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Delayed circulatory failure due to the induction of nitric oxide synthase by lipoteichoic acid from *Staphylococcus aureus* in anaesthetized rats

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1 This study investigates the effect of lipoteichoic acid (LTA) from the cell wall of *Staphylococcus aureus*, a micro-organism without endotoxin, on haemodynamics and induction of nitric oxide synthase (iNOS) in the anaesthetized rat.

2 Intravenous injection of LTA (10 mg kg^{-1}) resulted in a decrease in blood pressure from 123 ± 1 mmHg to 83 ± 7 mmHg after 270 min ($P<0.001$) and a reduction of the pressor response to noradrenaline (1 μg kg^{-1}) from 33 ± 1 mmHg.min to 23 ± 3 mmHg.min after 270 min ($P<0.05$).

3 The delayed circulatory failure (hypotension and vascular hyporeactivity) caused by LTA was prevented by pretreatment of rats with dexamethasone (10 mg kg^{-1}, 60 min prior to LTA) or the nitric oxide synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA, 10 mg kg^{-1}h^{-1}, i.v. infusion starting 30 min prior to LTA).

4 In contrast, treatment of rats with polymyxin B (0.05 mg kg^{-1}), an agent which binds endotoxin (lipopolysaccharides, LPS), did not affect the delayed circulatory failure caused by LTA. Polymyxin B, however, attenuated the hypotension and vascular hyporeactivity to noradrenaline afforded by endotoxaemia (2 mg kg^{-1} LPS, i.v.) for 270 min.

5 The delayed circulatory failure caused by LTA was associated with a time-dependent increase in (i) the expression of iNOS protein in the lung (Western blot analysis), and (ii) iNOS activity. This increase in iNOS protein and activity was prevented by pretreatment of LTA-rats with dexamethasone (10 mg kg^{-1}).

6 Intravenous injection of LTA resulted in an increase in serum tumour necrosis factor (TNF)-α (maximum at 90 min after LTA), which was attenuated by pretreatment of rats with dexamethasone (10 mg kg^{-1}, 60 min prior to LTA). The magnitude of the rise in TNF-α caused by LTA was similar to the one elicited by LPS (10 mg kg^{-1}, i.v.).

7 Thus, an enhanced formation of nitric oxide following the induction of iNOS contributes importantly to the delayed vascular failure (hypotension and vascular hyporeactivity) caused by LTA in the anaesthetized rat. We suggest that the endogenous release of TNF-α contributes to the induction of iNOS caused by LTA *in vivo*.

Keywords: Nitric oxide; lipoteichoic acid; gram-positive shock; circulatory shock; hypotension; noradrenaline

Introduction

Nitric oxide (NO) is a potent endogenous vasodilator produced from the guanidino nitrogen group of L-arginine by nitric oxide synthase (NOS). Different isoforms of NOS have recently been sequenced, cloned and expressed (for review, see Nathan, 1992; Knowles and Moncada, 1994). Under physiological conditions, the release of NO from endothelial cells by the constitutive NOS (eNOS) causes dilatation of the underlying vascular smooth muscle and contributes importantly to the regulation of organ blood flow and blood pressure (Moncada *et al.*, 1991). In addition, bacterial lipopolysaccharides (LPS, endotoxin) and a number of cytokines, such as tumour necrosis factor (TNF)-α, cause the expression of a distinct, inducible isoform of NOS (iNOS) in a wide variety of cells including macrophages, endothelial and vascular smooth muscle cells (for review, see Moncada *et al.*, 1991; Thiemermann, 1994). In contrast to eNOS, iNOS is calcium-independent and produces large amounts of NO for relatively long periods. The NO released following induction of iNOS in activated macrophages is cytostatic/cytotoxic to certain microbial pathogens and tumour cells. Overproduction of NO following iNOS induction in vascular smooth muscle cells makes an important contribution to the circulatory failure (hypotension and vascular hyporeactivity to vasoconstrictor agents) in endotoxin shock and to the delayed vascular decompensation in haemorrhagic shock (for review, see Thiemermann, 1994).

There are numerous studies which have used LPS in animals and man to elucidate the sequence of the pathophysiological events which lead to the 'systemic inflammatory response syndrome' (SIRS) and ultimately circulatory shock. Although these studies help to gain a better understanding of the pathophysiology of gram-negative shock, they may only provide a limited insight into the pathophysiology of circulatory shock caused by gram-positive bacteria, for these organisms do not contain endotoxin. However, the prevalence of sepsis resulting from gram-positive organisms has risen markedly in the last decade, and it is possible that cases of gram-positive sepsis may predominate in the years to come (Bone, 1994). A gram-positive organism contains various not very well characterized substances, that may initiate the release of cytokines, SIRS, or shock (Bone, 1993). Moreover, little is known about the pathophysiological importance of NO in gram-positive sepsis. Lipoteichoic acid (LTA), a cell-wall component from gram-positive bacteria, causes an enhanced

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release of NO due to induction of iNOS in cultured vascular smooth muscle cells (Auguet *et al.*, 1992; Lonchampt *et al.*, 1992) and in murine peritoneal macrophages (Cunha *et al.*, 1993). LTA also stimulates the release of cytokines, such as TNF-α, from human isolated blood monocytes (Lindermann *et al.*, 1988; Bhakdi *et al.*, 1991) and murine peritoneal macrophages (Kuwano *et al.*, 1993). This study investigates the effect of LTA isolated from the gram-positive bacterium, *Staphylococcus aureus*, on haemodynamics and induction of iNOS in anaesthetized rats.

Methods

Haemodynamic measurements

Animal experiments were performed in accordance with the Home Office regulations. Male Wistar rats (200–300 g, Glaxo Laboratories Ltd, Greenford, Middlesex) were anaesthetized with thiopentone sodium (Trapanal, 3% solution, 120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration, and rectal temperature was maintained at 37°C with a homeothermic blanket (BioSciences, Sheerness, Kent). The right carotid artery was cannulated with a catheter filled with heparinised saline (50 iu ml^{-1} heparin in 0.9 M NaCl) and connected to a pressure transducer (model P23XL, Spectramed, Stratham, NH, U.S.A) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate, which were registered on a polygraph recorder (model 7D, Grass Instruments, Quincy, MA, U.S.A). The right femoral and right jugular veins were cannulated for the administration of drugs.

After the surgical procedure, cardiovascular parameters were allowed to stabilize for 20 min. After recording baseline haemodynamic parameters, the pressor response to noradrenaline (1 μg kg^{-1}, i.v.) was determined. Subsequently, animals received vehicle (0.2 ml 0.9% NaCl) or LTA from *Staphylococcus aureus* (10 mg kg^{-1}) as a slow intravenous injection over 2 min. The pressor responses to noradenaline were reassessed at 60, 120, 180, 240, 300, 360 min after the administration of LTA. At the end of the experiment, lung, spleen, liver, heart and brain were removed, snap frozen in liquid nitrogen and stored at −80°C for the measurement of iNOS activity (see below).

To investigate the role of NO in the haemodynamic changes elicited by LTA, rats were (i) pretreated with the glucocorticosteroid dexamethosone (10 mg kg^{-1}, i.v., 60 min prior to LTA), a potent inhibitor of the induction of iNOS (Radomski *et al.*, 1990), or (ii) treated with the competitive NOS inhibitor N^G-monomethyl-L-arginine (L-NMMA, 10 mg kg^{-1} h^{-1}, i.v. starting 30 min prior to the injection of LTA and continuing until the end of the experiments). In these experiments, the pressor responses to noradrenaline (1 μg kg^{-1}, i.v.) were assessed 10 min before the administration of LTA and 60 and 270 min after LTA. To exclude the possibility that endotoxaemia accounts for the haemodynamic effects afforded by LTA, a separate group of animals was treated with LTA together with polymyxin B (0.05 mg kg^{-1}, i.v.), which binds and inactivates endotoxin. To ensure that this dose of polymyxin B is sufficient to attenuate the haemodynamic effects caused by endotoxin (positive control), rats received polymyxin B (0.05 mg kg^{-1}, i.v.) together with LPS (2 mg kg^{-1}, i.v.).

In a separate set of experiments, rats were treated with either vehicle (control) or LTA (10 mg kg^{-1}, i.v.) for 90, 180 or 270 min. At the end of each experiment, blood samples were obtained to measure white blood cell counts, TNF-α levels in serum and nitrite concentration in plasma. Subsequently, all animals were killed and the lung was removed and snap frozen in liquid nitrogen in order to evaluate the time-dependent changes in iNOS protein expression [Western (immuno)blot analysis] and iNOS activity.

Serum TNF-α levels

TNF-α levels were measured in the serum with a mouse TNF-α enzyme-linked immunoabsorbent assay (ELISA) kit from Genzyme (Cambridge, MA, U.S.A) which has also been used successfully to quantitate natural rat TNF-α (Pizarro *et al.*, 1993). The samples were measured according to the instruction delivered with the ELISA kit by the supplier. For comparison, TNF-α levels were also measured in the serum obtained from LPS-treated rats at 90 min and 180 min after injection of LPS (10 mg kg^{-1}, i.v.).

Plasma nitrite concentration

Nitrite is the primary oxidation product of NO and, hence, the nitrite concentration in plasma was determined as an indicator of changes in NO production. Nitrite was assayed by adding 0.8 ml Griess reagent (4% sulphonilamide and 0.2% naphtylenediamide in 10% phosphoric acid) to 0.2 ml plasma. After centrifugation, the difference in optical density between 540 mm and 600 nm was measured with a spectrophotometer. Nitrite concentrations (μM) were calculated by comparison with the optical density of standard solutions of sodium nitrite prepared in plasma.

NOS activity assay

Frozen organs (lung, spleen, liver, heart, brain) were homogenized on ice with an Ultra-Turrax T 25 in a Tris-buffer (pH = 7.4) composed of (mM) Tris-HCl 50, EDTA 0.1, EGTA 0.1, 2-mercaptoethanol 12 and phenylmethylsulphonylfluoride 1. Conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the homogenates as previously described (Szabó *et al.*, 1993a). Briefly, tissue homogenates (30 μl, approximately 100 μg protein) were incubated in the presence of L-arginine/[^{3}H]-L-arginine (10 μM, 7.4 kBq per tube), NADPH (1 mM), calmodulin (300 u ml^{-1}), tetrahydrobiopterin (5 μM), L-valine (50 mM) and calcium (1 mM) for 30 min at room temperature in Tris-buffer (total reaction volume 100 μl). Reactions were stopped by addition of 1 ml ice-cold HEPES buffer (pH = 5.5) containing HEPES (20 mM), EDTA (2 mM) and EGTA (2 mM). Reaction mixtures were applied to DOWEX 50W (sodium form) columns, and the eluted [^{3}H]-L-citrulline activity was measured by scintillation counting (model, Beckman Instruments Inc, Fullerton, California). Experiments performed in the absence of NADPH determined the extent of [^{3}H]-L-citrulline formation independent of NOS activity. Experiments in calcium-free buffer containing NADPH and EGTA (1 mM) determined the calcium-independent (i.e., induced) NOS activity. Protein concentrations were measured spectrophotometrically in 96-well plates by the method of Bradford with bovine serum albumin used as standard (Bradford, 1976).

Western (immuno)blot analysis

Lungs were homogenized on ice with an Ultra-Turrax T 25 homogenizer in an extraction buffer (pH = 7.4) consisting of 50 mM Tris-HCl, 10 mM EDTA, 1% v/v Triton X-100, and the protease inhibitors pepstatin A 50 μM, leupeptin 0.2 mM and phenylmethylsulphonylfluoride 1 mM. The homogenates were centrifuged (5000 *g*) for 15 min at 4°C and the supernatant was boiled for 10 min with gel-loading buffer (Tris 20 mM, EDTA 2 mM, SDS 2% w/v, glycerol 20% v/v, 2-mercaptoethanol 10% v/v, bromophenol blue 2 mg ml^{-1}, pH = 6.8) in a ratio of 1:1 (v/v). Cell extracts from bovine aortic endothelial cells in culture and murine macrophages (J774 cell line) activated with LPS (1 μg ml^{-1} for 24 h) were used as a positive control for the presence of eNOS and iNOS protein, respectively. The proteins in the samples were resolved by one dimensional gel electrophoresis (7.5% SDS gel) with molecular weight markers (SDS-7B; Sigma). After transfer to nitrocellulose by electrophoresis, the membranes

were primed overnight at 4°C with a polyclonal antibody raised to macrophage iNOS developed in rabbits (a generous gift from Eurodiagnostica, Sweden) or a monoclonal mouse antibody raised to constitutive, endothelial NOS (Affinity Research Products Ltd., U.K.). The blots were then incubated as appropriate with either anti-rabbit or anti-mouse IgG linked to horseradish peroxidase. All antibodies were used at a 1:1000 dilution. Subsequently, the Western blots were developed with diaminobenzamine used as a substrate.

Drugs

Unless stated otherwise, all compounds were purchased from Sigma (Dorset). Heparin (Multiparin) was obtained from Evans Medical (Middlesex) U.K. and thiopentone sodium (Intraval Sodium) from Rhône Mérieux Ltd. (Harlow, Essex). Solutions for injection were prepared in nonpyrogenic saline (0.9% NaCl; Baxter Healthcare Ltd., Thetford, Norfolk) and care was taken to prevent endotoxin contamination.

Data analysis

All data are presented as mean ± standard error of the mean (s.e.mean) of n independent experiments. The vasopressor response to noradrenaline was evaluated determining the area under the curve and was expressed in mmHg·min. Statistical analysis was performed by (one- or two-way) analysis of variance (ANOVA). If appropriate, this was followed by Bonferroni's test (one-way ANOVA) or Fisher's test (two-way ANOVA) for multiple comparison of single means.

Results

Circulatory failure induced by LTA

Injection of LTA (10 mg kg^{-1}) resulted in an initial fall in MAP from 123 ± 1 mmHg (time 0, control) to 101 ± 3 mmHg at 60 min ($P<0.01$). This was followed by a second fall in MAP from 104 ± 6 mmHg at 150 min to 83 ± 7 mmHg at 270 min. Thereafter, the MAP only gradually declined reaching 79 ± 4 mmHg at 360 min after LTA injection. The decrease in MAP observed in LTA-treated rats was significantly greater than the one observed in vehicle-treated rats (Figure 1a), in which the MAP showed a small, gradual decline from 122 ± 3 mmHg (at time 0) to 106 ± 7 mmHg at 360 min after injection of vehicle. There was no significant effect of LTA on heart rate (results not shown). In addition, the pressor response elicited by noradrenaline (1 μg kg^{-1}) was significantly reduced at 60, 120, 300 and 360 min in LTA-treated rats, but not in rats receiving vehicle (Figure 1b).

All subsequent experiments designed to elucidate the mechanism of the circulatory failure elicited by LTA were limited to 270 min, as both hypotension and vascular hyporeactivity to noradrenaline were near maximal at this time point. Injection of polymyxin B (0.05 mg kg^{-1}) together with LTA (10 mg kg^{-1}) neither inhibited hypotension to LTA (Figure 2a) nor the vascular hyporeactivity to noradrenaline (Figure 2b). When given together with LPS (2 mg kg^{-1}),

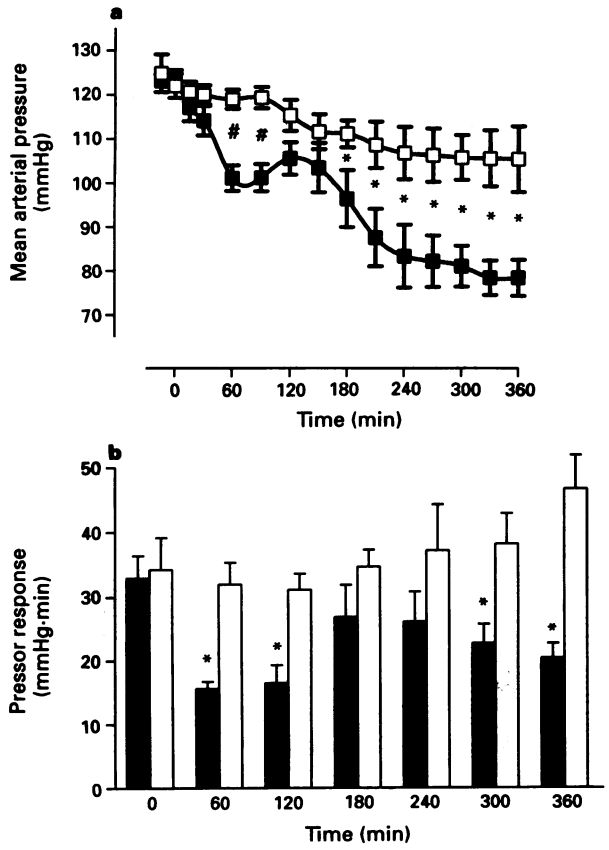

Figure 1 Hypotension (a) and attenuation of the pressor response to noradrenaline (b) (1 μg kg^{-1}, i.v.) elicited by lipoteichoic acid (LTA, 10 mg kg^{-1}, i.v.). Rats were treated with LTA (a: ■; b: solid columns, $n=8$) or vehicle (a: □; b: open columns, $n=5$). Results are expressed as mean ± s.e.mean. *$P<0.05$, *$P<0.01$ LTA vs. vehicle (two-way ANOVA followed by Fisher's test).

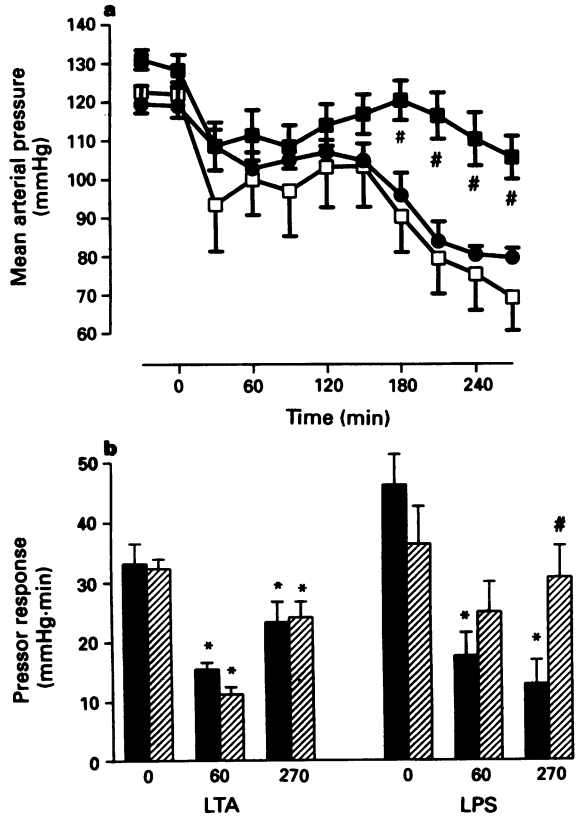

Figure 2 The delayed hypotension (a) and the vascular hyporeactivity to noradrenaline (b) elicited by endotoxin (lipopolysaccharide, LPS, 10 mg kg^{-1}, i.v.), but not by lipoteichoic acid (LTA, 10 mg kg^{-1}, i.v.) is inhibited by polymyxin B (0.05 mg kg^{-1}, i.v.). Rats were treated with LPS (a) □, b: solid columns), LPS and polymyxin B (a: ■, b: hatched columns), LTA (a: not shown -see Figure 3a, b: solid columns), or LTA and polymyxin B (a: ●, b: hatched columns). Results are expressed as mean ± s.e.mean. # $P<0.01$ (a), $P<0.05$ (b) LPS vs. LPS plus polymyxin B (two-way ANOVA followed by Fisher's test) and *$P<0.05$ vs time 0 (B only; one-way ANOVA followed by Bonferroni's test).

however, polymyxin B (0.05 mg kg^{-1}) attenuated both the fall in MAP as well as the vascular hyporeactivity to noradrenaline (at time 270 min) elicited by LPS (Figure 2). Although LPS in the presence of polymyxin B still caused some immediate decrease in MAP and vascular hyporeactivity to noradrenaline (60 min), this was no longer statistically significant compared to time 0 (just prior to LPS injection).

Inhibition of LTA-induced circulatory failure

Pretreatment of the animals with dexamethasone (10 mg kg^{-1}, i.v., 1 h prior to LTA) prevented the early and the delayed fall in MAP elicited by LTA (Figure 3a). In addition, dexamethasone pretreatment prevented the hyporeactivity to noradrenaline observed at 60 and 270 min after LTA injection (Figure 3b). Surprisingly, in control (vehicle treated)-rats pretreated with dexamethasone, the pressor responses to noradrenaline (measured as area under the curve) increased over time from 32 ± 6 mmHg·min (time 0), 35 ± 7 mmHg·min at 60 min to 51 ± 8 mmHg·min at 270 min ($P<0.05$) after injection of vehicle. There was no significant difference in the pressor response to noradrenaline between dexamethasone pretreated rats receiving vehicle or LTA.

Infusion of the NOS inhibitor L-NMMA (10 mg kg^{-1} h^{-1}) caused within 30 min a significant increase in MAP from 126 ± 4 mmHg to 141 ± 4 mmHg ($P<0.05$). The subsequent administration of LTA resulted in a fall in MAP to 118 ± 3 mmHg at 60 min ($P<0.01$). However, the delayed hypotension caused by LTA was significantly attenuated by infusion of L-NMMA (Figure 3a). In addition, infusion of L-NMMA also prevented the LTA-induced vascular hyporeactivity to noradrenaline (Figure 3b).

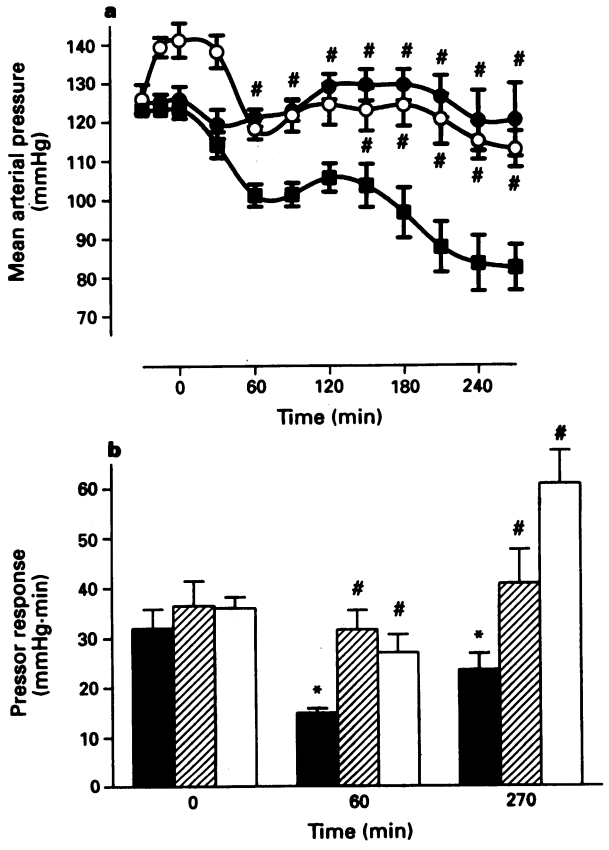

Figure 3 Inhibition of the hypotension (a) and vascular hyporeactivity to noradrenaline (b) elicited by lipoteichoic acid (LTA, 10 mg kg^{-1}, i.v.) by treatment of rats with dexamethasone (10 mg kg^{-1}, i.v., 1 h before LTA) or N^G-monomethyl-L-arginine (L-NMMA, 10 mg kg^{-1} h^{-1}, i.v., continuous infusion starting 30 min before LTA). Rats were treated with LTA (a: ■, b: solid columns), LTA and L-NMMA (a: ○, b: hatched columns), or LTA and dexamethasone (a: ●, b: open columns). Results are expressed as mean ± s.e.mean. #$P<0.01$ LTA vs LTA plus treatment (dexamethasone or L-NMMA) (two-way ANOVA followed by Fisher's test), and *$P<0.05$ vs time 0 (b only; one-way ANOVA followed by Bonferroni's test).

Induction of NOS by LTA

At 360 min after LTA injection, there was a significant increase in total NOS and calcium-independent iNOS activity in organ homogenates prepared from lung, spleen and liver (Figure 4). In contrast, no iNOS activity was found in homogenates from either brain or heart of rats treated for 360 min with LTA. The constitutive, neuronal NOS activity measured in brain homogenates was similar in LTA- and vehicle-treated rats (Figure 4).

The time-course of the expression of iNOS activity elicited by LTA is depicted in Figure 5. No significant increase in iNOS activity was observed in lungs obtained from rats treated with LTA for 90 min. However, a significant and progressive increase in iNOS activity was observed in lungs obtained from rats treated with LTA for 180 or 270 min. Similarly, plasma nitrite levels were not significantly elevated at 90 min after LTA administration, while a significant increase in plasma nitrite was observed at 180 and 270 min (Figure 5). Western blot analysis demonstrated a protein of approximately 130 kDa which was recognised by the iNOS antibody. A time-dependent expression of a 130 kDa protein recognised by the iNOS antibody was demonstrated by Western blot analysis in homogenates from lung obtained from rats treated with LTA (Figure 6). The iNOS antibody showed some cross reactivity with eNOS, but the protein recognised by the iNOS antibody was not recognised by the eNOS antibody. Furthermore, treatment of rats with LTA did not result in any alteration in the expression of eNOS protein (results not shown). Dexamethasone (10 mg kg^{-1}, i.v., 1 h prior to LTA) inhibited the expression of iNOS protein in the lung (Figure 6). Moreover, dexamethasone prevented the increase in iNOS activity caused by LTA (at 270 min) in the lung (LTA alone: 113 ± 10, and LTA plus dexamethasone: 2 ± 6 pmol L-citrulline 30 min^{-1} mg^{-1} protein; $P<0.001$).

Increase in serum TNF-α levels caused by LTA

Serum TNF-α levels were below the detection limit (35 pg ml^{-1}) in serum obtained from rats prior to the injection of LTA. However, after the injection of LTA, serum TNF-α levels rose markedly to 3834 ± 330 pg ml^{-1} at 90 min

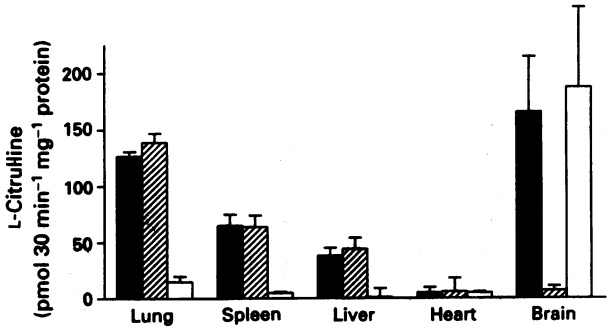

Figure 4 Induction of nitric oxide synthase activity (iNOS) by lipoteichoic acid (LTA, 10 mg kg^{-1}, i.v.) The conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in homogenates from organs obtained from rats 6 h after treatment with vehicle (open columns) or LTA (solid columns). Hatched columns represent iNOS activity in the absence of calcium and in the presence of EGTA (1 mM) in homogenates from organs obtained from LTA-treated rats. Results are expressed as mean ± s.e.mean.

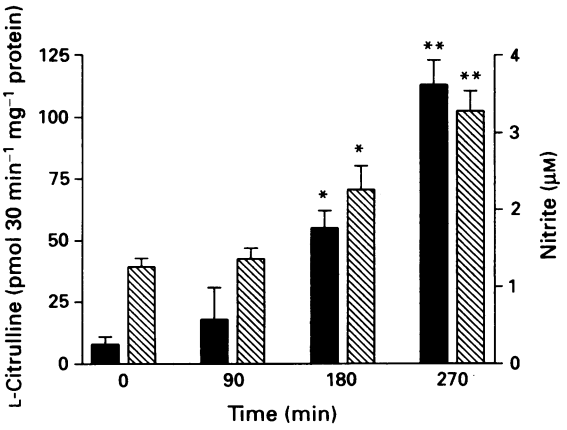

Figure 5 Time course of the induction of nitric oxide synthase activity (iNOS, solid columns) and the increase in plasma nitrite concentration (hatched columns) by lipoteichoic acid (LTA, 10 mg kg^{-1}, i.v.). The conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the absence of calcium and in the presence of EGTA (1 mM) in homogenates from lungs. Lungs and plasma were obtained at time 0 (control) or 90, 180 or 270 min after injection of LTA. Results are expressed as mean ± s.e.mean. *$P<0.05$ and **$P=0.01$ LTA vs. time 0 (control) (one-way ANOVA followed by Bonferroni's test).

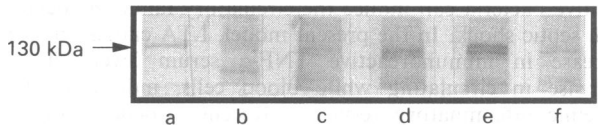

Figure 6 Time course of the expression of inducible nitric oxide synthase (iNOS) protein by lipoteichoic acid (LTA, 10 mg kg^{-1}, i.v.) determined by Western (immuno)blot analysis of homogenates from lung. The iNOS antibody recognised a protein at a molocular weight of approximately 130 kDa. The immunoblot presents in lane (a) endotoxin (1 μg ml^{-1}) activated murine macrophages (J774 cell line) (positive control), (b) time 0 (control rats), (c) LTA for 90 min, (d) LTA for 180 min, (e) LTA for 270 min, and (f) LTA for 270 min plus dexamethasone (10 mg kg^{-1}, i.v. 1 h before LTA). This immunoblot is representative for 3 separate experiments.

after which they rapidly declined to 498 ± 225 pg ml^{-1} at 180 and to 102 ± 12 pg ml^{-1} at 270 min ($n=6$). Pretreatment of rats with dexamethasone (10 mg kg^{-1}, i.v., 60 min prior to LTA) markedly attenuated the maximum increase in serum TNF-α levels caused by LTA (639 ± 275 pg ml^{-1}, $n=4$, $P<0.01$). Like LTA, injection of LPS (10 mg kg^{-1}) caused a time-dependent increase in serum TNF-α levels from undetectable levels (time 0) to 3553 ± 111 pg ml^{-1} at 90 min and 498 ± 183 pg ml^{-1} at 180 min ($n=3$–4).

Fall in white blood cell counts caused by LTA

Intravenous injection of LTA (10 mg kg^{-1}) caused a significant decrease in total white blood cell counts from 4.1 ± 0.3 × 10^6 ml^{-1} (time 0) to 1.8 ± 0.1 × 10^6 ml^{-1} at 90 min after LTA ($P<0.01$, $n=6$). This leukopenia was maintained throughout the experiment (180 min: 2.1 ± 0.2 × 10^6 ml^{-1}; 270 min: 3.0 ± 0.2 × 10^6 ml^{-1}).

Discussion

This study demonstrates that LTA from *Staphylococcus aureus* causes hypotension and vascular hyporeactivity to noradrenaline (circulatory failure) in the anaesthetized rat. Furthermore, LTA treatment resulted in a time-dependent increase in the expression of iNOS protein and activity, which coincided with an increase in plasma nitrite concentration. Thus, there is an enhanced formation of the vasodilator nitric oxide following the induction of iNOS after LTA. The time course of iNOS induction and plasma nitrite elevation reflect the delayed alterations in haemodynamics seen after LTA. The circulatory failure elicited by LTA occurs in two distinct phases. The first fall in blood pressure after LTA injection levels off after 60 min and is followed by a delayed, second drop in blood pressure starting approximately 150 min after LTA. Similarly, the pressor response to noradenaline was only significantly attenuated during the first (60 to 120 min) and the delayed (from 270 min) fall in blood pressure. Thus, only the delayed circulatory failure after LTA is associated with an elevated NO production following the induction of iNOS. Dexamethasone inhibited the induction of iNOS protein and activity elicited by LTA, explaining its beneficial haemodynamic effects on the circulatory failure elicited by LTA. The NOS inhibitor L-NMMA prevented the second fall in blood pressure and the delayed vascular hyporeactivity, providing further evidence that an enhanced formation of NO contributes to the delayed circulatory failure elicited by LTA.

The early hypotension and vascular hyporeactivity to vasoconstrictor agents in animals with LPS-shock is due to an enhanced formation of NO by the constitutive NOS (Julou-Schaeffer *et al.*, 1990; Kilbourn *et al.*, 1990; Thiemermann & Vane, 1990; Szabó *et al.*, 1993a). Here, NO release due to activation of the constitutive NOS also accounts for the acute vascular hyporeactivity caused by LTA in the anaesthetized rat, for (i) this vascular hyporeactivity was abolished by L-NMMA and (ii) no induction of iNOS in the lung was demonstrated within the first 90 min after LTA injection. Infusion of L-NMMA attenuated, but not abolished, the immediate hypotension caused by LTA. As L-NMMA caused a significant rise in blood pressure prior to injection of LTA, this effect of L-NMMA may in part be due to a functional antagonism. Dexamethasone prevented both the acute hypotension and acute vascular hyporeactivity elicited by LTA. As dexamethasone prevents the formation of prostanoids (Flower & Blackwell, 1979) and platelet-activating factor (Braquet *et al.*, 1987), an enhanced release of these inflammatory mediators may well mediate the early haemodynamic effects caused by LTA *in vivo*.

How gram-positive organisms initiate an inflammatory response is not yet clear. Animal models of sepsis have usually employed endotoxin, a constituent of the cell wall of gram-negative bacteria, which is regarded as the primary initiator of gram-negative septic shock (Suffredini *et al.*, 1989; Danner *et al.*, 1991). In the present study, LTA from the cell wall of *Staphylococcus aureus* induced a sustained decrease in blood pressure and a hyporeactivity to noradrenaline, that was not affected by the LPS neutralising agent, polymyxin B. In contrast, the delayed circulatory failure elicited by LPS was inhibited by polymyxin B. Thus, a rise in plasma LPS caused by contamination of LTA or by bacterial translocation from the intestine, is unlikely to account for the delayed hypotension and hyporeactivity to noradrenaline observed after LTA. Similarly, by using a well-characterized canine model of septic shock, Natanson *et al.* (1989) demonstrated that *Staphylococcus aureus* in the absence of endotoxaemia, induced the same cardiovascular abnormalities of septic shock as did *Escherichia coli*. Heat-killed *Staphylococcus epidermis* (a gram-positive organism) also induces a shock-like state without causing endotoxaemia in anaesthetized rabbits (Wakabayashi *et al.*, 1991). Thus, the present results further demonstrate that circulatory failure associated with septic shock can occur in the absence of endotoxin. Moreover, the present study shows that one component of the cell wall from gram-positive bacteria, LTA, may alone be sufficient to elicit cardiovascular abnormalities which are similar to those observed in septic shock.

Little is known about the mechanism by which gram-

positive bacteria can induce the circulatory failure associated with septic shock. In the present model, LTA causes an early increase in immunoreactive TNF-α serum levels and a decrease in circulating white blood cells, indicative of a systemic inflammatory response. Recent evidence indicates that inflammatory cytokines, such as TNF-α and interleukin (IL)-1, are key mediators of some of the pathophysiological events in septic shock caused by gram-positive bacteria. In a murine model of septic shock employing gram-positive bacteria, a causal relationship between the release of TNF-α and mortality has been reported (Freudenberg & Galanos, 1991) and, in the anaesthetized rabbit, release of interleukin-1 has been implicated in the hypotension induced by *Staphylococcus epidermis* (Aiura *et al.*, 1993). In the anaesthetized rat, IL-1 receptor antagonists and antibodies against TNF-α reduce not only the circulatory failure elicited by LPS, but also the induction of iNOS (Szabó *et al.*, 1993b; Thiemermann *et al.*, 1993). Interestingly, in the present study, expression of iNOS protein and activity followed the initial elevation in serum TNF-α and both effects were markedly attenuated by pretreatment with dexamethasone. Therefore, we suggest that the release of inflammatory cytokines such as TNF-α caused by LTA initiates translation and *de novo* iNOS protein synthesis resulting in an elevated release of NO.

The incidence of sepsis by gram-positive organisms has risen markedly and may predominate in the coming years (Bone, 1994). Therefore, it is important to develop animal models without endotoxaemia in order to elucidate the pathophysiological events leading to the circulatory failure caused by gram-positive organisms. These models are also useful to evaluate the effects of novel therapeutics which are potentially useful in septic shock. The demonstration that novel therapeutics exert beneficial effects in septic shock caused by gram-negative and gram-positive bacteria is of particular importance, for these agents are often (in clinical trials) given to patients regardless of the type of infectious organism. Thus, an enhanced release of NO following the induction of iNOS by LTA from *Staphylococcus aureus* is important for the delayed circulatory failure associated with septic shock.

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