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Dependence of endotoxin-induced vascular hyporeactivity on extracellular L-arginine

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1 The dependence on extracellular L-arginine of vascular hyporeactivity induced by bacterial lipopolysaccharide (LPS) was studied *in vivo* in rats infused with LPS and *in vitro* in endothelium-denuded rat thoracic aortic rings exposed to LPS.

2 Infusion of LPS during 50 min at a dose of 10 mg kg^{-1} h^{-1} produced a significant impairment of the pressor effect of noradrenaline, while in tissues collected 60 min after the start of LPS infusion, no significant alteration in either plasma arginine concentration or aortic arginine content was found compared to saline-infused controls (where plasma arginine was 78.5 ± 7 μM and aortic arginine 394 ± 124 nmol g^{-1} tissue).

3 Incubation of isolated, endothelium-denuded aortic rings with LPS (10 μg ml^{-1}) in the absence of L-arginine for 4 h at 37°C produced a 6 fold ($P<0.01$) rightward shift in the noradrenaline concentration-effect curve compared to polymyxin B (1 μg ml^{-1}, a LPS neutralizing agent) and reduced by 15% the maximum observed tension.

4 The presence of L-arginine (100 μM) during the incubation with LPS and throughout the following contraction experiments caused a 15 fold ($P<0.01$) increase in the EC_{50} of noradrenaline and greater depression (45%) of the maximum observed tension compared to polymyxin B-treated controls. Responses in control, non LPS-treated rings were unaffected by the presence of L-arginine.

5 The addition of L-arginine to rings incubated with LPS in the absence of L-arginine and maximally precontracted with noradrenaline (10 μM) induced a dose-dependent relaxation. The EC_{50} of L-arginine was 8.0 ± 0.3 μM.

6 The reactivity of LPS-treated rings to noradrenaline both in the absence and presence of L-arginine was restored to control levels by N^G-nitro-L-arginine methyl ester (L-NAME, 300 μM), an inhibitor of NO production and by methylene blue (3 μM), an inhibitor of guanylate cyclase.

7 Incubation of isolated aortae in the absence of L-arginine did not significantly decrease the tissue arginine content, whether LPS (10 μg ml^{-1}) was present or not. Similarly, the presence of L-arginine (100 μM) in the incubation medium did not modify the tissue arginine content.

8 These results show that the LPS-induced impairment of vasoconstriction elicited by noradrenaline is dependent on extracellular L-arginine, although the tissue arginine content is not depleted after LPS pretreatment, and that circulating L-arginine is sufficient to activate maximally the vascular L-arginine/NO pathway in endotoxaemic rats.

Keywords: Extracellular L-arginine; nitric oxide; NO synthase; lipopolysaccharide; septic shock

Introduction

Loss of vascular responsiveness to several vasoconstrictor agents is associated with high mortality in septic shock (Groenenveld & Thijs, 1986). Administration of *Echerichia coli* lipopolysaccharide (LPS) to rats *in vivo* (Wakabayashi *et al.*, 1987) produces hyporesponsiveness to the pressor effects of catecholamines and other agonists (Schaller *et al.*, 1985) and hyporeactivity in arteries studied *ex vivo* (Julou-Schaeffer *et al.*, 1990; Schneider *et al.*, 1992). Hyporeactivity to catecholamines can also be induced *in vitro* in vessels incubated with LPS (McKenna, 1988; Fleming *et al.*, 1990). Vascular hyporeactivity produced by LPS in these different conditions is reversed by inhibitors of nitric oxide (NO) production from L-arginine (Julou-Schaeffer *et al.*, 1990; Fleming *et al.*, 1990; Gray *et al.*, 1991), suggesting that NO, which stimulates cytosolic guanylate cyclase, is a major mediator of the hyporeactivity. This hypothesis has been further supported by the finding that LPS induces an NO synthase activity in the rat medial- adventitial layer (Knowles *et al.*, 1990; Rees *et al.*, 1990) and subsequent guanosine 3′:5′cyclic-monophosphate (cyclic GMP) accumulation in vascular smooth muscle cells (Fleming *et al.*, 1991a). Unlike the constitutive endothelial NO synthase, the induced NO synthase activity was calcium/calmodulin independent in smooth muscle cells.

L-Arginine, the substrate of NO synthase, is present in endothelial cells, where NO production does not require the addition of exogenous L-arginine. In addition to a system capable of transporting L-arginine from the extracellular space (Mann *et al.*, 1989; 1990), endothelial cells have the ability to synthesize L-arginine (Hecker *et al.*, 1990). In these cells, NO production by the constitutive NO synthase is therefore not limited by the supply of extracellular L-arginine, except after prolonged incubation without L-arginine (Gold *et al.*, 1989). In contrast, relatively little is known about regulation of L-arginine supply in smooth muscle cells. It has been reported that prolonged incubation with L-arginine decreases contraction elicited by phenylephrine in rat aortic rings (Schini & Vanhoutte, 1991). In addition, *ex vivo* and *in vitro* aortic hyporeactivity induced by LPS is enhanced by the addition of L-arginine to endothelium-denuded aortae in the organ bath (Julou-Schaeffer *et al.*, 1990; 1991). Both studies suggest that after NO synthase induction, smooth muscle

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cells may have a higher dependence on extracellular L-arginine for NO production than endothelial cells containing the constitutive enzyme. However, infusion of L-arginine in LPS-treated rats did not enhance *in vivo* hyporeactivity to intravenously injected noradrenaline (Gray *et al.*, 1991).

The aim of the present work was to investigate the effect of LPS on circulating and tissue levels of L-arginine and the relationship between extracellular L-arginine supply and hyporesponsiveness to noradrenaline (NA) in LPS-exposed aortic rings. Since small amounts of LPS contained in physiological solutions may be sufficient to induce NO synthase in the rat aorta (Rees *et al.*, 1990), some control experiments were run in the presence of polymyxin B in order to neutralize any small contaminant concentrations of LPS (Flynn *et al.*, 1987; Stokes *et al.*, 1989). Activation of the L-arginine/NO pathway and guanylate cyclase was assessed with the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) and the guanylate cyclase inhibitor, methylene blue.

Methods

In vivo *experiments*

Male Wistar rats (12 weeks, 250–300 g) were anaesthetized by intraperitoneal injection of sodium pentobarbitone (60 mg kg^{-1}, Sanofi). Arterial blood pressure was monitored with a pressure transducer (Gould Statham P23 ID) connected via a cannula containing heparinized saline (150 iu ml^{-1}, NaCl 0.9%) to the right carotid artery. The blood pressure was recorded with a pen recorder (Beckmann R511A). The right femoral vein and the right jugular vein were cannulated for NA administration and for LPS infusion (10 mg kg^{-1} h^{-1}) respectively. The body temperature was maintained at 37 ± 0.5°C.

In order to assess the effect of LPS on NA-induced increases of arterial blood pressure, NA (100, 300 and 1000 ng kg^{-1}) pressor responses were measured after a 30 min stabilization period and after 50 min of infusion of LPS (10 mg kg^{-1} h^{-1}) or saline (NaCl 0.9%). In preliminary experiments it was seen that neither blood pressure nor LPS-induced hyporeactivity to NA changed after 50 min infusion. The blood pressure was allowed to return to the basal (pre-NA) value between each dose of NA and before collection of blood samples (2 ml). The return to basal blood pressure took approximately 2 to 3 min and the assessment of reactivity to the three doses of NA was achieved within 10 min.

Blood samples were collected into heparinized tubes 60 min after the start of LPS infusion and immediately centrifuged. At the same time, the animals were exsanguinated and aortae were removed. Trichloroacetic acid (TCA) was added to the plasma and to the aortae at a final concentration of 6% and the samples were frozen until determination of arginine levels.

In vitro *contraction studies*

Male Wistar rats (12 weeks, 250–300 g) were killed by cervical dislocation. Thoracic aortae were removed, cleared of adherent tissue and the endothelium was removed by gently rubbing with blunt forceps. They were used for measurements of arginine content or cut into rings 2 mm in length for contraction studies. Both aortae and rings were incubated for 4 h at 37°C in an incubator gassed with 5% CO_2/95% air in L-arginine-free Eagle's minimal essential medium (MEM) with LPS (10 μg ml^{-1}) or polymyxin B (1 μg ml^{-1}), with or without L-arginine (100 μM). After incubation with LPS or polymyxin B, rings were mounted under 2 g tension in organ baths containing physiological salt solution (PSS) of the following composition (in mM): NaCl 118.4, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 1.3, KH_2PO_4 1.2, $NaHCO_3$ 25.0, glucose 11.7 at 37°C bubbled with 95% O_2/5% CO_2 (pH 7.4). L-Arginine (100 μM) was added to the PSS of some rings as indicated. All experiments were carried out in the presence of indomethacin (10 μM), cocaine (30 μM) and corticosterone (40 μM). Indomethacin, cocaine and corticosterone were present to prevent the interference of cyclo-oxygenase products, neuronal and non neuronal NA uptake, respectively. Tension was measured with an isometric force transducer.

After an equilibration period of 60 min, during which time the PSS was changed each 15 min, the basal tension was re-adjusted to 2 g. The absence of functional endothelium was verified by the lack of effect of acetylcholine (1 μM) on rings precontracted with NA (1 μM). After a second washing period of 60 min, during which tension returned to baseline levels, a cumulative dose-response curve to NA (10^{-9}–10^{-5} M) was obtained in some experiments. In other experiments, the rings were maximally contracted with a single dose of NA (10 μM) and activation of the L-arginine/NO pathway was assessed with L-NAME and methylene blue, or a cumulative dose-response curve to L-arginine (1–300 μM) was obtained. Note that all contraction studies were made 6 h after initial contact with LPS, and that tissue arginine content in isolated control and LPS-treated aortae were determined similarly after 6 h (see below).

Determination of arginine levels

Aortae taken from rats infused intravenously with LPS (10 mg kg^{-1} h^{-1}) or saline were homogenized in TCA (6%) with a Potter glass/glass homogenizer, sonicated (Ultrason-Annemasse, Type 75 TS France) and centrifuged at 10,000 *g* for 15 min. Blood samples were also centrifuged at 10,000 *g* for 15 min. The supernatants were extracted with water-saturated diethyl ether, the aqueous layer was lyophilized and reconstituted in 4 ml of 0.1 M citrate buffer, pH 5.3. The samples were then chromatographed on 0.7 × 0.6 cm Dowex-Na^+ columns (Dowex 50 W, 200–400 × 8, H^+). Each column was washed with 20 ml of citrate buffer in order to remove citrulline and ornithine, and arginine was eluted with 4 ml of 0.2 N NaOH (Gold *et al.*, 1989). In controls, >95% of known arginine concentrations were recovered by this method. The arginine containing solutions were lyophilized and reconstituted in 1 or 2 ml distilled H_2O for aortic tissue and plasma, respectively. Arginine was assayed by the Sakaguchi reaction (formation of a coloured complex with α-naphthol) modified by the addition of thymine to stabilize the coloured complex, according to Van Pilsum *et al.* (1956). A 0.5 ml aliquot of a 1:1 (vol/vol) solution of thymine (20 mg ml^{-1} in 10% NaOH) and α-naphthol (0.04% in ethanol) was added to the sample, followed by the addition of 0.2 ml NaOCl (1% solution) with immediate mixing. Exactly 1 min later, 0.2 ml of a $Na_2S_2O_3$ solution (0.03 g ml^{-1} in H_2O) was added. The reaction was monitored at 515 nm (Beckman model 25 Spectrophotometer). Standard titration curves for L-arginine were linear from 0 to 100 μM, with a calculated (Miller & Miller, 1988) threshold level of detection of 4 μM and a sensitivity of 3 μM. Both the weight of individual aortic samples (approximately 90 mg) and appropriate dilutions of plasma samples were chosen to allow determinations of absolute arginine levels to be made in a range close to the midpoint of the control titration curve.

Arginine content of isolated aortae was similarly determined. Aortae were either used immediately after dissection or treated as described for contraction studies, i.e. arginine content was determined after 6 h incubation (see above).

Materials

Noradrenaline bitartrate (Sigma Chemical Co.) was stored as a 10 mM stock solution in buffer containing Na_2SO_3 (7.9 mM) and HCl (34 mM), and diluted as required. Acetylcholine chloride (ACh, 10 mM, Sigma Chemical Co.) and cocaine (30 mM) were stored frozen. Methylene blue (Sigma Chemical Co.) was made up as a 10 mM stock solution, and diluted as required. All other substances were freshly prepared before

each experiment. Polymyxin B sulphate (Sigma Chemical Co.), L-arginine hydrochloride (Calbiochem), and N^G-nitro-L-arginine methyl ester (L-NAME, Sigma Chemical Co.) were dissolved in PSS or in Eagle's minimum essential medium (MEM, Eurobio). Indomethacin (Sigma Chemical Co.) was dissolved in 0.5% Na_2CO_3 solution. Lipopolysaccharide (LPS, *E. coli* 055: B5, Difco) was dissolved in MEM for *in vitro* studies and in saline (NaCl 0.9%) for *in vivo* infusion. Corticosterone (Sigma Chemical Co.) was dissolved in 70% ethanol.

Statistical analysis

NA pressor responses *in vivo* were compared by two-way analysis of variance (ANOVA). Where ANOVA showed a significant difference, the results were further analysed with an *a posteriori* Newmann-Keuls test. Aortic and plasma contents of L-arginine *in vivo* were compared by Student's *t* test. For *in vitro* experiments, EC_{50} (concentration which gives 50% of the maximal response) values were calculated by logit-log regression. EC_{50} and maximal contractile responses were compared by Student's *t* tests for unpaired data. The change in tension induced by L-arginine in NA-contracted aortic rings and the arginine aortic content were analyzed with a one-way ANOVA.

Results

In vivo *experiments*

The mean arterial blood pressure in anaesthetized rats was not significantly changed after a 50 min infusion of LPS (10 mg kg^{-1} h^{-1}) compared to saline-infused controls (Table 1). Infusion of LPS caused a significant decrease in NA pressor responses at all three NA doses used compared to control rats infused with saline (Table 1). Neither the plasma nor the aortic arginine content were altered in a significant way by infusion of LPS (Table 1).

Contraction studies in vitro

Effect of the presence of L-arginine throughout the experiment Concentration-response curves to NA are illustrated in Figure 1 and the corresponding EC_{50} and maximal contraction values are given in Table 2. Controls were run in the presence and in the absence of polymyxin B (1 μg ml^{-1}). Polymyxin B was used to neutralize any contaminant LPS and did not modify significantly the concentration-response curves to NA (Table 2). The presence of L-arginine (100 μM) throughout the experiment, i.e. during the initial 4 h incubation period and the following contraction experiment, did not produce any significant alteration in concentration-response curves to NA obtained in control rings (both with or without polymyxin B).

In the absence of L-arginine, treatment with LPS induced a significant ($P<0.01$) rightward shift of the dose-response curve to NA (EC_{50} was increased 6 fold, compared to polymyxin B, $P<0.01$). However, in this case, the slight decrease in maximal contractile response seen after LPS treatment was not significant (Table 2). When L-arginine was present throughout the experiment, LPS induced a greater rightward shift of the concentration-response curve to NA: EC_{50} was increased 15 fold, $P<0.01$, $n=5$ compared with control rings with or without polymyxin B and 2.5 fold ($P<0.01$) compared with LPS-treated rings without arginine. LPS also induced a significant ($P<0.01$) decrease in the maximal contractile response when L-arginine was present throughout (Figure 1).

Effect of the addition of L-arginine, L-NAME and methylene blue to NA-contracted aortae After completion of NA concentration-effect curves, L-arginine (1 mM) was added to maximally contracted rings (Figure 2). Addition of L-arginine to control rings (incubated with or without polymyxin B) maximally contracted with NA (10 μM) had no effect on

Table 1 Effects of lipopolysaccharide (LPS) *in vivo* on blood pressure, responsiveness to noradrenaline (NA) and arginine content

		Control	*LPS*	
BP (mmHg)	Before infusion	115 ± 8	109 ± 4	
	After infusion	112 ± 3	102 ± 3	
Increase in BP (mmHg)	NA 100 ng kg^{-1}	17 ± 2	8 ± 1	*
	NA 300 ng kg^{-1}	35 ± 4	15 ± 2	**
	NA 1000 ng kg	58 ± 5	33 ± 4	**
Arginine content	Plasma (μM)	78.5 ± 7	81.9 ± 6	NS
	Aortic (μmol kg^{-1})	394 ± 124	279 ± 98	NS

Mean arterial blood pressure (BP, mmHg), responsiveness to NA (increase in BP, mmHg) and circulating and aortic arginine contents in control and LPS-treated rats. Values are the mean ± s.e.mean of 5 experiments.
*$P<0.05$, **$P<0.001$, NS: not significant $P>0.05$ compared to respective controls.

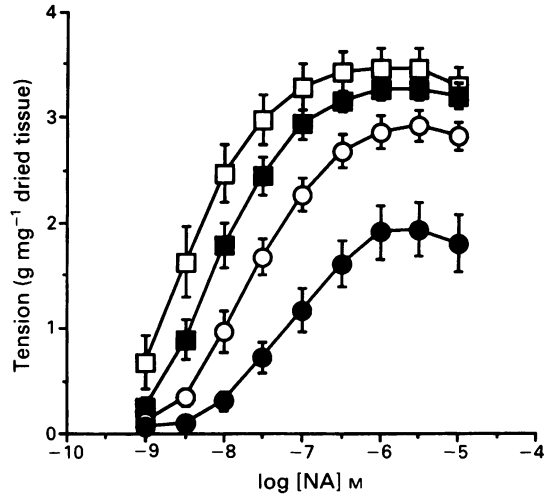

Figure 1 Influence of L-arginine on vascular hyporeactivity to noradrenaline (NA) induced by lipopolysaccharide (LPS). Concentration-response curves elicited by NA in endothelium-denuded rat aortic rings incubated for 6 h without (open symbols) or with (closed symbols) L-arginine (100 μM) and exposed to either polymyxin B (1 μg ml^{-1}, □ ■) or LPS (10 μg ml^{-1}, ○ ●).

Table 2 Effect of L-arginine on aortic contractile responsiveness to noradrenaline

Treatment	*EC_{50}* (μM)		*Maximal contraction* (g mg^{-1} dried tissue)	
None	5.3 ± 1.2		3.0 ± 0.1	
None + L-arginine	12.5 ± 2.3	NS	3.1 ± 0.4	NS
Polymyxin	4.3 ± 1.3		3.3 ± 0.2	
Polymyxin + L-arginine	9.9 ± 2.3	NS	3.2 ± 0.1	NS
LPS	25.0 ± 6.2		2.8 ± 0.1	
LPS + L-arginine	65.9 ± 10.8	**	1.8 ± 0.3	**

EC_{50} values and the maximal tension were determined in aortic rings without endothelium treated with or without polymyxin B or LPS, in the absence or presence of L-arginine. Values are the mean ± s.e.mean of 5 experiments.
**$P<0.01$; NS $P>0.05$ compared to respective values in the absence of L-arginine.

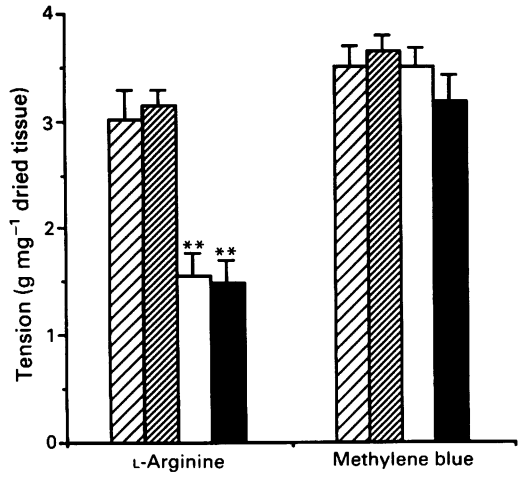

Figure 2 Effect of the consecutive addition of L-arginine (1 mM) and methylene blue (3 μM) to aortic rings exposed to a maximally active noradrenaline (NA) concentration (10 μM), after completion of the concentration-response curves for rings incubated with polymyxin B (1 μg ml^{-1}, ▨ ▨) or with LPS (10 μg ml^{-1}, □ ■), in the absence (▨ □) or in the presence (▨ ■) of L-arginine (100 μM). The effect of L-arginine was measured 5 min after its addition but 30 min after the addition of methylene blue. Values are the mean of 5–7 experiments; vertical lines show s.e.mean. $^{**}P<0.001$ compared to polymyxin B.

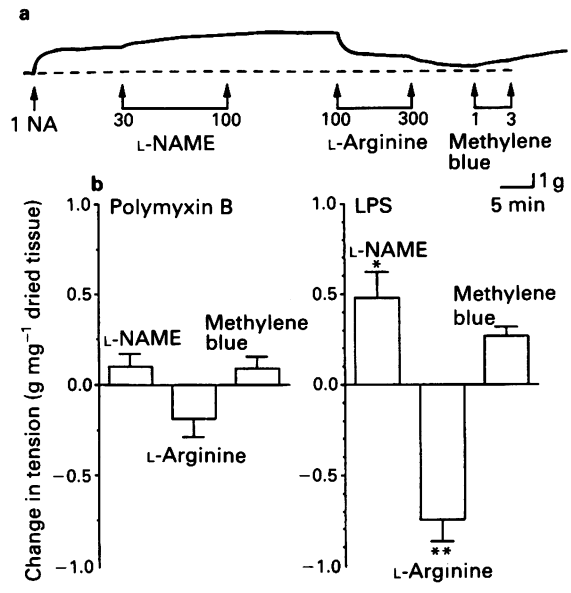

Figure 3 Effects of addition of N^G-nitro-L-arginine methyl ester (L-NAME), L-arginine and methylene blue to noradrenaline (NA)-precontracted rings incubated without L-arginine. (a) Representative tension trace showing the effect of L-NAME (100 μM) and subsequent additions of L-arginine and methylene blue to NA contracted, LPS-treated aortic ring. The numbers indicate the drug concentrations in μM. (b) Mean changes in tension elicited by L-NAME (100 μM) L-arginine (300 μM) and methylene blue (3 μM) in NA-precontracted (1 μM), polymyxin B- (left panel) or LPS-treated (right panel) endothelium denuded aortic rings using the protocol illustrated in (a). The tensions elicited by NA (zero value) were 2.3 ± 0.4 and 2.4 ± 0.2 g mg^{-1} dried tissue in polymyxin B and LPS-exposed rings, respectively. The effects of L-NAME and methylene blue were obtained 30 min after the addition of the drug and 5 min after the addition of L-arginine. Values are the mean of 5–6 experiments; vertical lines show s.e.mean. $^{*}P<0.05$; $^{**}P<0.01$; compared to NA contraction.

tension, as was the case for subsequent addition of L-NAME (1 mM). This was true whether the control rings were incubated with or without L-arginine throughout the experiment. In contrast, the addition of L-arginine (1 mM) to LPS-treated rings incubated without L-arginine reduced tension (by 33%, $P<0.001$) to a stable level equivalent to that obtained in LPS-treated rings incubated in the continuous presence of L-arginine.

Further addition of L-arginine (1 mM) to LPS-treated aortae incubated with 100 μM L-arginine failed to induce any relaxation. The relaxation obtained with L-arginine, present during the incubation or added to contracted aortae, was abolished by addition of L-NAME (1 mM) or methylene blue (1 μM) (Figure 2) in LPS-treated rings. After the addition of either L-NAME or methylene blue, tension was not different in the four groups.

Figure 3 illustrates the effects of L-NAME and further consecutive additions of L-arginine and methylene blue in NA-precontracted aortic rings which were incubated without L-arginine. The recorded tension of maximally NA-contracted aortae was not significantly different between LPS-treated and control rings. In control rings incubated with polymyxin B, L-NAME produced only a slight but non significant increase in tension (Figure 3b). However, it significantly ($P<0.05$) enhanced tension in LPS-treated rings (Figure 3a,b). This effect of L-NAME was reversed by subsequent addition of L-arginine (300 μM), which again was without effect in control rings. Methylene blue reversed the L-arginine induced relaxation in LPS-treated rings, but was without effect in control rings.

Concentration dependence of the effect of L-arginine for tissue incubated without L-arginine Figure 4 illustrates dose-dependent effects of L-arginine in NA-precontracted aortic rings incubated without L-arginine. For LPS-treated rings, increasing concentrations of L-arginine added cumulatively caused relaxation. This relaxation was reversed by subsequent addition of 3 μM methylene blue (tension reached 2.4 g mg^{-1} dried tissue; not shown). In LPS-treated rings, L-arginine-induced relaxation was significant at a concentration as low as 1 μM and reached a maximum at concentrations higher than 30 μM. Half maximal relaxation was obtained at 8.0 ± 3.0 μM (Figure 4).

In control aortae, L-arginine had slight effects, relaxation being significant only in the presence of the highest L-arginine concentrations (> 100 μM) in rings incubated without polymyxin B ($P<0.002$), but not in the presence of polymyxin B. At low concentrations (1–3 μM), L-arginine also produced a small, non significant increase in tension in control rings with or without polymyxin B.

Tissue arginine levels during in vitro *experiments*

Incubation of endothelium-denuded aortae *in vitro* without L-arginine for 6 h induced no significant change in arginine content, whether LPS was present in the medium (for 4 h) or absent (neutralized by polymyxin B). The actual values were 435 ± 32 nmol g^{-1} tissue before incubation, 415 ± 45 and 358 ± 47 nmol g^{-1} tissue, for polymyxin B-treated and LPS-treated aortae ($n = 5$), respectively. In addition, the presence of L-arginine (100 μM) in the incubation medium did not significantly modify the arginine content of control, polymyxin B-treated aortae (498 ± 65 nmol g^{-1} tissue, $n = 5$) compared to LPS-treated aortae (460 ± 38 nmol g^{-1} tissue, $n = 5$).

Discussion

The present work documents a key role for extracellular L-arginine in regulating the extent of vascular hyporeactivity produced by LPS. In endotoxaemic rats, the circulating level

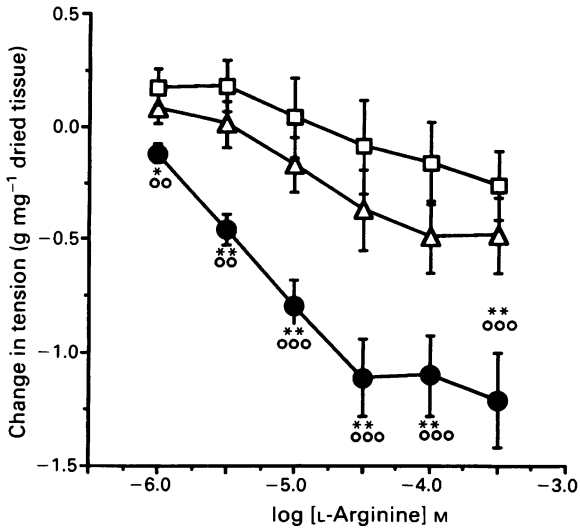

Figure 4 L-Arginine concentration-response curves in maximally noradrenaline (NA)-contracted (10 μM) aortic rings incubated without L-arginine. Control (△); LPS-treated (10 μg ml^{-1}, ●); polymyxin B-treated (1 μg ml^{-1}, □). Cumulative doses of L-arginine were added every 5 min and the maximal effect of each concentration measured 5 min after addition of each dose. Values are the mean of 5–6 experiments; vertical lines show s.e.mean. $^{*}P<0.05$; $^{**}P<0.01$; with respect to control; $^{\circ}P<0.05$; $^{\circ\circ}P<0.01$; $^{\circ\circ\circ}P<0.001$; with respect to polymyxin B.

of arginine (80 μM) was not decreased compared to control animals, and was close to concentrations producing maximal relaxation in LPS-exposed vessels such as the aorta (this study) or a small resistance mesenteric artery (Schneider *et al.*, 1992). The finding that injection of L-arginine failed to decrease blood pressure and to intensify hyporeactivity to NA in LPS-treated rats *in vivo* (Gray *et al.*, 1991) might seem unexpected in view of the relaxant effect of L-arginine in LPS-exposed vessels. This difference between results obtained *in vivo* and *in vitro* is not due to the absence of endothelium in aortic rings used in the present study, as the presence or the absence of endothelium in LPS-treated vessels was previously reported not to change the effect of L-arginine on both cyclic GMP levels and contractile responses to NA (Fleming *et al.*, 1991b). However, this apparent discrepancy may be explained by the present observation that the circulating arginine concentration was unaffected by LPS and sufficient to induce maximal vascular hyporeactivity in LPS-treated rats.

The findings that neither circulating nor tissue arginine contents varied after LPS infusion is not due to a lack of sensitivity of the assay used, as the calculated threshold level of detection and sensitivity of the assay were 4 and 3 μM L-arginine, respectively (see Methods). Furthermore, other amino acids (including ornithine and glutamine) and more than 100 other naturally occurring biological compounds do not interfere with the assay, which is based on detection of guanidine residues (Van Pilsum *et al.*, 1956). While other guanidine-containing compounds might contribute to the determined arginine content, if present and not removed by the chromatographic procedure (see Methods), comparable arginine tissue levels (400–800 μM depending on the tissue) have been reported with this or another technique (Gold *et al.*, 1989; Baydoun *et al.*, 1990).

The continuous presence of L-arginine (100 μM) in the isolated organ bath throughout the experiment or its addition during the contraction experiments, after 6 h in its absence, resulted in the same level of contraction in LPS-treated aortic rings. This relaxation or inhibition of contraction elicited by L-arginine was due to production of NO or a NO-like factor and subsequent cyclic GMP synthesis, since it was inhibited by both the NO synthase inhibitor L-NAME and the guanylate cyclase inhibitor methylene blue, indicating that NO synthase was induced during the 4 h incubation with LPS (note that the contraction studies were made 2 h afterwards, or for control rings, after 6 h). It has also been reported that LPS induces NO synthase activity in the rat aortic medial-adventitial layer (Rees *et al.*, 1990) and in freshly dissociated aortic smooth muscle cells (Fleming *et al.*, 1991a). Consistent with previous data obtained in the aorta (Julou-Schaeffer *et al.*, 1990; Fleming *et al.*, 1991a) and in small resistance arteries (Schneider *et al.*, 1992) from endotoxaemic rats, the present results suggest that extracellular L-arginine concentration is a rate limiting factor for NO synthase activity in vessels exposed to LPS *in vitro*. It was previously reported that these effects of L-arginine on cyclic GMP content and reactivity of arteries are stereospecific since they were not produced by D-arginine (Fleming *et al.*, 1991b).

The presence of low concentrations of LPS in saline solutions may be sufficient to induce the L-arginine/NO pathway (Rees *et al.*, 1990). Controls were therefore run in parallel with and without polymyxin B, which neutralizes LPS but may also produce other effects. No statistically significant difference was found between these two control groups, although a small but significant relaxation was induced by the highest L-arginine concentration in rings incubated without polymyxin B. Thus in the incubation conditions used here, production of an L-arginine derived relaxing factor in control aortic rings was very slight, if at all, consistent with the previously reported lack of constitutive NO synthase activity in aortic smooth muscle cells (Fleming *et al.*, 1991a).

The present results also show that the dependence of smooth muscle relaxation on extracellular L-arginine was not due to exhaustion of tissue arginine content consecutive to NO production from the amino acid or to its release into the extracellular fluid. The tissue arginine content was not significantly altered after 6 h incubation in PSS without L-arginine, whether LPS was present in the bath or not. Furthermore, consistent with previous determinations in the same tissue (Gold *et al.*, 1989), aortic arginine content (300–400 nmol g^{-1} tissue) was high compared to the concentration (30–100 μM) of L-arginine inducing maximal relaxation. This large tissue arginine store was apparently only partly available as substrate for the LPS-induced NO synthase, as shown by the relatively small LPS-induced reduction of the response to NA in rings incubated with exogenous L-arginine (Figure 1). Most of the substrate for maximal activity of the induced NO synthase was of extracellular origin, as shown by the large relaxation induced by the addition of L-arginine in LPS-treated rings incubated without L-arginine (Figure 2). These data support the view that extracellular L-arginine concentration is a rate limiting factor for NO synthase activity in vessels exposed to LPS *in vitro*.

The extracellular L-arginine dependence of NO production observed in endothelium-denuded aortic rings exposed to LPS seems different from what happens in vascular endothelial cells. In these cells, agonists are able to activate NO production by the constitutive NO synthase in the absence of extracellular L-arginine, perhaps because endothelial cells are able to recycle L-citrulline to L-arginine (Hecker *et al.*, 1990). However, it was recently reported that after incubation in absence of L-arginine, agonists that increase NO release also stimulate L-arginine uptake in endothelial cells, even though their intracellular arginine content seems to be high enough to permit maximal NO production by the enzyme (Bogle *et al.*, 1991). Thus, the source of L-arginine used by NO synthase in different cells requires further investigaton.

The major endogenous source of circulating L-arginine *in vivo* is renal synthesis from L-citrulline (Featherston *et al.*, 1973). Upon infusion of citrulline, both renal citrulline uptake and renal production of L-arginine increase (Dhanakoti *et al.*, 1990). During LPS stimulation, induced tissue NO synthase activity converts L-arginine to NO and to L-citrulline. Any L-citrulline released into the circulation might

therefore be used in renal synthesis of L-arginine or locally converted to L-arginine through the pathway described in endothelial cells (Hecker *et al.*, 1990), thus contributing to maintain the arginine content at a constant level.

In summary, the results of this investigation indicate that the vascular NO production induced by LPS is dependent to a large extent on extracellular L-arginine and that the circulating arginine concentration is high enough to induce a maximal L-arginine relaxation in endotoxaemic rats.

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