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SPECIAL REPORT

Role of adventitial nitric oxide in vascular hyporeactivity induced by lipopolysaccharide in rat aorta

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This study was designed to elucidate the role of the adventitia in NO-mediated vascular effects of lipopolysaccharide (LPS). After incubation of rat aorta with LPS, the adventitia generated 3.5 times more nitrite plus nitrate than a corresponding segment of media. Control media covered by adventitia from LPS-treated aortic rings exhibited a 4 fold elevated level of cyclic GMP. Medial layers from LPS-treated aortic rings (like LPS-treated adventitia-intact rings) exhibited a decrease in sensitivity to noradrenaline (NA) that was reversed by 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (1 μM) or N^{ω}-nitro-L-arginine methylester (0.3 mM). However, in contrast to LPS-treated adventitia-intact rings, medial layers showed no reduction in maximal contraction to NA and virtually no relaxation to L-arginine. These data indicate that in blood vessels exposed to LPS, the adventitia is a more powerful source of NO than the media. The adventitia-derived NO can reach soluble guanylyl cyclase in the medial layer and contribute greatly to vascular hyporeactivity and L-arginine-induced relaxation observed in blood vessels exposed to LPS.

Keywords: Adventitia; hyporeactivity; L-arginine; lipopolysaccharide; noradrenaline; nitric oxide; rat aorta

Introduction In blood vessels, bacterial products such as lipopolysaccharide (LPS) cause expression of inducible nitric oxide (NO) synthase (iNOS) consequently leading to NO overproduction and development of hyporeactivity to vasoconstrictor agonists (for review see Stoclet *et al*., 1993; Szabó, 1995; Thiemermann, 1997). It is assumed that iNOS is mainly located in vascular smooth muscle cells (VSMC) (Rees *et al*., 1990; Knowles *et al*., 1990) because they are the predominant cell type of the vascular wall and express iNOS in response to proinflammatory stimuli (Busse & Mülsch, 1990; Fleming *et al*., 1991; Schini *et al*., 1994). However, to the best of our knowledge, no data have been provided on the role of the adventitia in such iNOS-mediated vascular effects of LPS. Here we show that in rat aorta exposed to LPS, the adventitia generates several times more nitrite and nitrate (NO_x) than the VSMC layer. Moreover, adventitia-derived NO can play an important part in vasodilatation and hyporeactivity to constrictors observed in these vessels.

Methods Male Wistar rats (11–13 weeks) bred from genitors provided by Iffa Credo (Abresles, France) were killed by cervical dislocation. Thoracic aortae were removed and cleaned under sterile conditions. Whole aortae (about 30 mm long) were placed in 3 ml of Minimal essential medium (MEM; Gibco, France) with LPS (*E. coli* 055:B5; Difco, Detroit, U.S.A.; 10 μg ml^{-1}) and incubated for 20 h at 37°C in an incubator gassed with 95% air/5% CO_2. The incubation time of 20 h was chosen from preliminary experiments showing that the maximal iNOS activity (NO_x production) in aorta occurs between 15 and 24 h of incubation with LPS. After the 20 h incubation period, the endothelium was removed by gently rubbing the aortic intimal surface and in some cases, the adventitia were separated as an intact invert tube by microdissection. For control experiments we used freshly isolated aortae rather than aortae incubated for 20 h to avoid potential induction of iNOS by the trace amount of endotoxin usually present in the medium. For NO_x determination, adventitia or media isolated from whole LPS-treated aorta (about 30 mm long) was placed in 1 ml Krebs solution (composition in mM: NaCl 119, KCl 4.7, $MgSO_4$ 1.17, $CaCl_2$ 1.25, KH_2PO_4 1.18, $NaHCO_3$ 25 and glucose 11) containing 1 mM L-arginine (L-arg) and incubated at 37°C for an additional 3 h. Concentrations of NO_x in the incubation medium were measured after conversion of nitrate into nitrite using nitrate reductase followed by nitrite determination with Griess reagent (Green *et al*., 1982). The detection limit for NO_x was about 1 μM. The amounts of NO_x produced under these conditions were then calculated either per aortic segment (30 mm long) per 1 h or per μg DNA per 1 h. Cyclic GMP content was measured in control medial layer rings (3 mm long) under three experimental conditions: (1) in media alone, (2) in media covered by control adventitia (attempting to reconstruct vascular wall), and (3) in media covered by adventitia from aortic rings pretreated with LPS for 20 h. Rings were incubated for 30 min in Krebs solution supplemented with 1 mM L-arg, 0.1 mM isobutylmethylxanthine (IBMX) and superoxide dismutase (SOD, 100 u ml^{-1}; Sigma). Cyclic GMP (guanosine 3′:5′-cyclic monophosphate) content was determined by radioimmunoassay according to the method described by Cailla *et al*. (1976), modified for the separation of free/bound cyclic GMP with activated charcoal. [^{125}I]-cyclic GMP and antibodies against cyclic GMP were supplied by Dr B. Lutz-Bucher (CNRS URA 1446, Strasbourg, France). DNA content was measured as described by Brunk *et al*. (1979). For contraction studies, aortic rings or rings of medial layer (3 mm long) were mounted under a passive tension of 1 g in organ baths filled with Krebs solution at 37°C and bubbled with 95% O_2/5% CO_2. Tension was measured with an isometric force transducer. The absence of functional endothelium was assessed by the inability of acetylcholine (1 μM) to induce relaxation of rings precontracted with noradrenaline (NA, 1 μM). Subsequently, L-arg

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(0.1 mM) and in some experiments S-nitroso-N-acetylpenicillamine (SNAP, 10 μM) were added. After a second washing period of 60 min, addition of cumulative concentrations of NA (1 nM to 3 μM) was performed in the absence or in the presence of N^{ω}-nitro-L-arginine methylester (L-NAME; 300 μM) or 1H (1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ; 1 μM; Tocris-Cookson, Bristol, U.K.). L-NAME and ODQ were preincubated for 15 min. Results are expressed as mean $\pm$ s.e.mean of n experiments. EC_{50} values for NA were determined by log-logit regression. Statistical comparisons were made with the non parametric Mann-Whitney test. P values less than 0.05 were considered to be statistically significant.

Results No detectable NO_x products were found in the medium after incubation of adventitia or media from control aorta (not shown). Adventitia of aorta pretreated with LPS *in vitro* generated 3.5 times more NO_x per aortic segment than corresponding media and 10 times more per μg DNA (Figure 1). Control media incubated together with adventitia from LPS treated aorta exhibited a 4 fold elevated level of cyclic GMP in comparison to control media incubated alone (653 ± 59 and 170 ± 49 fmol μg^{-1} DNA respectively; $P<0.001$; $n=5$). No significant change in cyclic GMP level was observed when a control media was incubated together with a control adventitia (101 ± 21 fmol μg^{-1} DNA; $n=5$). L-Arg (0.1 mM) produced a profound relaxation ($62 \pm 6\%$, $n=15$) in LPS-pretreated endothelium-denuded aortic rings contracted with NA (Figure 2). The rings exhibited a significant decrease in sensitivity and in maximal contraction to NA in comparison to controls (Table 1). The presence of a soluble guanylyl cyclase inhibitor, ODQ (1 μM) or NOS inhibitor, L-NAME (0.3 mM) in the organ bath shifted the concentration-response curve to NA towards control values (Table 1). No significant effects of ODQ or L-NAME on the concentration-response curve to noradrenaline was observed in control endothelium-denuded aortic rings (not shown). Contraction experiments were also performed on the medial layers. In control medial layers, the maximal contractile responses to NA were significantly less than in control aortic rings with intact adventitia. This might be attributed to mechanical trauma of VSMC during removal of the adventitia. However, the sensitivity of the medial layers to NA was not significantly different from control rings with

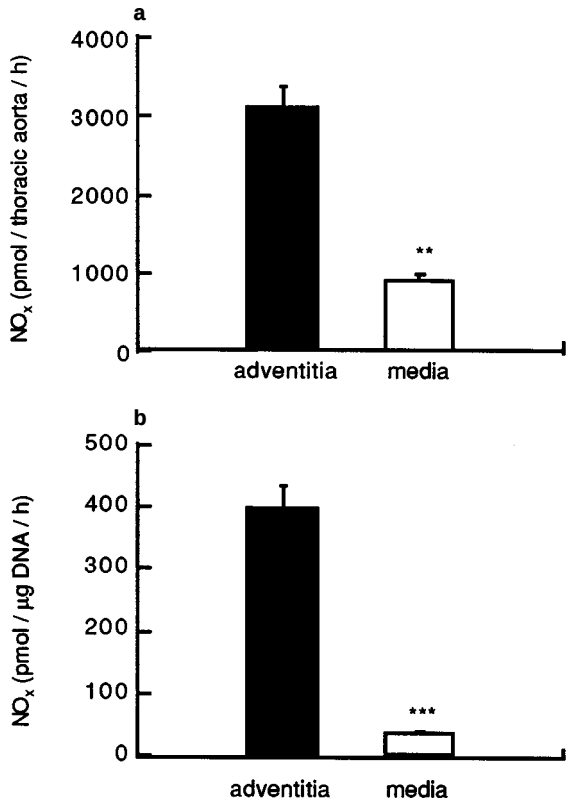

Figure 1 Rate of production of nitrite plus nitrate (NO_x) by adventitia and media obtained from LPS-treated (10 $\mu g\ ml^{-1}$; 20 h) rat thoracic aorta. NO_x was determined after incubation of vascular preparation in 1 ml of Krebs solution with 1 mM L-arg for 3 h. The rate of NO_x production by the layers calculated (a) per segment of thoracic aorta (30 mm long) or (b) per μg DNA. Data expressed as mean $\pm$ s.e.mean of 5 experiments. $^{**}P<0.01$; $^{***}P<0.001$.

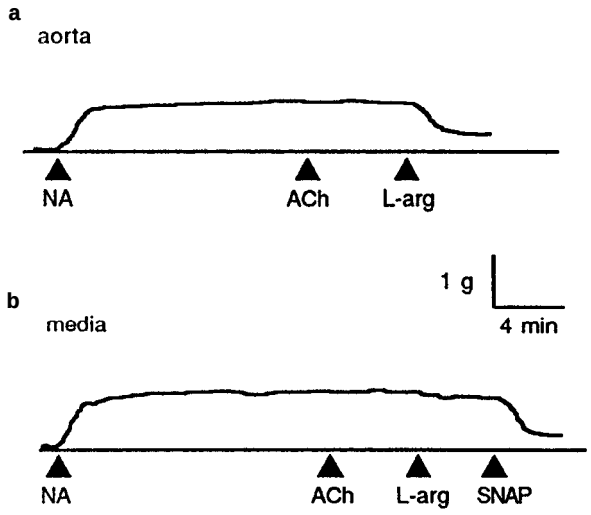

Figure 2 Effect of acetylcholine (ACh; 1 μM), L-arginine (L-arg, 100 μM) and S-nitroso-N-acetylpenicillamine (SNAP; 10 μM) on LPS pretreated (10 $\mu g\ ml^{-1}$; 20 h), noradrenaline (1 μM) contracted endothelium-denuded aortic rings (a) or on medial layers from these rings (b). Representative traces of at least 9 experiments.

Table 1 EC_{50} values and maximum contractile response (E_{max}) to noradrenaline of rat aorta and aortic medial layer

	Whole aorta			*Aortic medial layer*		
	EC_{50} (nM)	E_{max} (g)	n	EC_{50} (nM)	E_{max} (g)	n
Control	6.4 ± 1.0	3.09 ± 0.23	(4)	6.2 ± 1.3	1.34 ± 0.14	(7)
LPS	155 ± 30**	1.68 ± 0.17**	(15)	59 ± 11**	1.24 ± 0.12	(12)
LPS + L-NAME	33 ± 16*†	2.58 ± 0.21†	(4)	4.8 ± 1.1††	1.44 ± 0.22	(6)
LPS + ODQ	8.6 ± 1.7†††	2.56 ± 0.21†	(7)	4.7 ± 1.9††	1.22 ± 0.16	(7)

EC_{50} is the concentration causing half-maximal contraction. $^{*}P<0.05$; $^{**}P<0.01$ compared to respective controls. $\dagger P<0.05$; $\dagger\dagger P<0.01$; $\dagger\dagger\dagger P<0.001$ compared to LPS treatment. Non parametric Mann-Whitney test.

intact adventitia (Table 1). Experiments on NA-contracted medial layers isolated from LPS-treated aortic rings revealed that L-arg (0.1 mM) produced only little or even no relaxation ($4\pm3\%$, $n=12$, in comparison to $62\pm6\%$ in LPS-treated aortic rings; $P<0.001$). Addition of the NO donor, SNAP (10 μM), gave a profound relaxation in the same conditions (Figure 2). The medial layers from LPS-treated aortic rings displayed a significant decrease in sensitivity to NA in comparison to control media. However, the EC_{50} for NA in media from LPS-treated rings was about 2.5 times less than in LPS-treated aorta (Table 1). Additionally, these media exhibited no decrease in maximal response to NA in comparison to control media. The impaired reactivity of the media to NA was totally restored by ODQ or L-NAME (Table 1).

Discussion It is well established that expression of iNOS and subsequent NO overproduction plays a critical role in vascular failure induced by LPS (Stoclet *et al*., 1993; Szabó, 1995; Thiemermann, 1997). Earlier we observed that the adventitia contributes to an elevated cyclic GMP level within whole LPS-treated aorta of the rat (Gray *et al*., 1991). Here we show that in these vessels, adventitia is a source of NO, potentially much more powerful than media. Indeed, a segment of adventitia generated significantly more NO_x than the equivalent segment of media (Figure 1a). The calculation of NO_x per amount of DNA in both tunicas allowed us to conclude that after challenge with LPS, one adventitial cell generated significantly much more NO_x (10 times) than one VSMC (Figure 1b). The adventitia is a tissue composed of various cell types (Rhodin, 1980), including potential producers of NO such as fibroblasts (Jorens *et al*., 1992) and macrophages (MacMicking *et al*., 1997). The identification of the major NO-producing cell type and potential cross-talk between cells within the vascular wall after inflammatory stimuli require further investigations.

Experiments on 'reconstructed aorta' which was composed of control media and adventitia from LPS-treated aorta showed an elevated level of cyclic GMP in the media, consistent with adventitia-derived NO reaching and activating soluble guanylyl cyclase within the VSMC layer. The contribution of adventitia-derived NO to vascular hyporeactivity was evaluated in comparative studies performed on aortic rings (with intact adventitia) and in medial layers derived from such rings. In contrast to LPS-treated aortic rings, their medial layers did not relax or relaxed very slightly upon addition of L-arg (Figure 2). However, these medial rings showed a profound relaxation to SNAP indicating unaltered reactivity of the vascular preparations to NO. According to previous studies, the relaxation elicited by exogenous L-arg in rings without endothelium is a characteristic feature of expression of extra-endothelial iNOS activity (Schott *et al*., 1993). Our data provide evidence that adventitial cells play a crucial role in this phenomenon.

The analysis of contractile responses of LPS-treated aortic rings and corresponding medial layers to increasing concentrations of NA suggests that adventitia, through generation of large amounts of NO, can contribute greatly to the impairment of contractility of LPS-exposed blood vessels. This result may be of clinical importance, since administration of L-arg to septic shock patients results in an immediate decrease of blood pressure (Lorente *et al*., 1993). The present findings might also direct a search for an efficient therapy of shock-like conditions toward manipulation of the NO pathway in adventitial cells. In conclusion, within blood vessels exposed to LPS, adventitia is apparently potentially a more powerful source of NO than VSMC. The adventitia-derived NO can reach functionally important targets, such as soluble guanylyl cyclase within medial layer and have a significant influence on vascular contractile function. The latter conclusion is consistent with the recent work showing that transgene expression of endothelial NOS in the adventitia influences the contractility (Kullo *et al*., 1997).

Abbreviations: iNOS, inducible NO synthase; IBMX, isobutylmethylxanthine; L-arg, L-arginine; L-NAME, N^{ω}-nitro-L-arginine methylester; LPS, lipopolysaccharide; NA, noradrenaline; NO_x, nitrite plus nitrate; ODQ, 1H-(1,2,4) oxadiazolo (4,3-a) quinoxalin-1-one; SNAP, S-nitroso-N-acetylpenicillamine; SOD, superoxide dismutase; VSMC, vascular smooth muscle cells

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