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Evidence for N-acetylcysteine-sensitive nitric oxide storage as dinitrosyl-iron complexes in lipopolysaccharide-treated rat aorta

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1 The aim of this study was to assess whether or not vasoactive nitric oxide (NO) stores exist within vascular tissue after lipopolysaccharide (LPS)-treatment.

2 Rat thoracic aortic rings (for contraction experiments) or whole thoracic aortae (for electron paramagnetic resonance (e.p.r.) spectroscopy) were incubated for 18 h at 37°C in the absence (control) or in the presence of LPS (10 $\mu g\ ml^{-1}$), with or without L-arginine (L-Arg, 1 mM), the substrate of NO synthase (NOS) or N^{ω}-nitro-L-arginine methyl ester (L-NAME, 1 mM), an inhibitor of NOS.

3 Incubation of rat aortic rings with LPS and L-Arg resulted in a significant decrease of the maximum contractile response to noradrenaline (NA, 3 μM). Addition of L-NAME (3 mM) enhanced contraction towards control values. After precontraction with NA and L-NAME, addition of N-acetyl-L-cysteine (NAC, 0.1 to 10 mM) evoked a concentration-dependent relaxation in rings incubated with LPS and L-Arg, but not in control rings, rings incubated with LPS in the absence of L-Arg or rings incubated with LPS in the presence of L-Arg and L-NAME. Removal of the endothelium did not significantly modify the relaxation induced by NAC. Methylene blue (3 μM), an inhibitor of the activation of guanylyl cyclase by NO, completely abolished the relaxing effect of NAC.

4 The presence of protein-bound dinitrosyl non-haem iron complexes (DNIC) was detected by e.p.r. spectroscopy in aortae incubated with LPS and L-Arg, but not in control aortae. Furthermore in LPS-treated aortae, addition of NAC (20 mM) gave rise to the appearance of an e.p.r. signal characteristic of low molecular weight DNIC.

5 These results provide evidence that, within vascular tissue, NO generated from L-Arg by LPS-induced NOS activity can be stored as protein-bound DNIC in non-endothelial cells. Upon addition of NAC, low molecular weight DNIC are released from these storage sites and induce vascular relaxation probably through guanylyl cyclase activation.

Keywords: Lipopolysaccharide; nitric oxide; dinitrosyl non haem iron complexes; electron paramagnetic resonance spectroscopy; N-acetyl-L-cysteine; vascular contraction

Introduction

Nitric oxide (NO) which is synthesized by NO synthase enzymes (NOS) from L-arginine (L-Arg), is recognized as an ubiquitous messenger involved in many physiological and pathophysiological processes (Moncada & Higgs, 1995). An overproduction of NO is involved in the vascular failure induced by bacterial wall components like lipopolysaccharide (LPS) (Julou-Schaeffer *et al.*, 1990). This appears to be the consequence of the activity of an inducible form of NO synthase (iNOS) in vascular tissue (Stoclet *et al.*, 1993).

There are several potential molecular targets that can be either activated or inhibited as a consequence of reacting with NO (Nathan, 1992; Henry *et al.*, 1993). The most significant property of NO in terms of its vascular biological activity is its reversible binding with haeme-iron containing soluble guanylyl cyclase, leading to the activation of cyclic GMP synthesis and vascular relaxation (Ignarro, 1991).

NO also reacts with non-haeme iron to form protein-bound dinitrosyl non-haem iron complexes (DNIC) (Vanin, 1991; Butler *et al.*, 1995). Such complexes are formed following activation of the iNOS pathway in macrophages (Lancaster & Hibbs, 1990; Pellat *et al.*, 1990) and in their target cells (Drapier *et al.*, 1991), as well as in pancreatic islet β-cells (Corbett *et al.*, 1991) and hepatocytes (Stadler *et al.*, 1993). It has been proposed that DNIC might contribute to the cytotoxic action of NO, through inhibition of some key mitochondrial enzymes of the respiratory chain (for review see Henry *et al.*, 1993).

In vascular tissues, treatment with exogenous low molecular weight DNIC results in the formation of DNIC with proteins which appear to behave as NO stores (Mülsch *et al.*, 1991). These complexes decompose on addition of low molecular weight thiols such as N-acetyl-L-cysteine (NAC), releasing dinitrosyl-iron dithiolate which promotes guanylyl cyclase activation and induces vascular relaxation. However, whether LPS, a well known trigger of NO overproduction through iNOS induction, leads to the formation of NO storage as DNIC with potential vasoactive properties remains to be determined.

Therefore the aim of the present study was to assess whether or not such NO stores exist within LPS-treated vascular tissue.

Methods

Aortic preparations

Male Wistar rats (280-350 g) were pretreated with heparin (250 iu i.p.) 15 min before being killed by cervical dislocation. The thoracic aorta was removed, cleaned of fat and connective tissues in aseptic conditions. Whole thoracic aortae (approximately 30 mm length) were used for electron paramagnetic resonance (e.p.r.) studies. Thoracic aortic rings (2 to 3 mm length) were used for contraction experiments. Both aortae and rings were incubated at 37°C in an incubator gassed with

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5% CO_2/95% air in minimal essential medium (MEM) in the absence or in the presence of LPS (10 $\mu g\ ml^{-1}$), L-Arg (1 mM) or N^{ω}-nitro-arginine methylester (L-NAME, 1 mM). A standard incubation time of 18 h was chosen for the present experiments, since preliminary studies showed that the e.p.r.-detectable signal for DNIC was stable between 16 and 24 h after addition of LPS and L-Arg.

Contraction experiments

After the 18 h incubation period, rings were mounted under a passive tension of 2 g in standard organ baths filled with 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) continuously kept at 37°C and bubbled with 95% O_2/5% CO_2. In some experiments, the endothelium was removed by gently rubbing the intimal surface of the rings with curved forceps before mounting. Tension was measured with an isometric force transducer.

After an equilibration period of 60 min (during which time the Krebs solution was changed every 20 min), aortic rings were precontracted with noradrenaline (NA, 1 μM). The presence of functional endothelium was assessed by the ability of acetylcholine (1 μM) to induce at least 10% relaxation of rings precontracted with NA (1 μM). Rings were considered to be denuded of functional endothelium when acetylcholine failed to induce any relaxing response under these conditions. NOS induction was verified by the ability of L-Arg (100 μM) to induce relaxation. After a second washing period of 60 min (during which time the Krebs solution was changed every 20 min), aortic rings were precontracted by successive addition of NA (1 nM to 3 μM) and L-NAME (1 and 3 mM). In some experiments, methylene blue (3 μM) was subsequently added. When the contraction reached a steady-state level, NAC (0.1 to 10 mM) was added to the bath in cumulative manner. Experiments with NAC were conducted in the dark.

E.p.r. studies

After the 18 h incubation period, the control or LPS-pretreated thoracic aorta was opened longitudinally and placed with care in a flat e.p.r. ampoule filled with Krebs solution. E.p.r. spectra were recorded with a Brucker 300E spectrometer at room temperature. E.p.r. parameters were as followes: microwave frequency 9.79 GHz, microwave power 102 mW, modulation amplitude 0.61 mT and modulation frequency 100 kHz. Addition of NAC (final concentration 20 mM) was made with a microcatheter inserted into the e.p.r. ampoule.

Expression of results and statistical analysis

Results from contraction experiments are expressed as mean ± s.e.mean of at least 5 experiments. The contractile effects of NA and L-NAME are expressed in g of tension per mg of air-dried tissue. The relaxing effect of NAC is expressed as percentage relaxation of the tone induced by NA plus L-NAME (in the absence or in the presence of methylene blue). Statistical significances were tested by the analysis of variance. P values less than 0.05 were considered to be statistically significant.

Drugs and reagents

LPS (*E. coli* 055:B5, $LD_{50} = 32.76\ mg\ kg^{-1}$, Difco, Detroit, U.S.A.) was dissolved in MEM. MEM (endotoxin level lower than 0.02 $ng\ ml^{-1}$) was purchased from Gibco BRL Life Technologies (Cergy-Pontoise, France). The drugs used for the 18 h incubation period (L-Arg hydrochloride and L-NAME) were dissolved in MEM. They were purchased from Sigma Chemical Co (Saint Quentin-Fallavier, France) as well as NA bitartrate, acetylcholine hydrochloride and methylene blue. NA was stored as a 10 mM stock solution in Na_2SO_3 (7.9 mM)/HCl (34 mM) and subsequently diluted in the Krebs solution. Acetylcholine, L-Arg and L-NAME (used for the contractile experiments) were dissolved in Krebs solution. Methylene blue was dissolved in MilliQ water (Millipore). NAC (Fluimucil for parenteral use) was obtained from Zambon Laboratory (Antibes, France) as a 5 g 25 ml^{-1} solution of EDTA/NaOH (pH 6.5) and further diluted in Krebs solution.

Results

Contraction experiments

As illustrated in Figure 1, incubation of rat aortic rings with LPS (10 $\mu g\ ml^{-1}$) and L-Arg (1 mM) for 18 h resulted in a significant ($P < 0.001$) decrease of the contractile response to a maximally active concentration of NA (3 μM) as compared to controls. Addition of L-NAME (3 mM) produced an increase in tension in controls as well as in LPS-treated rings. The contractile effect of L-NAME was significantly ($P < 0.001$) greater in LPS-treated rings than in controls. Therefore, the contraction level of LPS-treated rings after addition of L-NAME was not significantly different from the level obtained in controls.

The effect of NAC was studied on rings precontracted with NA (3 μM) and L-NAME (3 mM). NAC (0.1 to 10 mM) did not significantly modify the tension of control rings incubated for 18 h in the absence of LPS, nor did it modify the tension of rings incubated with LPS in the absence of L-Arg or in the presence of L-Arg and L-NAME (Figure 2a). However, in rings incubated for 18 h with LPS and L-Arg, NAC evoked a concentration-dependent relaxation which was not significantly different from the relaxation observed in endothelium-denuded aortic rings (Figure 2b). Methylene blue (3 μM) completely prevented the relaxing effect of NAC (Figure 2c). In the latter case, addition of methylene blue after L-NAME produced an increase in tension of about 15%.

E.p.r. studies

At room temperature, thoracic aorta incubated with LPS and L-Arg for 18 h exhibited a distinct anisotropic e.p.r. signal with $g_{\perp} = 2.04$ and $g_{\parallel} = 2.015$ (Figure 3a), attributed to DNIC with sulphur groups of proteins (Mülsch *et al.*, 1991). This was not observed in control aorta (Figure 3b). Addition of NAC (20 mM) to LPS-pretreated aorta led to the appearance of

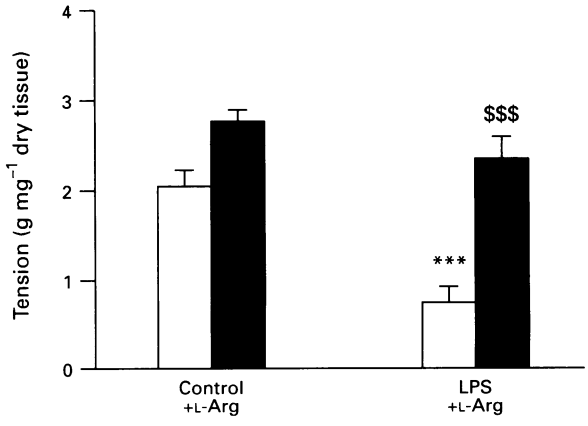

Figure 1 Histograms showing the contractile effects of 3 μM noradrenaline (NA, open columns) and 3 μM NA + 3 mM N^{ω}-nitro-L-arginine methyl ester (L-NAME) (solid columns) in rat aortic rings incubated for 18 h in MEM with L-arginine (L-Arg, 1 mM) in the absence (Control) or presence in lipopolysaccharide (LPS, 10 $\mu g\ ml^{-1}$). ***$P < 0.001$: significant decrease in the contractile effect of NA in comparison to control rings. $$$$P < 0.001$: significant increase of the contractile effect of L-NAME in comparison to control rings.

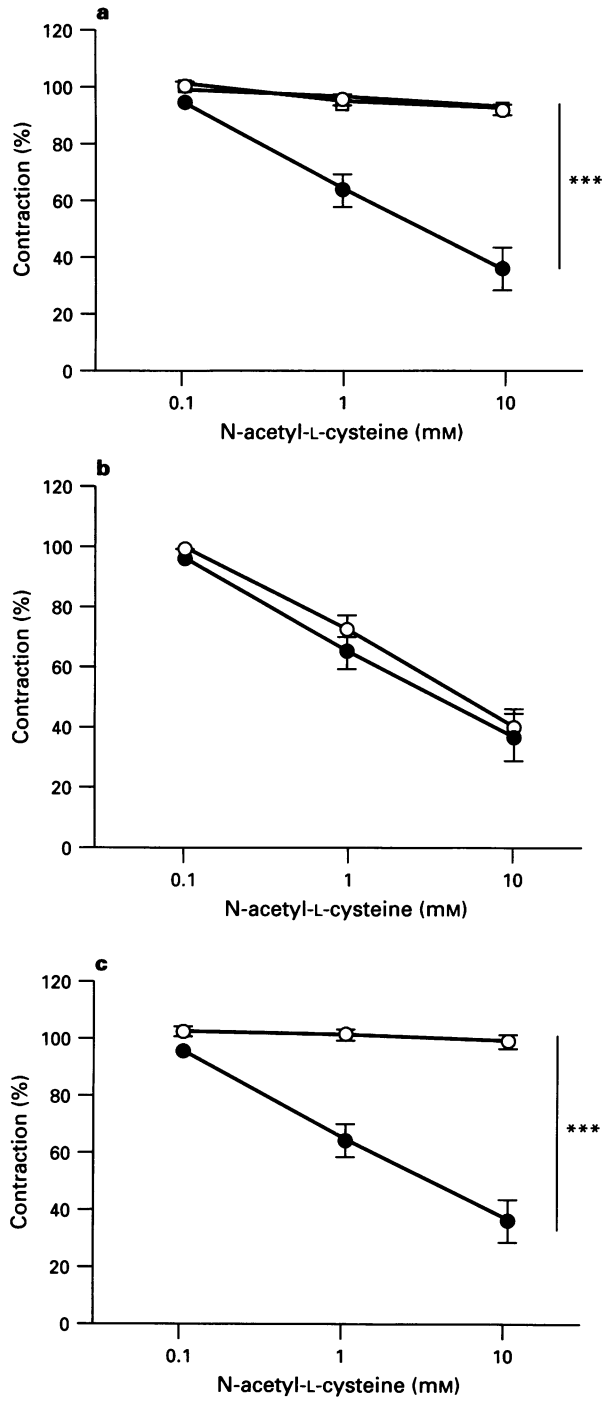

Figure 2 (a) Concentration-response curve for N-acetyl-L-cysteine obtained in noradrenaline (NA, 3 μM) plus N$^{\omega}$-nitro-L-arginine methyl ester (L-NAME) (3 mM) precontracted rat aortic rings incubated for 18 h in MEM with L-arginine (L-Arg, 1 mM) in the absence (△) or presence of lipopolysaccharide (LPS, 10 μg ml^{-1}, ●) or incubated with LPS (10 μg ml^{-1}) in the absence of L-Arg (□), or in the presence of L-Arg (1 mM) and L-NAME (1 mM, ○). (b) Effect of N-acetyl-L-cysteine on aortic rings incubated for 18 h in MEM with L-Arg (1 mM) and LPS (10 μg ml^{-1}), precontracted with NA (3 μM) plus L-NAME (3 mM); concentration-response curves were obtained in aortic rings with functional endothelium (●) or in rings from which endothelium was removed after the 18 h incubation (○). (c) Effect of N-acetyl-L-cysteine on aortic rings incubated for 18 h in MEM with L-Arg (1 mM) and LPS (10 μg ml^{-1}), precontracted with NA (3 μM) plus L-NAME (3 mM); concentration-response curves were obtained in the absence (●) or presence of methylene blue (3 μM, ○). ***$P<0.001$.

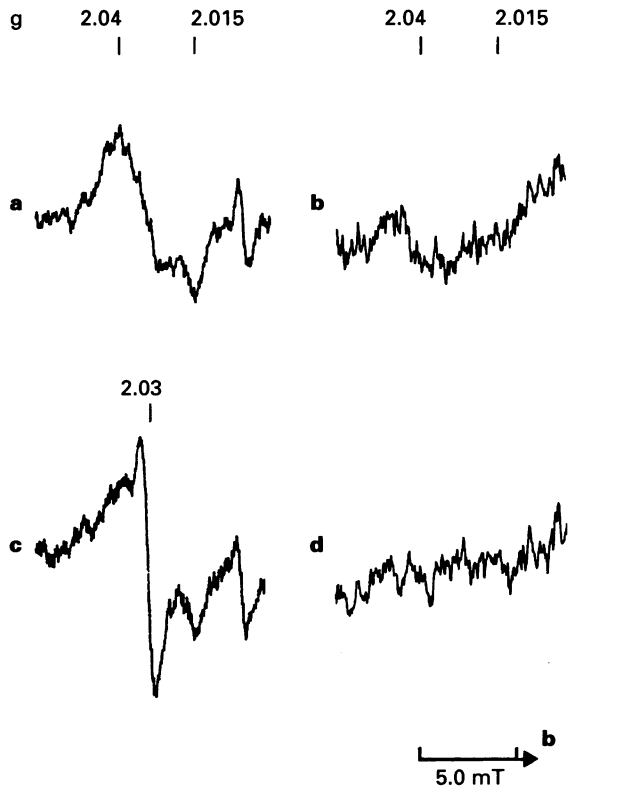

Figure 3 Electron paramagnetic resonance (e.p.r.) spectra of whole rat thoracic aorta incubated for 18 h in the presence (a,c) or absence (b,d) of lipopolysaccharide (LPS, 10 μg ml^{-1}) and L-arginine (1 mM) recorded before (a,b) or after (c,d) addition of 20 mM N-acetyl-L-cysteine. E.p.r. spectra were recorded at room temperature in a flat quartz ampoule. E.p.r. parameters were as indicated in the Methods section.

narrow e.p.r. singlet with g = 2.03 which is characteristic of low molecular weight DNIC, in addition to the anisotropic signal (Figure 3c). In control aorta, addition of NAC did not modify the e.p.r. spectrum (Figure 3d).

Discussion

This study shows that, in rat aorta, incubation with LPS and L-Arg was associated with an NAC-dependent relaxation which was prevented by methylene blue and with the formation of e.p.r-detectable DNIC that were partly converted to low molecular weight DNIC in the presence of NAC.

LPS induced a depression of the contractile response to NA in rat aortic rings, which was reversed by the NOS inhibitor L-NAME. The contractile effect of L-NAME in these rings was significantly greater than in control. The effect of L-NAME in controls was related to the inhibition of the relaxing effect of NO produced by the endothelial constitutive NOS. Altogether, this suggests that the vascular hyporeactivity to NA was due to an overproduction of NO via LPS-induced iNOS activity in rat aorta (Stoclet *et al.*, 1993). In this model, although the hyporeactivity to NA occurs even in the absence of endothelium (Fleming *et al.*, 1990), the endothelium accelerates the induction process and increases the sensitivity of the preparation of LPS (Fleming *et al.*, 1993).

This LPS-induced NOS activity in rat aorta was associated with a relaxing effect of NAC. NAC is a membrane-permeable low molecular weight thiol which is thought to have antioxidant properties and to scavenge oxygen-free radicals (Villa & Ghezzi, 1995). Some of the effects of NAC could therefore be related to a potentiation of the effect of NO, by destruction of oxygen-free radicals which are involved in NO breakdown

(Zhang *et al.*, 1994). The effects of NAC could also be due to its reaction with NO to form nitrosothiol intermediates which are potent activators of guanyly cyclase (Ignarro *et al.*, 1981). In the present study, the relaxing effect of NAC is thought to be related to NO and cyclic GMP generation in that it was abolished in the presence of methylene blue, an inhibitor of the activation of soluble guanylyl cyclase by NO. Since the relaxing effect of NAC was observed in the presence of a maximally active concentration of the NOS inhibitor L-NAME, it is unlikely that it is related to NOS activity. However, the relaxing effect of NAC was dependent on LPS-induced NOS activity during the incubation period, since no effect was observed when the rings were incubated with LPS in the absence of the substrate of the enzyme, L-Arg, or in the presence of the substrate and an inhibitor of NOS activity, L-NAME. These results therefore suggest that the mechanism of the relaxant effect of NAC is related to the release of NO (or NO-related species) from tissular stores formed during incubation with LPS. The observation that removal of the endothelium after the incubation period did not modify the relaxation induced by NAC suggests that NAC-sensitive NO stores are localized in non-endothelial cells, probably in the smooth muscle and/or the adventitial layer.

In various cell types, the induction of iNOS is associated with the formation of DNIC (Lancaster & Hibbs, 1990; Pellat *et al.*, 1990; Drapier *et al.*, 1991; Corbett *et al.*, 1991; Stadler *et al.*, 1993; Nüssler *et al.*, 1993). It has been speculated that the formation of DNIC is invariably associated with a cytotoxic action of NO through inhibition of iron sulphur proteins of the mitochondrial respiratory chain (Lancaster & Hibbs, 1990). However, this statement is presently under reassessment, since a wide variety of thiol-containing proteins may form DNIC upon reaction with iron(II) and NO (Vanin *et al.*, 1992; Kennedy *et al.*, 1993; Schwartz *et al.*, 1995). The results presented here show the appearance of an e.p.r. signal characteristic of DNIC only in LPS-treated aorta. The target proteins, the intracellular localization of DNIC and their potential contribution to the cytotoxic effect of NO remain to be investigated. However, the results presented here are not in favour of a DNIC-related cytotoxicity since the contractility of LPS-treated rings in the presence of NA and L-NAME was not impaired.

In LPS-treated aorta, NAC induced the appearance of an isotropic e.p.r. signal with a g factor of 2.03. At ambient temperature, this signal is characteristic of low molecular weight DNIC due to rapid rotation of these species in liquid phase and averaging of g factor anisotropy exhibited by high molecular weight DNIC (Mülsch *et al.*, 1991). Comparing the intensities of the e.p.r. signals according to Vanin *et al.* (1975), one estimates the ratio of low molecular weight DNIC and high molecular weight DNIC content as being 1:20 after addition of 20 mM NAC. Therefore, NAC may accept Fe-NO groups from protein-bound forms (through ligand-exchange mechanisms) and transfer them to cellular targets. It has been shown that DNIC with L-cysteine activates soluble guanylyl cyclase (Mülsch *et al.*, 1991), relaxes blood vessels such as the rat aorta (Verdernikov *et al.*, 1992) and exerts a hypotensive effect in rats (Kleschyov *et al.*, 1985). In accordance with this mechanism, the relaxant effect of NAC was prevented in the present study by methylene blue, an inhibitor of the activation of soluble guanylyl cyclase by NO.

Although these results provide strong evidence for the contribution of DNIC to NAC-sensitive NO storage in LPS aorta, there exist other potential forms of NO derivative that might release NO by reaction with low molecular weight thiols; these include S-nitrosothiols (Moro *et al.*, 1994; Stamler, 1994), N^{ω}-hydroxy-L-Arg-NO adducts (Hecker *et al.*, 1995), or peroxynitrite adducts with biological alcohol functional groups (Moro *et al.*, 1995).

Taken together, this study provides evidence that within vascular tissue, NO generated from L-Arg by LPS-induced NOS activity can be stored as DNIC in non-endothelial cells. By reaction with low molecular weight thiols such as NAC, NO can be released from these storage sites as low molecular weight DNIC which induce vascular relaxation probably through the activation of soluble guanylyl cyclase.

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