Role of tumour necrosis factor in the induction of nitric oxide synthase in a rat model of endotoxin shock

¹Christoph Thiemermann, Chin-Chen Wu, Csaba Szabó, Mauro Perretti & John R. Vane

The William Harvey Research Institute, St. Bartholomew's Hospital Medical College, Charterhouse Square, London, EC1M 6BQ

- 1 This study investigates the role of tumour necrosis factor (TNF) in the induction of nitric oxide synthase (NOS) by bacterial endotoxin (lipopolysaccharide; LPS) in a rat model of endotoxin shock.
- 2 In anaesthetized rats, pretreatment with a monoclonal antibody for TNF (TNF_{ab}; 20 mg kg⁻¹, s.c., at 16 h prior to LPS) ameliorated the fall in mean arterial blood pressure (MAP) in response to LPS (2 mg kg⁻¹, i.v.). For instance, endotoxaemia for 180 min resulted in a fall in MAP from 114 ± 6 (control) to 84 ± 5 mmHg ($P < 0.01$; $n = 7$). In contrast, animals pretreated with TNF_{ab} prior to LPS injection maintained significantly higher MAP when compared to LPS-control (MAP at 180 min; 118 ± 3 mmHg; $P < 0.01$, $n = 5$).
- 3 Three hours of endotoxaemia was also associated with a significant reduction of the contractile effects of noradrenaline (NA) (10^{-8} – 10^{-6} M) on the thoracic aorta *ex vivo*. This hyporeactivity to NA was partially restored by *in vitro* treatment of the vessels with N^G-nitro-L-arginine methyl ester (L-NAME, 20 min, 3×10^{-4} M). Pretreatment of rats with TNF_{ab} (20 mg kg⁻¹; at 16 h prior to LPS) significantly ($P < 0.05$) attenuated the LPS-induced hyporeactivity of rat aortic rings *ex vivo*. L-NAME did not enhance the contractions of aortic rings obtained from TNF_{ab} pretreated LPS-rats.
- 4 At 180 min after LPS there was a significant elevation of the induced NOS activity in the lung (5.14 ± 0.57 pmol citrulline mg⁻¹ min⁻¹, $n = 8$). TNF_{ab} pretreatment significantly attenuated this induction of NOS in response to LPS by $37 \pm 6\%$ ($n = 5$; $P < 0.05$).
- 5 We conclude that the formation of endogenous TNF contributes to the induction of the calcium-independent isoform of NOS in response to LPS *in vivo*. Thus, the beneficial effects of agents which inhibit either the release or the action of TNF in circulatory shock may be, in part, due to inhibition of NOS induction.

Keywords: Nitric oxide; endotoxin shock; vasodilatation; contraction; aorta; noradrenaline

Introduction

Tumour necrosis factor (TNF) is a primary mediator of circulatory shock (Tracey *et al.*, 1986; 1987; see Billiau & Vanderkerckhove, 1991, for review). Administration of TNF alone, or in combination with low (otherwise ineffective) doses of endotoxin mimics several cardiovascular features of circulatory shock, including hypotension, peripheral vasodilatation, and organ damage (for review see Billiau & Vanderkerckhove, 1991). Elevated plasma concentrations of TNF are found in endotoxaemia (Beutler *et al.*, 1985; Waage, 1987; Michie *et al.*, 1988; Feuerstein *et al.*, 1990; Klosterhafen *et al.*, 1992). In addition, antibodies directed against TNF (Tracey *et al.*, 1987; Mathison *et al.*, 1988; Hinshaw *et al.*, 1990; Silva *et al.*, 1990; Walsh *et al.*, 1992), or agents which inhibit the release of TNF, such as pentoxifylline (Schade, 1990), exert protective effects in animal models of endotoxin shock.

An enhanced formation of NO in response to LPS is an important mediator of hypotension, peripheral vasodilatation and vascular hyporeactivity to vasoconstrictor agents in endotoxaemia (Julou-Schaeffer *et al.*, 1990; Thiemermann & Vane, 1990; Kilbourn *et al.*, 1990; Wright *et al.*, 1992; Meyer *et al.*, 1992; Szabó *et al.*, 1993a). LPS induces a calcium-independent nitric oxide (NO) synthase (NOS) in various cells (including macrophages and vascular smooth muscle cells *in vitro*), as well as in whole organs such as lung, liver and spleen *in vivo*, resulting in an enhanced formation of NO (see Moncada *et al.*, 1991; Kilbourn & Griffith, 1992; Nathan, 1992). Corticosteroids, which inhibit the induction of this calcium-independent isoform of NO synthase (NOS)

in response to LPS (Radomski *et al.*, 1990; Knowles *et al.*, 1990) exert beneficial effects in circulatory shock (Wright *et al.*, 1992; Szabó *et al.*, 1993a).

Like LPS, TNF also induces the calcium-independent isoform of NOS *in vitro* (Drapier *et al.*, 1988; Kilbourn & Belloni, 1990). Systemic administration of TNF increases NO production (Kosaka *et al.*, 1992) and causes NO-mediated vasodilatation (Kilbourn *et al.*, 1990) and vascular hyporeactivity to vasoconstrictors *in vivo* (Vicaute & Baundry, 1992) and *ex vivo* (Takahashi *et al.*, 1992; Foulkes & Shaw, 1992).

Thus, there is strong experimental evidence suggesting that (i) TNF is a key mediator of endotoxin shock; (ii) TNF can induce NOS *in vitro* and (iii) NOS induction leading to an enhanced formation of NO contributes to the cardiovascular failure in endotoxin shock. The importance of TNF in the induction of NOS *in vivo*, however, has not yet been adequately studied. Here we investigate whether the endogenous production of TNF contributes to the induction of NOS in response to LPS in the anaesthetized rat.

Methods

Haemodynamic measurements

Male Wistar rats (250–290 g; Glaxo Laboratories Ltd., Greenford, Middx.) were anaesthetized with thiopentone sodium (Trapanal; 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 (Bio-Sciences, Sheerness, Kent). The right carotid artery was cannulated and connected to a pressure transducer (P23XL,

¹ Author for correspondence.

Spectramed, Statham, U.S.A.) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate (HR) which were displayed on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, Mass, U.S.A.). Left or right femoral veins were cannulated for the administration of drugs.

Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 15 min. After recording baseline haemodynamic parameters, animals received *E. coli* LPS (2 mg kg⁻¹, i.v.; in 0.3 ml saline) as a slow injection over 2 min and were monitored for 180 min. The above protocol was used in vehicle-treated control rats and in rats pretreated subcutaneously with monoclonal antibody for TNF_α (TNF_{ab}; 20 mg kg⁻¹, 16 h prior to LPS). Such treatment produces maximal plasma concentrations at 16 h (Perretti & Flower, 1993). Moreover, this pretreatment with TNF_{ab} neutralizes the TNF_α-mediated cytotoxic effect of plasma obtained from rats at 60 min after *E. coli* LPS (1 mg kg⁻¹, i.p.) in L-929 fibroblasts (Perretti & Peers, unpublished observation).

Organ bath experiments

At 180 min after the injection of LPS, thoracic aortae were obtained from rats pretreated either with vehicle (control) or with TNF_{ab}. In addition, control aortic rings were obtained from male Wistar rats anaesthetized as above and subsequently exsanguinated. The vessels were rapidly removed, cleared of adhering periadventitial fat and cut into rings of 2 mm width. The endothelium was removed by rubbing. The rings were mounted in 20 ml organ baths filled with warmed (37°C), oxygenated (95% O₂/5% CO₂) Krebs solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH₂PO₄ 1.2, MgSO₄ 1.17, CaCl₂ 2.5, NaHCO₃ 25 and glucose 5.6. Isometric force was measured with Biegestab K30 type 351 transducers (Hugo Sachs Elektronik, Germany) and recorded on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, Mass, U.S.A.). A tension of 2 g was applied and the rings were equilibrated for 60 min, changing the Krebs solution every 15 min. In every experimental group, dose-response curves to noradrenaline (10⁻⁹–10⁻⁶ M) were obtained before and after N^G-nitro-L-arginine methyl ester (L-NAME; 20 min, 3 × 10⁻⁴ M).

Nitric oxide synthase assay

Lungs from animals treated with LPS (in the absence [control] or in the presence of TNF_{ab} pretreatment) were removed at 180 min and frozen in liquid nitrogen. Lungs from sham-operated rats were also prepared for determination of baseline NOS activity. Lungs were stored for no more than 2 weeks at -80°C before assay. Frozen lungs were homogenized on ice with an Ultra-Turrax T 25 homogenizer (Janke & Kunkel, IKA Labortechnik, Staufen i. Br., Germany) in a buffer composed of (mM): Tris-HCl 50, EDTA 0.1 mM, EGTA 0.1 mM, 2-mercaptoethanol 12 mM and phenylmethylsulphonyl fluoride 1 mM (pH 7.4). Conversion of [³H]-arginine to [³H]-citrulline was measured in the homogenates as described by Mitchell *et al.* (1991) and Szabó *et al.* (1993a). Briefly, tissue homogenates (50 µl, approx. 100 µg protein) were incubated in the presence of [³H]-L-arginine (10 µM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 µM) and calcium (2 mM) for 35 min at 37°C in HEPES buffer (pH 7.5). Reactions were stopped by dilution with 1 ml of ice cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na⁺ form) columns and the eluted [³H]-L-citrulline activity was measured by scintillation counting (Beckman, LS3801; Fullerton, CA, U.S.A.). Experiments performed in the absence of NADPH determined the extent of [³H]-L-citrulline formation independent of a specific NOS activity. Experiments in the presence of NADPH, without calcium and with EGTA (5 mM), deter-

mined the calcium-independent (i.e. induced) NOS activity.

Protein concentration was measured spectrophotometrically in 96-well plates with Bradford reagent (Bradford, 1976), using bovine serum albumin as standard.

Materials

Calmodulin, bacterial lipopolysaccharide (*E. coli* serotype 0.127:B8), NADPH, N^G-nitro-L-arginine methyl ester, noradrenaline bitartrate, and Dowex 50W anion exchange resin were obtained from Sigma Chemical Co. (Poole, Dorset). Monoclonal antibody for murine TNF_α (raised in hamster; TN3.19.12) was supplied by Celltech (Slough, Bucks). All solutions were made in saline. L-[2,3,4,5-³H] arginine hydrochloride was obtained from Amersham (Buckinghamshire, U.K.). Tetrahydrobiopterin (6R-L-erythro-5,6,7,8-tetrahydrobiopterin) was obtained from Dr B. Schircks Laboratories (Jona, Switzerland).

Statistical evaluation

All values in the figures and text are expressed as mean ± standard error of the mean of *n* observations, where *n* represents the number of animals, or the number of blood vessels studied. Student's paired or unpaired *t* tests were used to compare means among or between groups, respectively. A *P*-value less than 0.05 was considered to be statistically significant.

Results

TNF_{ab} protects against LPS-induced cardiovascular changes in the anaesthetized rat

Baseline values for MAP and HR of the vehicle- and TNF_{ab}-pretreated animal groups were 114 ± 6 (*n* = 7) and 118 ± 6 (*n* = 5) mmHg and 423 ± 9 (*n* = 7) and 422 ± 17 (*n* = 5) beats min⁻¹, respectively and were not significantly different between groups. Administration of LPS (2 mg kg⁻¹, i.v.) induced a fall in MAP from 114 ± 6 mmHg to 84 ± 5 mmHg (*n* = 7, *P* < 0.01) at 60 min. Thereafter, MAP remained significantly reduced throughout the 180 min experimental period; e.g. at 180 min, MAP was 84 ± 5 mmHg (*n* = 7) (Figure 1a). LPS injection did not cause a significant change in HR (Figure 1b). LPS-treated animals which had been pretreated with TNF_{ab} maintained higher MAP values when compared to rats treated with LPS alone at 90–180 min (Figure 1a). TNF_{ab} pretreatment of LPS-rats did not affect HR (Figure 1b).

TNF_{ab} prevents the LPS-induced vascular hyporeactivity to noradrenaline ex vivo

In rat aortic rings obtained from sham-operated rats (control), NA caused a dose-dependent increase in vascular tone. Rat aortic rings obtained from rats subjected to a 180 min period of endotoxaemia showed a significant reduction of the contractile responses to NA (*P* < 0.01 at 10⁻⁹–10⁻⁶ M; Figure 2a). TNF_{ab} pretreatment of LPS-rats significantly attenuated the vascular hyporeactivity to NA of rat aortic rings *ex vivo* (*P* < 0.05 at 10⁻⁸–10⁻⁶ M; Figure 2a). However, contractions of the rat aortic rings produced by NA obtained from TNF_{ab} pretreated LPS-rats were still significantly reduced when compared to control responses (*P* < 0.05 at 10⁻⁸–10⁻⁷ M; Figure 2a).

In vitro treatment of vessels with L-NAME, an inhibitor of NO synthase (Moore *et al.*, 1990) (3 × 10⁻⁴ M), did not enhance the contractions to NA in control rings (Figure 2b). In contrast, L-NAME significantly enhanced the contractile responses to NA in aortic rings obtained from vehicle-treated LPS-rats (*P* < 0.05 at 10⁻⁸–10⁻⁶ M) (Figure 2c). However, even after L-NAME the contractile responses of these vessels

Figure 1 Tumour necrosis factor antibody (TNF_{ab}) ameliorates the delayed hypotension in endotoxic shock in the anaesthetized rat. Depicted are the changes in (a) mean arterial blood pressure and (b) heart rate in rats subjected to *E. coli* lipopolysaccharide (LPS, 2 mg kg⁻¹, i.v.). Groups of animals were pretreated either with vehicle (●, *n* = 7), or TNF_{ab} (20 mg kg⁻¹, s.c. for 16 h; ○, *n* = 5). LPS was administered at time 0. Data are expressed as means ± s.e.mean of *n* observations. *P < 0.05 and **P < 0.01 represent significant differences when compared to control at the same time point.

to NA were still significantly smaller when compared to control (*P* < 0.05 at 10⁻⁹–10⁻⁶ M). In contrast, L-NAME did not enhance the NA-induced contractions of rat aortic rings obtained from TNF_{ab}-pretreated LPS-rats (Figure 2d).

TNF_{ab} inhibits the induction of nitric oxide synthase by LPS in vivo

A small, calcium-independent NOS activity was detectable in lung homogenates obtained from sham-operated control animals. After 180 min of endotoxaemia there was a substantial induction of NOS activity in lung homogenates (Figure 3). The activity of this induced NOS was significantly reduced (by 37 ± 6%, *n* = 5, *P* < 0.05) in lungs obtained from TNF_{ab} pretreated LPS-rats (Figure 3).

Discussion

The present study demonstrates that TNF_{ab} attenuates the induction of NOS by LPS in lung homogenates *ex vivo* and it protects against the vascular hyporeactivity of rat aortic rings *ex vivo*. The inhibition of NOS induction by TNF_{ab} was associated with a significant protection against the fall in MAP. Indeed, at 180 min there was a complete recovery of MAP, as compared to LPS alone. Thus, our data suggest that TNF is one of the endogenous mediators of NOS induction by LPS in the lung and aorta of the anaesthetized rat.

LPS treatment results in reduced contractile responsiveness

Figure 2 Tumour necrosis factor antibody (TNF_{ab}) protects against the development of vascular hyporeactivity to noradrenaline (10⁻⁸–10⁻⁶ M) after lipopolysaccharide (LPS) injection *ex vivo* (a) Dose-response curves to noradrenaline (10⁻⁹–10⁻⁶ M) in aortic rings without endothelium obtained from control rats (■, *n* = 5), from LPS-treated rats (●, *n* = 12) and from LPS-treated rats pretreated with TNF_{ab} (○, *n* = 9). (b–d) Dose-response curves to noradrenaline (10⁻⁹–10⁻⁶ M) in aortic rings without endothelium obtained from control rats (■), from LPS-treated rats (●) and from LPS-treated rats pretreated with TNF_{ab} (○) before (solid line) and after (dotted line) *in vitro* L-NAME (3 × 10⁻⁴ M) treatment. Data are expressed as means ± s.e.mean.

Figure 3 Tumour necrosis factor antibody (TNF_{ab}) inhibits induction of the calcium-independent nitric oxide synthase in lung homogenates *ex vivo*. Calcium-independent (induced) nitric oxide synthase (NOS) activity was measured in lung homogenates obtained from control rats (C, *n* = 3) and from rats pretreated with vehicle (LPS, *n* = 7) or TNF_{ab} (20 mg kg⁻¹, s.c. 16 h prior to LPS; TNF_{ab}/LPS *n* = 5) at 180 min after LPS administration (2 mg kg⁻¹, i.v.). **Represents a significant (*P* < 0.01) increase in NOS activity after 180 min of endotoxaemia (LPS) when compared to controls (C); † represents a significant (*P* < 0.05) reduction in the LPS-induced NOS activity in TNF_{ab}-treated animals. Data are expressed as means ± s.e.mean.

to NA in blood vessels such as the rat thoracic aorta *ex vivo*, which is mediated by an enhanced formation of NO by the inducible NOS (Julou-Schaeffer *et al.*, 1990; Rees *et al.*, 1990). The present findings demonstrate that pretreatment of LPS-treated rats with TNF_{ab} significantly attenuated the hyporeactivity to NA in aortic rings *ex vivo*. However, L-NAME only partially restored the contractile response to NA in LPS-treated rats, suggesting that the vascular hyporeactivity to NA following systemic LPS administration is only partially mediated by NO. These results reinforce the contribution made by mechanisms independent of NOS induction to the vascular hyporeactivity to NA. For instance, LPS induces an isoform of cyclo-oxygenase (COX-2) resulting in an enhanced formation of cyclo-oxygenase metabolites including vasodilator prostanoids (Masferrer *et al.*, 1992). The subsequent enhanced formation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) in the vascular smooth muscle and an impairment of the NA-mediated signal transduction (see Suba *et al.*, 1992) may also contribute to the vascular hyporeactivity in response to endotoxin. Nevertheless, TNF_{ab} significantly attenuated the hyporeactivity to NA in aortic rings *ex vivo*, and L-NAME did not further enhance the contractions to NA in rats pretreated with TNF_{ab} prior to LPS. This suggests that TNF_{ab} may completely prevent NOS induction in the aorta.

Thus, the endogenous release of TNF in endotoxaemia contributes to the induction of NOS by LPS *in vivo*. Although the inhibition of NOS induction by TNF_{ab} in the lung was only partial, pretreatment with TNF_{ab} prevented the fall in MAP observed at 180 min after LPS. Similarly, dexamethasone administration prior to LPS injection results in an incomplete inhibition of NOS induction in the lung, but abolishes NOS induction in the aorta (Szabó *et al.*, 1993a). Moreover in anaesthetized rats, the calcium antagonist, nifedipine (Szabó *et al.*, 1993b) and the platelet-activating factor antagonist, WEB 2086 (Szabó *et al.*, 1993c) only partially inhibit NOS induction in the lung. However, both drugs cause a near-complete prevention of the delayed fall in blood pressure associated with endotoxaemia in the anaesthetized rat.

The present data provide a rational basis for recent observations demonstrating that the platelet-activating factor

antagonist, WEB 2086 (Szabó *et al.*, 1993c) and certain prostaglandins such as prostacyclin and prostaglandin E₂ (Marotta *et al.*, 1992) inhibit NOS induction, for PAF antagonists (Floch *et al.*, 1989; Rabinovici *et al.*, 1990) and prostaglandin E₂ (Renz *et al.*, 1988; Ferreri *et al.*, 1992) inhibit the release of TNF in response to LPS. In addition, the present data support the view that elevations of plasma TNF levels in the absence of endotoxaemia are involved in the induction of NOS in haemorrhagic shock (Thiemermann *et al.*, 1993).

Although TNF_{ab} attenuated both NOS induction and the delayed fall in MAP in rats subjected to LPS, TNF_{ab} did not affect the immediate hypotension caused by LPS injection. This is consistent with the view that approximately 30–60 min are required for an increase in plasma concentrations of TNF after LPS injection (Feuerstein *et al.*, 1990; Klosterhafen *et al.*, 1992). In contrast, the immediate hypotension in response to LPS can be prevented by NO synthase inhibitors (Thiemermann & Vane, 1990; Lambert *et al.*, 1992) and platelet-activating factor antagonists (Casals-Stenzel, 1987; Szabó *et al.*, 1993c). As PAF can release NO (Schmidt *et al.*, 1989; Moritoki *et al.*, 1992), it is conceivable that the immediate hypotension to LPS in the rat is due to an enhanced formation of NO which is triggered by activation of the constitutive NOS by PAF.

Glucocorticosteroids such as dexamethasone inhibit the LPS-mediated induction of NOS *in vitro* and *in vivo* (Radomski *et al.*, 1990; Knowles *et al.*, 1990). Glucocorticosteroid pretreatment also ameliorates the delayed fall in MAP after LPS administration (Wright *et al.*, 1992; Szabó *et al.*, 1993a). As glucocorticosteroids inhibit the release of TNF (Waage, 1987; Zuckerman *et al.*, 1989), the present study suggests that this may be one of the mechanisms by which glucocorticosteroids inhibit the induction of NOS.

NO, when produced in large amounts (e.g. following NOS induction), acts as a cytotoxic effector molecule (Stuehr & Nathan, 1989) which can mediate or enhance cellular damage in endotoxaemia. Thus, NO is likely to participate in the TNF-induced cellular damage. Indeed, TNF-induced endothelial cell damage can be blocked by NO synthase inhibitors (Estrada *et al.*, 1992). In addition, both NO (Geng *et al.*, 1992) and TNF (Stadler *et al.*, 1992) inhibit mitochondrial respiration. It is conceivable that NO also participates in the inhibitory effect of TNF on mitochondrial respiration.

Although the prevention of the LPS-induced circulatory failure with antibodies directed against TNF appears to be an important approach for the therapy of endotoxin shock, it should be stressed that the beneficial cardiovascular effects of TNF antibodies, when administered following LPS, are limited (Tracey *et al.*, 1987; Mathison *et al.*, 1988; Hinshaw *et al.*, 1990; Silva *et al.*, 1990). Similarly, inhibition of NOS induction with corticosteroids (Radomski *et al.*, 1990; Knowles *et al.*, 1990), prevents the cardiovascular failure caused by LPS, but does not exert beneficial cardiovascular effects once NOS induction has occurred (Wright *et al.*, 1992; Szabó *et al.*, 1993a; Paya *et al.*, 1993). This is consistent with the hypothesis that NOS induction contributes importantly to the delayed cardiovascular failure in endotoxaemia (Wright *et al.*, 1992; Szabó *et al.*, 1993a) and haemorrhagic shock (Thiemermann *et al.*, 1993). This observation also suggests that at a later stage of endotoxin shock, when the induction of NOS has already occurred, inhibitors of the inducible isoform of NOS may be therapeutically important.

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