *et al.*, 1990; Knowles *et al.*, 1990), an effect that may contribute to beneficial actions of corticosteroid pretreatment in experimental septic shock (Wright *et al.*, 1992).

In the present study, the relationship between the induction of NOS and the increase in vascular permeability produced in the rat jejunum and the colon over a 5 h period following administration of endotoxin has been determined in the rat. The effects of dexamethasone on the induction of NOS and vascular leakage produced by endotoxin have also been investigated. In addition, the actions of L-NMMA, administered at a time of detectable expression of the inducible NOS, on the intestinal vascular injury has been evaluated.

A preliminary account of this work has been presented to the British Pharmacological Society (Boughton-Smith *et al.*, 1992a).

Methods

Nitric oxide synthase activity

Lipopolysaccharide (LPS) from *E. coli* (3 mg kg^{-1}) or the vehicle, isotonic saline (2 ml kg^{-1}) was administered via the tail vein, to male Wistar rats (225–275 g) under halothane anaesthesia. This dose of LPS was selected from previous dose-response studies as near-maximal for the induction of NOS (Knowles *et al.*, 1990).

Nitric oxide synthase activity (NOS) in the jejunal and colonic tissues was measured as the conversion of L-[^{14}C]-arginine monohydrochloride to [^{14}C]-citrulline, based on the

[1] Present address: Department of Pharmacology, Fisons Pharmaceuticals, Loughborough, Leicestershire LE11 0RH.
[2]Author for correspondence.

method described by Knowles et al. (1990). Intestinal tissue was taken at the various time intervals over 5 h, and homogenized (20 s, Ultra-Turrax; 5 mm blade) in buffer (250 mg ml^{-1}) containing HEPES (40 mM), sucrose (32 mM), DL-dithiothreitol (1 mM), leupeptin (10 $\mu g\ ml^{-1}$), soybean trypsin inhibitor (10 $\mu g\ ml^{-1}$) and aprotonin (2 $\mu g\ ml^{-1}$).

Following centrifugation (10 000 g, 20 min 4°C), an aliquot of the supernatant (40 µl) was added to a tube containing 100 µl of pre-warmed (37°C) incubation buffer containing (final concentration) potassium phosphate (50 mM; pH 7.4); L-valine (6 mM); NADPH (100 µM); $MgCl_2$ (1 mM) and $CaCl_2$ (200 µM), L-arginine (20 µM) and L-[^{14}C]-arginine monohydrochloride (0.271 µCi, 11.8 GBq $nmol^{-1}$) and incubated for 10 min at 37°C. The reaction was terminated by the addition of 500 µl of a mixture of H_2O:Dowex-AG50W (1:1 v/v, 200–400, 8% cross-linked, Na^+ form), prepared by washing H^+ form of the resin with 1 M NaOH, four times and then washing with purified water until the pH was less than 7. The resin-incubate mix was dispersed and diluted by the addition of 860 µl of purified water. The resin was allowed to settle (30 min) and 975 µl of supernatant taken for scintillation counting (2 ml Pico-Fluor; Beckman LS400).

Characterization of nitric oxide synthase

Product formation that was inhibited by *in vitro* incubation with L-NMMA (300 µM) was taken as an index of NOS activity and calculated from the total of added substrate, as the formation of citrulline, nmol $min^{-1}\ g^{-1}$ of tissue.

The activity of NOS in the intestinal tissue was further characterized *in vitro* by incubation with EGTA (1 mM) to determine the dependence of the enzymic activity on calcium. The calcium-dependent activity under control conditions was taken as constitutive NOS, while that not inhibited by EGTA incubation following endotoxin challenge was taken as a calcium-independent inducible isoform of NOS (Salter et al., 1991).

Plasma leakage

Intestinal vascular permeability was determined as the leakage into the jejunal and colonic tissue of [^{125}I]-labelled human serum albumin ([^{125}I]-HSA) administered intravenously (0.2 ml; 0.5 µCi, 1.85 GBq) immediately after either LPS or isotonic saline. At various times (1 to 5 h) after LPS or saline administration, segments of jejunal and colonic tissue were ligated and removed. The intestinal tissues were rapidly washed, blotted dry and weighed. Blood (0.5 ml) from the abdominal aorta was collected into tubes containing trisodium citriate (0.318% final concentration) and plasma prepared by centrifugation (10 000 g × 10 min). The [^{125}I]-HSA content in segments of whole tissue and in aliquots of plasma (100 µl), was determined in a gamma spectrometer (Nuclear Enterprises NE1600). The total content of plasma in the intestinal tissues was expressed as µl g^{-1} of tissue.

Intravascular volume

Changes in intravascular volume in the intestinal tissue was determined in an additional group of rats by administering [^{125}I]-HSA (0.5 µCi) intravenously via the tail vein, at each time point, 2 min before tissue removal. The tissue and plasma content of radiolabel was determined and intravascular volume expressed as µl g^{-1} tissue. This value was subtracted from that obtained in the plasma leakage studies to obtain a measure of the intestinal plasma albumin leakage.

Effect of dexamethasone or L-NMMA

Groups of rats were pretreated with dexamethasone (1 mg kg^{-1}, s.c.) 2 h before intravenous administration of saline or LPS, in a dose derived from previous dose-response studies on the inhibition of the induction of NOS by endotoxin in the rat (Knowles et al., 1990). The NOS activity and vascular leakage of radiolabelled albumin were determined 4 h after LPS administration.

In a further study, L-NMMA (12.5–50 mg kg^{-1}, s.c.) was administered 3 h after LPS, at a time when induction of NO synthase was detectable. Vascular leakage of radiolabelled albumin in jejunum or colon was determined 1 h later (i.e. 4 h after LPS). In an additional group, L-arginine (300 mg kg^{-1}, s.c.) was administered 15 min prior to L-NMMA (50 mg kg^{-1}). In control experiments in the absence of LPS, the effects of L-NMMA (50 mg kg^{-1}, s.c.) on vascular leakage in jejunum and colon were determined 1 h after administration.

Drugs and materials

N^G-monomethyl-L-arginine (L-NMMA) was synthesized in the Department of Medicinal Chemistry, Wellcome Research Laboratories. *E. coli* lipopolysaccharide (0111:B4), and L-arginine hydrochloride were from Sigma Chemical Company (Poole, Dorset), L-[U-^{14}C] arginine monohydrochloride and [^{125}I]-labelled human serum albumin were from Amersham International (U.K.). Dexamethasone was supplied as the injectable form (Decadron, Merck Sharp & Dohme Herts). All other reagents were from the Sigma Chemical Company.

Statistical analysis

The data are expressed as the mean ± s.e.mean of (n) rats per experimental group. Statistical comparisons were made

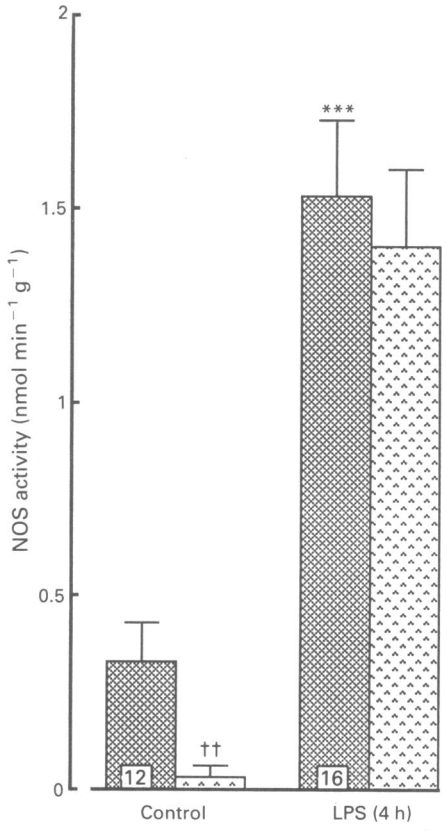

Figure 1 Increase in nitric oxide synthase (NOS) activity in rat jejunal tissue 4 h following challenge with LPS (*E. coli*, 3 mg kg^{-1}, i.v.). NOS activity, determined as the conversion of radiolabelled L-arginine to citrulline (nmol $min^{-1}\ g^{-1}$ tissue) that is abolished *in vitro* by N^G-monomethyl-L-arginine (L-NMMA, 300 µM), in supernatents of jejunal homogenates incubated in the absence (cross hatched columns) and presence (speckled columns) of EGTA (1 mM), is expressed as the mean values ± s.e.mean, of 12 and 16 experiments. A significant increase in NOS activity is given as ***$P < 0.001$, and significant inhibition of NOS activity by incubation with EGTA is shown by ††$P < 0.01$.

by Student's t test for paired and unpaired data as appropriate.

Results

Induction of nitric oxide synthase

Basal NOS activity, that was abolished by incubation *in vitro* with L-NMMA (300 μM), was detected in the supernatants of homogenates of segments of jejunum or colon, being 0.28 ± 0.08 ($n = 20$) and 1.18 ± 0.07 ($n = 12$) nmol min^{-1} g^{-1} of tissue, respectively. This activity in the supernatants from the jejunum (Figure 1) and colon (Figure 2) was abolished by incubation with EGTA (1 mM).

In jejunal tissue, elevated NOS activity was detected 4 h after LPS administration, and that was not significantly inhibited by incubation with EGTA, as shown in Figure 1. In the study on the time-course of NOS induction, administration of LPS (3 mg kg^{-1}, i.v.) had no effect on NOS activity in the colonic tissue when determined 1 and 2 h after challenge (Figure 2). However, 3 to 5 h after LPS administration, NOS activity was significantly ($P < 0.01$) increased in a time-dependent manner (Figure 2). This NOS activity, observed after 3 and 4 h, was only partially inhibited by incubation *in vitro* with EGTA (1 mM), the low level of EGTA-sensitive activity remaining corresponding to the level of calcium-dependent NOS activity seen under resting conditions (Figure 2). At 5 h after LPS administration, incubation with EGTA did not significantly inhibit the elevated colonic NOS activity (Figure 2).

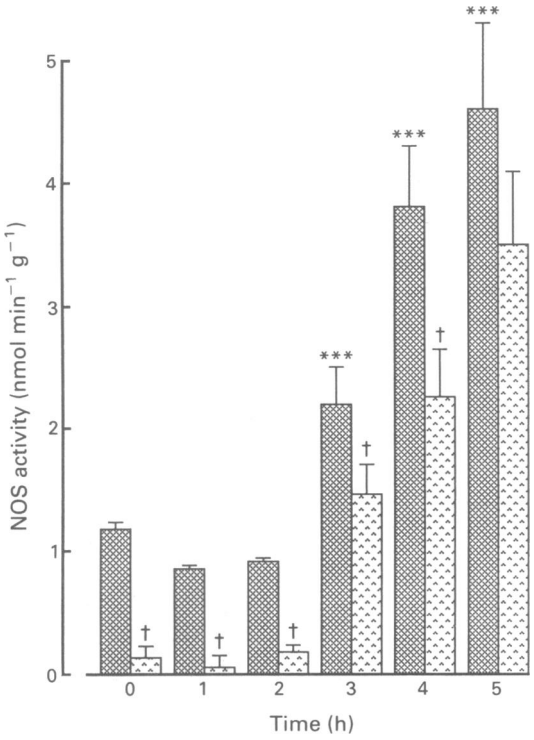

Figure 2 Induction of nitric oxide synthase (NOS) in rat colonic tissue over a 5 h period following challenge with LPS (*E. coli*, 3 mg kg^{-1}, i.v.). NOS activity, determined as the conversion of radiolabelled L-arginine to citrulline (nmol min^{-1} g^{-1} tissue), that is abolished *in vitro* by N^G-monomethyl-L-argine (L-NMMA, 300 μM), in supernatant of colonic homogenates incubated in the absence (hatched columns) and presence (speckled columns) of EGTA (1 mM), is expressed as the mean values ± s.e.mean, of 6–8 experiments for each time point. A significant increase in total NOS activity is given as $***P < 0.001$, and significant inhibition of this activity by incubation with EGTA is shown by $\dagger P < 0.01$.

Blood volume and plasma leakage

In control rats receiving bolus intravenous injection of isotonic saline (2 ml kg^{-1}), the intravascular blood volume in the jejunum and colon, determined by the tissue level of radiolabelled human serum albumin injected 2 min prior to tissue removal, did not significantly change over a 5 h period (Table 1). Likewise, intravascular blood volume in the jejunum and colon did not significantly change during the 5 h observation period following administration of LPS (3 mg

Table 1 Intravascular blood volume in rat jejunum and colon over a 5 h period following administration of *E. coli* lipopolysaccharide (LPS)

	Intestinal blood volume (μl g^{-1} tissue)			
	Control		*LPS*	
Time (h)	*Jejunum*	*Colon*	*Jejunum*	*Colon*
0	45 ± 2	66 ± 10	–	–
1	63 ± 9	87 ± 10	56 ± 1	59 ± 16
3	41 ± 8	55 ± 10	47 ± 10	60 ± 11
4	47 ± 6	52 ± 8	39 ± 3	64 ± 5
5	47 ± 2	64 ± 10	53 ± 5	39 ± 9

Intravascular blood volume in the intestinal segments was determined by injection of radiolabelled albumin 2 min prior to the removal of the tissue over a 5 h period in control rats and following administration of LPS (3 mg kg^{-1}, i.v.). Results, shown as blood volume (μl g^{-1} tissue), are mean ± s.e.mean of 4 experiments for each time point. There was no significant difference between control or LPS treatment in either the jejunum or colon at any time.

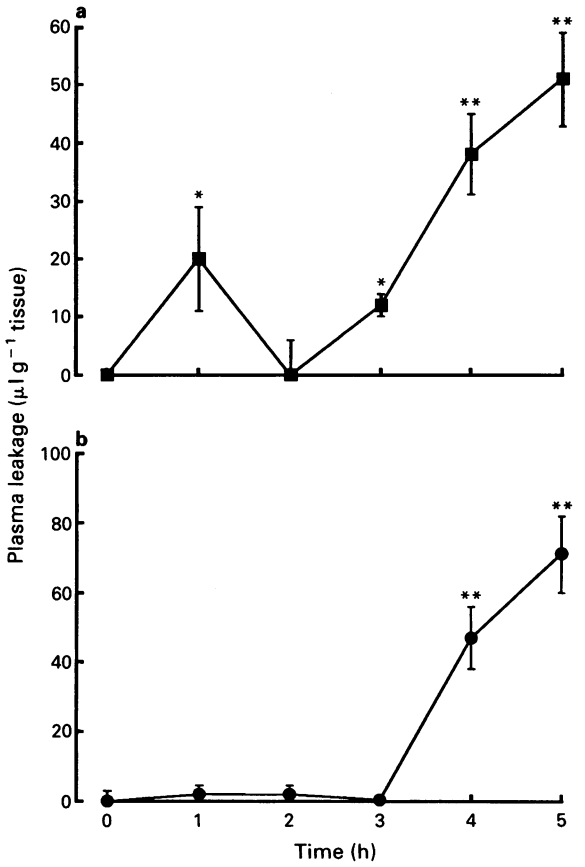

Figure 3 Extravasation of plasma into the rat jejunum (a) and the colon (b) over a 5 h period following challenge with *E. coli* lipopolysaccharide (LPS, 3 mg kg^{-1}, i.v.). Results, expressed as the leakage of radiolabelled albumin (μl g^{-1} tissue), are shown as the mean values ± s.e.mean of 6–8 experiments at each time point, where significant difference from control is given as $*P < 0.05$, $**P < 0.01$.

kg^{-1}, i.v.), and furthermore, were not significantly different from those under control conditions (Table 1).

Following administration of LPS (3 mg kg^{-1}, i.v.), there was no change in the plasma leakage into the colon, 1, 2 or 3 h after administration, which remained similar to the low resting levels. However, 4 and 5 h after LPS administration, there was a substantial ($P<0.01$) increase in the plasma leakage (Figure 3). There was also a significant increase in plasma leakage in the jejunum, 3, 4 and 5 h after LPS administration, with a transient initial increase also being observed 1 h after challenge which returned to the resting value after 2 h (Figure 3).

Effect of dexamethasone on nitric oxide synthase activity and plasma leakage

In control rats, pretreatment with dexamethasone (1 mg kg^{-1}, s.c.) 2 h prior to administration of isotonic saline (2 ml kg^{-1}, i.v.) had no significant effect on the calcium-dependent NOS activity in colonic or in jejunal tissue, determined 4 h later (Figures 4 and 5). However, the increase in NOS activity induced by LPS determined 4 h after challenge, was significantly ($P<0.001$) suppressed by pretreatment with dexamethasone in both colonic (Figure 4) and jejunal tissue (Figure 5).

Pretreatment with dexamethasone (1 mg kg^{-1}, s.c.), 2 h prior to LPS challenge abolished the increase in plasma leakage induced by LPS in both the colon and jejunum, determined 4 h after challenge (Figures 4 and 5). Dexamethasone administration had no effect on the plasma leakage in control rats 4 h following saline injection (Figures 4 and 5).

Effect of L-NMMA on plasma leakage

Administration of L-NMMA (12.5–50 mg kg^{-1}, s.c.), 3 h after LPS injection, caused a dose-dependent reduction in radiolabelled albumin leakage in both jejunum and colon, determined 1 h later, as shown in Figure 6. Pretreatment with L-arginine (300 mg kg^{-1}, s.c.) 15 min prior to L-NMMA (50 mg kg^{-1}) abolished ($P<0.001$) this inhibition of albumin leakage in the jejunum and colon (82 ± 8 and 75 ± 7 μl plasma g^{-1} tissue, respectively; $n=8$ for each, not significantly different from LPS alone). L-Arginine (300 mg kg^{-1}) did not itself affect the increases in albumin leakage induced by LPS over this time period ($n=8$ for each; $P>0.05$).

By contrast, under control conditions in the absence of LPS challenge, L-NMMA (50 mg kg^{-1}) did not significantly

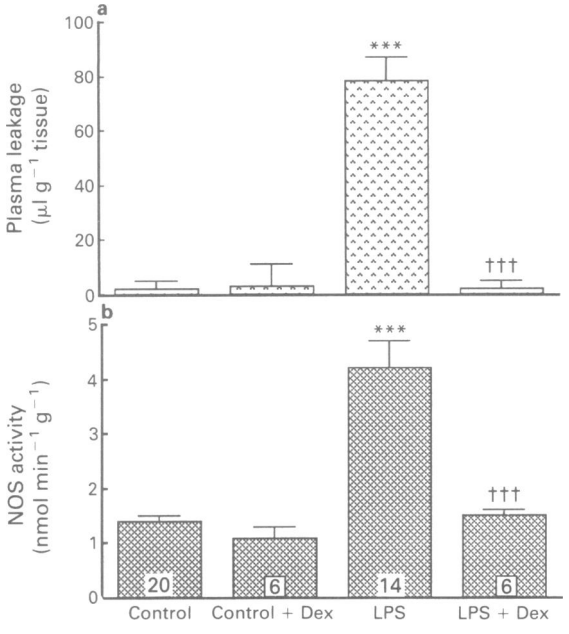

Figure 4 Extravasation of plasma (a) and induction of nitric oxide synthase (NOS) in the rat colon (b) following administration of *E. coli* lipopolysaccharide (LPS, 3 mg kg^{-1}, i.v.) and the actions of dexamethasone (Dex) pretreatment (1 mg kg^{-1}, s.c., 2 h before LPS challenge). Results, shown as NOS activity (nmol min^{-1} g^{-1} tissue) and leakage of radiolabelled albumin (μl g^{-1} tissue) 4 h following saline (2 ml kg^{-1}, i.v.) or LPS administration with and without dexamethasone pretreatment, are the mean values ± s.e.mean of the number of experiments shown in each column, where significant difference from control values is given as ***$P<0.001$ and inhibition of LPS-induced actions as †††$P<0.001$.

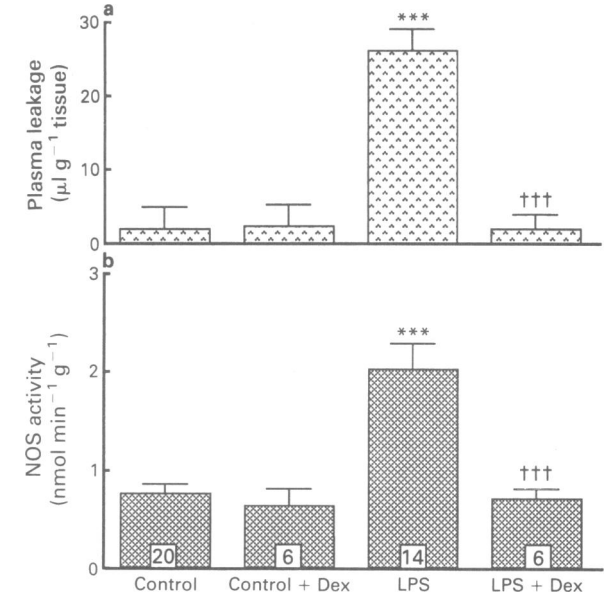

Figure 5 Extravasation of plasma (a) and induction of nitric oxide synthase (NOS) in the rat jejunum (b) following administration of *E. coli* lipopolysaccharide (LPS, 3 mg kg^{-1}, i.v.) and the actions of dexamethasone (Dex) pretreatment (1 mg kg^{-1}, s.c., 2 h before LPS challenge). Results, shown as NOS activity (nmol min^{-1} g^{-1} tissue) and leakage of radiolabelled albumin (μl g^{-1} tissue) 4 h following saline (2 ml kg^{-1}, i.v.) or LPS administration with and without dexamethasone pretreatment, are the mean values ± s.e.mean of the number of experiments shown in each column, where significant difference from control is given as ***$P<0.001$ and inhibition of LPS-induced actions as †††$P<0.001$.

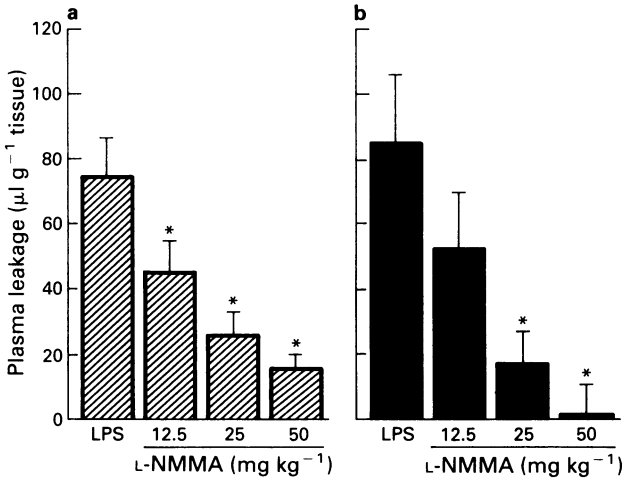

Figure 6 Effects of N^G-monomethyl-L-arginine (L-NMMA, 12.5–50 mg kg^{-1}, s.c.), or isotonic saline (0.4 ml) administered 3 h after challenge with *E. coli* lipopolysaccharide (LPS, 3 mg kg^{-1}, i.v.) on the increase in leakage of radiolabelled albumin (plasma leakage μl g^{-1} tissue) observed 1 h later (i.e. 4 h after LPS challenge) in the rat colon (a) and jejunum (b). Results are shown as mean values ± s.e.mean of 6–8 experiments in each group, where significant inhibition from LPS group is given as *$P<0.05$.

induce jejunal or colonic albumin leakage determined after 1 h (17 ± 12 and 3 ± 7 μl plasma g^{-1} tissue compared with saline alone, respectively; $n = 6$, $P > 0.05$).

Discussion

In the present study, endotoxin produced *in vivo*, after a lag period of 3 h, a time-dependent increase in vascular leakage of plasma albumin in the rat colon. The change in colonic vascular permeability was preceded by the induction of a calcium-independent NOS activity. Likewise, in the rat jejunum an increase in extravasation of albumin 4 and 5 h after challenge with endotoxin was observed at a time of the induction of the calcium-independent NOS. This temporal relationship is therefore compatible with the concept that excessive NO production by the inducible NOS is involved in the intestinal vascular injury produced by endotoxin after several hours.

The increase in vascular permeability observed in the rat colon several hours after LPS contrasts with our previous study on the acute effects of endotoxin on the colon (Hutcheson *et al.*, 1990). In that study, a high dose of LPS (50 mg kg^{-1}) produced an increase in plasma leakage in the stomach, duodenum, jejunum and ileum after 15 min, whereas the colon was not affected. These regional differences in sensitivity of the intestinal vasculature after acute or more prolonged exposure to endotoxin suggest the involvement of different mediators and mechanisms underlying the microvascular damage following such periods of challenge. Indeed, the intestinal damage induced by acute administration of high doses of endotoxin involves the release of a number of vasoactive mediators including platelet activating factor (PAF) and thromboxane A_2 (Wallace *et al.*, 1987; Whittle *et al.*, 1987; Boughton-Smith *et al.*, 1989). The acute release of these mediators may also account for the transient initial rise in jejunal vascular permeability, seen in the current study, 1 h after endotoxin challenge, whereas no such acute change was observed in colon.

The present study identifies the presence of a calcium-dependent constitutive NOS in the rat jejunal tissue, and confirms its presence in colonic tissue (Salter *et al.*, 1991). Although the presence of an inducible NOS enzyme could not be detected in the colon in a previous study using a spectrophotometric assay, the technique used critically depended on a haemoglobin-free tissue extract being obtained from perfused tissue (Salter *et al.*, 1991). In the current study using the conversion of radiolabelled L-arginine, the increase by endotoxin of NOS activity was more marked in colonic tissue than in the jejunum. Although this activity was predominantly calcium-independent, EGTA did cause a partial reduction in the elevated levels of NOS in colonic tissue at the earlier phases of induction after LPS challenge. This could reflect the contribution of the calcium-dependent constitutive enzyme to the total levels of NOS under these conditions, or to the induction or stimulation of a calcium-dependent isoform, as found previously in the ileum (Salter *et al.*, 1991). In the jejunum, EGTA abolished NOS activity under control conditions yet had no significant effect on the increase in the NOS activity observed 4 h after LPS, which therefore could indicate a reduction in constitutive NOS activity as a consequence of NOS induction, as seen in other tissues (Nishida *et al.*, 1992; de Belder *et al.*, 1993). It will be of interest to determine the cytosolic and particulate distribution of these NOS activities (Forstermann *et al.*, 1991; Mitchell *et al.*, 1991) in further studies and to define the full biochemical nature and immunohistochemical specificity of these NOS isoforms.

The induction by endotoxin and cytokines of a calcium-independent NOS *in vitro* is prevented by dexamethasone and by inhibitors of DNA transcription and translation, indicating that the effects of the corticosteroid are a consequence of inhibition of *de novo* synthesis of NOS (Radomski *et al.*, 1990; Knowles *et al.*, 1990; Di Rosa *et al.*, 1990). Furthermore, the induction of the calcium-independent NOS in the rat ileum by endotoxin administration *in vivo* was also prevented by dexamethasone pretreatment (Salter *et al.*, 1991) as confirmed in the present study on jejunal and colonic tissue. Since the anti-inflammatory corticosteroids can inhibit cytokine synthesis (Snyder & Unanue, 1982; Waage & Baake, 1988; Kern *et al.*, 1988), the actions of dexamethasone seen in the present study could also reflect inhibition of endotoxin-stimulated synthesis of the cytokines involved in the process of NOS induction under these *in vivo* conditions.

The prevention of NOS induction in both colon and jejunum by dexamethasone could be responsible for the concurrent abolition of the vascular permeability changes provoked by LPS in these tissues. Dexamethasone can also inhibit eicosanoid or PAF synthesis, through suppression of phospholipase A_2 activity as a consequence of stimulating lipocortin production (Blackwell *et al.*, 1980; Hirata *et al.*, 1980). However, pretreatment with high doses of dexamethasone caused only a modest reduction in intestinal damage and in the jejunal formation of PAF or thromboxane B_2 following acute endotoxin challenge (Boughton-Smith *et al.*, 1989). Such actions of dexamethasone may therefore not make a major contribution to the effects on vascular permeability seen in the current study.

Adhesion of neutrophils to vascular endothelium has been implicated in intestinal vascular injury (Hernandez *et al.*, 1987; Kubes *et al.*, 1991). Studies on the cat mesentery have demonstrated that acute inhibition of NO biosynthesis augments neutrophil adherence to vascular endothelium and provokes acute changes in intestinal vascular permeability, effects reversed by the NO donor, nitroprusside (Kubes *et al.*, 1991; Kubes & Granger, 1992). Such findings support the role of endogenous constitutive NO, probably located in the endothelium, in the regulation of the integrity of the microvasculature (Hutcheson *et al.*, 1990). The cellular sources of the constitutive and inducible forms of NOS observed in the current study on intestinal tissue are not yet known but the increase following endotoxin administration could reflect induction of the enzyme in vascular and epithelial tissue, or in resident or invading inflammatory cells. How neutrophil adhesion to the vascular endothelium would be affected by the high levels of NO produced by the induced NOS is not clear, but vascular damage under these conditions may be independent of such cellular interactions. The actions of corticosteroids on cell adhesion to the endothelium with the present experimental protocol also warrants attention as a possible contributory mechanism in the prevention of vascular injury by dexamethasone.

The process by which excessive NO production by an inducible NOS could produce increases in vascular permeability may partly involve an increase in blood flow. While an increase in blood flow alone would not itself result in increased vascular permeability, it would augment the actions of other pro-inflammatory mediators released by endotoxin, such as PAF or cytokines, which have a direct injurious action on the microvascular endothelium. Studies in rat skin have demonstrated that intradermal injection of endotoxin induced a time-dependent increase in blood flow that was inhibited by local administration of NOS inhibitors and by pretreatment with a corticosteroid (Warren *et al.*, 1992). Inhibitors of NO synthase can also attenuate the changes in vascular permeability and oedema formation induced by pro-inflammatory agents in rat skin, a process that may involve reduction in local blood flow (Hughes *et al.*, 1990; Ialenti *et al.*, 1992). In the present study, no substantial change in the intravascular volume of the jejunum or colon could be detected following endotoxin challenge. However, determination of intestinal blood flow will be needed to clarify the contribution of microcirculatory blood flow to the overall changes in plasma leakage associated with NO induction.

NO, or a subsequent product may also have a direct

injurious action on endothelial cells to produce the observed changes in intestinal vascular permeability. Induction of NOS in activated macrophages accounts for their cytotoxic action against bacterial and protozoal microorganisms and against tumour cells (Hibbs *et al.*, 1988; Marletta *et al.*, 1988; Drapier *et al.*, 1988; Stuehr *et al.*, 1989; Granger *et al.*, 1990; Adams *et al.*, 1990; Liew *et al.*, 1990). Furthermore, *in vitro* findings suggest that endothelial cell cytotoxicity produced by LPS and cytokines is dependent on the induction of NOS activity, and induced NO synthesis is implicated in damage to adenocarcinoma cells (Palmer *et al.*, 1992; O'Connor & Moncada, 1991). NO can interact with the superoxide anion to produce a reactive peroxynitrite radical which can subsequently lead to the production of the highly reactive hydroxyl radical (Beckman *et al.*, 1990). The hydroxyl radical can produce cell damage and cytotoxicity in a variety of cells, including endothelial cells, and has been implicated in intestinal vascular damage produced by ischemia-reperfusion in the intestine (Parks & Granger, 1983; Hernandez *et al.*, 1987).

The involvement of induced NO synthesis in the vascular injury provoked by LPS is supported by the finding that administration of the NO synthase inhibitor, L-NMMA at a time when elevation of NOS activity was just detectable, prevented the subsequent increase in plasma leakage in the jejunum and colon, an action abolished by prior administration of L-arginine. The doses of L-NMMA used did not themselves lead to any change in vascular permeability in the rat jejunum or colon over a 1 h period under control conditions in the absence of endotoxin, which contrasts with studies in the cat intestine with the more potent inhibitor, N^G-nitro-L-arginine methyl ester (Kubes & Granger, 1992). However, it is possible that these doses of L-NMMA used in the present study, which presumably act by inhibiting the activity of the induced NOS, are insufficient to abolish the constitutive NOS activity in the rat intestine, perhaps required for such permeability changes under control conditions and indeed they did not affect resting intestinal or colonic blood flow in control rats (Pique *et al.*, 1992b). Studies with selective inhibitors of the inducible NOS isoform will clarify the role of NO in such endotoxin-induced vascular damage.

The current findings thus suggest that the plasma extravasation, an index of inflammation and microvascular injury in the colonic and jejunal mucosa that follows the prolonged exposure to endotoxin *in vivo*, is temporally associated with the induction of intestinal tissue NOS. Induction of a calcium-independent NOS has also been demonstrated recently in colonic tissue from a rat model of inflammatory bowel disease, while the inducible NOS can be detected in inflamed colonic mucosa for ulcerative colitis patients (Boughton-Smith *et al.*, 1992c,d). Furthermore, elevated levels of luminal nitrate have been observed in a model of ileitis, and enhanced levels of the NO co-product, citrulline has been observed in colonic tissue from colitic patients (Miller *et al.*, 1993; Middleton *et al.*, 1993). Thus, the ability of dexamethasone to prevent both the induction of NOS and the concurrent changes in intestinal vascular permeability, as seen in the present study, may contribute in part to the therapeutic benefit of such corticosteroids in the treatment of inflammatory conditions of the intestine. Moreover, selective inhibitors of the inducible isoforms of NOS may prove of clinical value in such intestinal diseases.

F.L. was supported by a fellowship from the Royal Society.

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(Received June 7, 1993
Accepted July 14, 1993)