

## Induction of nitric oxide synthase by lipoteichoic acid from *Staphylococcus aureus* in vascular smooth muscle cells

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Inducible vascular nitric oxide synthase accounts for the contractile impairment observed in endotoxemia. We provide evidence that lipoteichoic acid (LTA) from *Staphylococcus aureus*, a micro-organism without endotoxin, also induces nitric oxide synthase. Our study demonstrates that on endothelium-free rings of rat aorta, LTA-like lipopolysaccharide induces a loss of contractility restored by Methylene blue and *N*<sup>G</sup>-nitro-L-arginine-methyl ester (LNAME). Moreover in cultured vascular smooth muscle cells, LTA produces a dose-dependent increase in intracellular cyclic GMP which is antagonized by LNAME and prevented by dexamethasone.

Lipoteichoic acid; Endotoxin; Nitric oxide (NO) synthase; Cyclic GMP; Rat aorta

### 1. INTRODUCTION

Septic shock results from an infective process induced by endotoxins constituting lipopolysaccharide (LPS), the major components of the outer membrane of Gram-negative bacteria. Circulating levels of endotoxin induce haemodynamic alterations including systemic arterial hypotension associated with hypo-responsiveness to pressor agents. The observed in vivo vascular hypo-responsiveness persists when vascular tissue removed from LPS-injected rat is examined ex vivo [1–4] or when artery rings are incubated in vitro with LPS [5,6]. It has been recently demonstrated in ex vivo [7] and in vitro studies [8,9] that the loss of vascular reactivity induced by LPS was due to an overproduction of nitric oxide (NO). However, if NO synthesis from L-arginine was originally discovered in vascular endothelial cells [10], the vascular smooth muscle cells seem to be the main source of NO activated by LPS [7–9]. In addition, a Ca<sup>2+</sup>-independent NO synthase that is sensitive to dexamethasone and induced by LPS has been shown in vascular smooth muscle cells [11].

However, cardiovascular dysfunctions associated with septic shock may also be observed in the absence of endotoxemia [12,13]. Similar haemodynamic changes are produced either by a Gram-negative bacteria (*Escherichia coli*) or by a Gram-positive bacteria (*Staphylococcus aureus*), a micro-organism without LPS, indicating a common pathway leading to cardiovascular abnormalities [14]. In the present study, we describe the

induction of NO synthase in vascular smooth muscle cells by lipoteichoic acid (LTA), a component of the membrane of Gram-positive bacteria [15]. The results suggest that induction of NO synthase in the vascular wall is a general phenomenon responsible for the loss of reactivity in bacteremia caused by Gram-positive or Gram-negative micro-organisms.

### 2. MATERIALS AND METHODS

#### 2.1. Contractile responses in rat aortic rings

Endothelium-free rings (2 mm wide) from rat aorta were incubated for 5 h in MEM medium, in the absence (control) or presence of either LPS or LTA. Then, rings were suspended under a tension of 2 g in organ baths containing Krebs-Henseleit solution as previously described [4,7]. After a 60 min equilibration period, contraction was induced by application of a maximal concentration of phenylephrine (PE, 10  $\mu$ M).

#### 2.2. Culture of smooth muscle cells

Smooth muscle cells were isolated by enzymatic (elastase and collagenase) digestion of rat thoracic aorta as previously described [16]. They were cultured for 4 days in DMEM with 10% fetal calf serum and used between passages 3 and 7.

#### 2.3. cGMP measurements

Cell monolayers were washed and the medium was replaced with 2 ml of DMEM containing 2 mM glutamine, antibiotics, 0.1 mM isobutylmethylxanthine (IBMX) with or without LPS or LTA. After 24 h, cGMP was extracted from cells by rapid aspiration of the medium and addition of 1 ml 0.1 N HCl to each well. The samples were frozen until cGMP determination by radioimmunoassay (NEN kit). To study the effect of LNAME, cells were incubated for 24 h in RPMI 1640 (L-arginine concentration is 1.2 mM) with or without LPS (0.1  $\mu$ g/ml) or LTA (10  $\mu$ g/ml). IBMX (0.1 mM) was added 30 min before cGMP extraction in the presence or not of LNAME (1.2 mM).

#### 2.4. Materials

*N*<sup>G</sup>-nitro-L-arginine methyl ester salt was purchased from Calbiochem. Phenylephrine, dexamethasone, endotoxin (lipopolysac-

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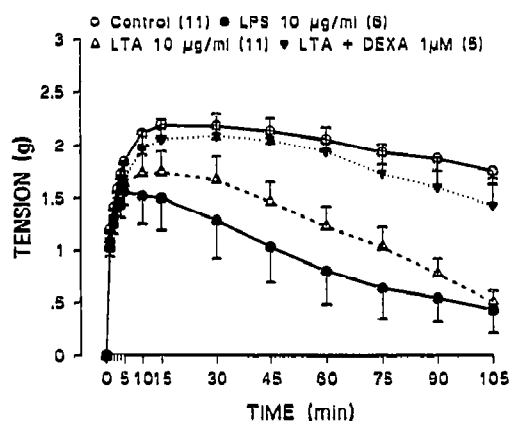


Fig. 1. Kinetics of the contraction induced by phenylephrine ( $10 \mu\text{M}$ ) on endothelium-free rings of rat aorta incubated for 5 h in the presence or absence of LPS ( $10 \mu\text{g/ml}$ ) or LTA ( $10 \mu\text{g/ml}$ ). LTA was also incubated in the presence of  $1 \mu\text{M}$  dexamethasone (DEXA). Mean  $\pm$  S.E.M. Figures in parentheses indicate the number of measurements.

charides; *E. Coli* 0111:B4), and lipoteichoic acid (*Staphylococcus aureus*) were from Sigma.

#### 2.5. Statistics

The data are given as means  $\pm$  S.E.M. and one-way analysis of variance (ANOVA) was used for statistical comparison of the results.

### 3. RESULTS AND DISCUSSION

Five hour incubation of endothelium-free rings of rat aorta in the presence of LPS ( $10 \mu\text{g/ml}$ ) induced a loss of tonicity to the contractile response of phenylephrine ( $10 \mu\text{M}$ ). This phenomenon has previously been attributed to the induction of smooth muscle NO synthase by endotoxin [7,9]. Incubation with LTA ( $10 \mu\text{g/ml}$ ) resulted in a comparable decrease in the contraction

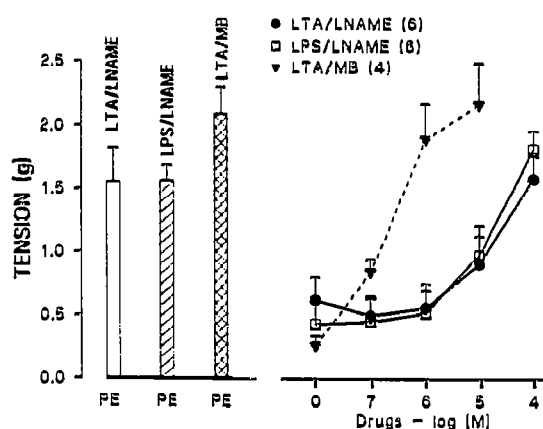


Fig. 2. The effects of LNAME and Methylene blue (MB) in reversing the loss of contraction tonicity to phenylephrine (PE) in endothelium-free rings incubated with LPS or LTA. The drugs were added in a dose-dependent manner after 105 min contraction to PE when contractile impairment was maximal (see Fig. 1). Histograms depict the maximal tension induced by phenylephrine. Mean  $\pm$  S.E.M. Figures in parentheses indicate the number of measurements.

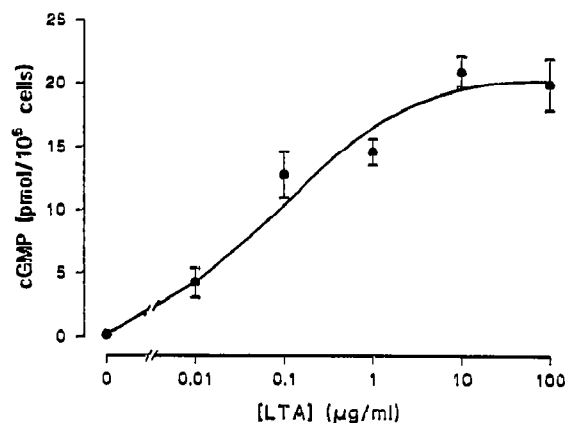


Fig. 3. Dose relationship for the cGMP accumulation induced by LTA in cultured smooth muscle cells. Means  $\pm$  S.E.M. of 3 experiments performed in triplicate.

elicited by phenylephrine (Fig. 1). The loss of vascular responsiveness was not observed after 1 h incubation with LPS or LTA up to  $100 \mu\text{g/ml}$  ( $n = 3$ ) indicating a delayed cellular process and suggesting an induction of a biological activity. Indeed, the effects of LTA were abolished by  $1 \mu\text{M}$  dexamethasone (Fig. 1) which is known to prevent the induction of NO synthase by endotoxin [9]. Furthermore *N*<sup>G</sup>-nitro-L-arginine-methyl ester (LNAME), a specific inhibitor of NO synthase, was equally potent in restoring the contractile defect to phenylephrine due to LPS or LTA (Fig. 2). Methylene blue was also able to counteract the action of LTA indicating the activation of soluble guanylate cyclase responsible for the vascular hypo-reactivity (Fig. 2). L-Arginine ( $300 \mu\text{M}$ ) rapidly reversed the effects of LNAME ( $30 \mu\text{M}$ ), but not those of Methylene blue ( $10 \mu\text{M}$ ) (data not shown).

These results were extended in cultured vascular smooth muscle cells. Incubation with LPS or LTA exhibited a time- and dose-dependent elevation in intracellular cGMP. LPS (from  $1 \text{ ng/ml}$  to  $10 \mu\text{g/ml}$ ) induced an elevation in cGMP content from  $0.83 \pm 0.04$  (control

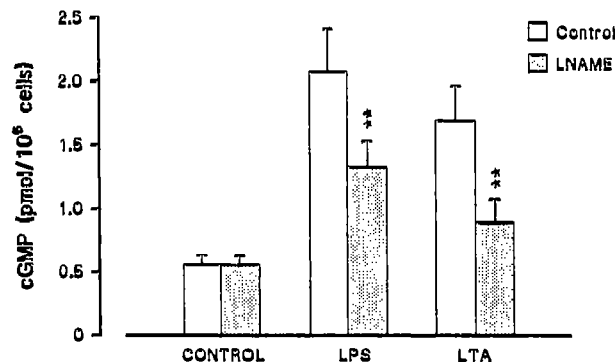


Fig. 4. Effect of LNAME ( $1.2 \text{ mM}$ ) on cGMP accumulation in smooth muscle cells in cultures incubated for 24 h in the presence of  $0.1 \mu\text{g/ml}$  LPS or  $10 \mu\text{g/ml}$  LTA. Means  $\pm$  S.E.M. of 3 experiments performed in triplicate.  $^{**}P < 0.01$ .

cells) to a maximal value  $40.8 \pm 5.8$  pmol/ $10^6$  cells ( $1 \mu\text{g/ml}$  LPS). In the same conditions LTA elicited an increase of cGMP production with an  $\text{EC}_{50}$  of about  $0.1 \mu\text{g/ml}$  (Fig. 3). The effects of LPS or LTA ( $0.1 \mu\text{g/ml}$ ) were inhibited by 90% in cells which were co-treated with dexamethasone ( $1 \mu\text{M}$ ). A 30 min incubation with LNAME ( $1.2 \text{ mM}$ ) reduced the increased cGMP accumulation in both LPS- and LTA-treated cells (Fig. 4).

These results provide the first evidence that LTA, like LPS, expresses NO synthase in vascular smooth muscle cells. This indicates that an inducible NO/L-arginine pathway may participate to the severe hypotension induced by sepsis either caused by Gram-positive or Gram-negative bacteria. Moreover, our study demonstrates a direct action of the bacterial membrane components LPS and LTA on vascular smooth muscle cells.

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