British Journal of Pharmacology (1998) 124, 540–546
http://www.stockton-press.co.uk/bjp

Nitric oxide and the haemodynamic profile of endotoxin shock in the conscious mouse

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1 The release of cytokines following administration of endotoxin and the contribution of nitric oxide (NO) to the subsequent haemodynamic profile were investigated in the conscious mouse.

2 Administration of endotoxin (*E. Coli*, 026 : B6, 12.5 mg kg^{-1}, i.v.) elevated the concentration of tumour necrosis factor-α (TNF-α) in the plasma within 0.5 h, reaching a maximum at 2 h and returning to control concentrations by 4 h. In addition, the concentration of interleukin-6 (IL-6) in the plasma was also elevated within 1 h, reaching a maximum at 3 h and remaining elevated throughout the 12 h of study.

3 Endotoxin (12.5 mg kg^{-1}, i.v.) induced the expression of a Ca^{2+}-independent (inducible) NO synthase in the mouse heart and elevated the concentrations of nitrite and nitrate in the plasma within 4 h, reaching a maximum at 12 h. This was accompanied by a progressive fall in blood pressure over the same period.

4 The vasopressor effect of noradrenaline (0.5–4 μg kg^{-1} min^{-1}, i.v.) administered as a continuous infusion was significantly attenuated 7 h after endotoxin (12.5 mg kg^{-1}, i.v).

5 The NO synthase inhibitor N^G-monomethyl-L-arginine HCl (L-NMMA; 1–10 mg kg^{-1}, i.v. bolus) reversed the fall in blood pressure when administered 7 h after endotoxin (12.5 mg kg^{-1}, i.v.).

6 In an attempt to maintain a constant blood concentration, L-NMMA was administered as a continuous infusion (10 mg kg^{-1} h^{-1}, i.v.), beginning 4 h after a lower dose of endotoxin (6 mg kg^{-1}, i.v.). Such treatment prevented the fall in blood pressure and the elevation of nitrite and nitrate in the plasma throughout the 18 h of observation.

7 The fall in blood pressure following endotoxin (3 mg kg^{-1}, i.v.) was significantly reduced throughout the 18 h of observation in homozygous mutant mice lacking the inducible NO synthase.

8 In summary, we have developed a model of endotoxin shock in the conscious mouse in which an overproduction of NO by the inducible NO synthase is associated with the haemodynamic disturbances. This model, which exhibits many of the characteristics of septic shock in man, will enable the study of the pathology of this condition in more detail and aid the investigation of potential therapeutic agents both as prophylactics and, more importantly, as treatments.

Keywords: Nitric oxide; endotoxin; septic shock; conscious mouse; iNOS mutant; blood pressure; cytokines; L-NMMA

Introduction

Septic shock in man is characterized by cardiovascular collapse following microbial invasion of the body. The progressive hypotension, hyporeactivity to vasopressor agents and vascular leak leads to circulatory failure with multiple organ dysfunction and death (Suffredini *et al*., 1989; Root & Jacobs, 1991). A variety of host mediators have been implicated in the pathogenesis of shock, in particular, cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6. Indeed, administration of TNF-α and IL-1 in animals and man produces the pathological features of shock (for review see Parillo, 1993; Bone, 1996). Overproduction of nitric oxide (NO) may be a common mechanism by which microbial products and several cytokines bring about their deleterious actions on the cardiovascular system (Vallance & Moncada, 1993; Thiemermann, 1994; Rees, 1995).

In vascular endothelial cells, shear stress induced by blood flow and receptor activation by vasoactive mediators elevates intracellular calcium, thus stimulating the constitutive endothelial NO synthase (eNOS; Moncada *et al*., 1991). The relatively small amount (pmol) of NO generated provides an important physiological vasodilator tone and reduces platelet and white cell activation (Moncada *et al*., 1991; Radomski, 1995). Furthermore, the generation of endothelium-derived NO may contribute to the protection of the microvasculature from damage in the early phase of septic shock by counterbalancing the action of mediators that induce vasoconstriction and increased permeability (Whittle, 1995).

A different, inducible, Ca^{2+}-independent NO synthase (iNOS) is expressed, in vascular endothelial, smooth muscle and other cells, following activation by endotoxin and/or cytokines. This enzyme generates larger quantities (nmol) of NO over long periods resulting in loss of vascular tone, diminished response to vasoconstrictors (Julou-Schaeffer *et al*., 1990; Rees *et al*., 1990a) and vascular leakage (Whittle, 1995). In addition, this overproduction of NO may contribute to the impaired oxygen utilization and acidosis characteristic of septic shock by inhibiting key enzymes that regulate cellular respiration (Brown *et al*., 1995; Fink, 1996).

Whilst there are many short-term studies of endotoxin shock in anaesthetized animals (Thiemermann & Vane, 1990; Wright *et al*., 1992; Nava *et al*., 1992) few have examined the development of shock in conscious animals (Meyer *et al*., 1992; Schlag *et al*., 1994; Gardiner *et al*., 1995) in which the time

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course of the syndrome can be examined over several hours and the final outcome evaluated. Because of this we have developed a long-term model of septic shock in the conscious mouse in order to investigate the pathophysiology of this condition and to evaluate potential therapeutic approaches.

Methods

Female CD-1 mice (Charles River, U.K. 25–35 g) or MF1 (Wellcome Foundation Ltd, U.K., 22–30 g) were housed in a temperature-controlled room with water and food *ad libitum*. Blood pressure, NO synthase activity in the heart and plasma concentrations of TNF-α, IL-6 and nitrite and nitrate were determined 0–18 h following administration of endotoxin (*E. Coli*, 026:B6; 12.5 mg kg^{-1}, i.v.). A lower dose of endotoxin (6 mg kg^{-1} and 3 mg kg^{-1} in CD1 and MF1 strain of mouse, respectively) was subsequently used to reduce the severity of the model and allow the study of potential therapeutic approaches. All studies were in accordance with the United Kingdom Home Office regulations for the care and use of animals (Animals (Scientific Procedures) Act, 1986).

Induction of endotoxin shock

The mice were anaesthetized briefly with isofluorane (2%). A cannular was implanted in the femoral artery and femoral vein, tunnelled subcutaneously to exit at the top of the back and connected to a swivel tether system for continuous monitoring of blood pressure and drug administration respectively. Normal physiological saline (154 mM) containing heparin (25 u ml^{-1}) was administered as a continuous infusion via the femoral artery (50 μ lh^{-1}) to maintain patency of the blood pressure cannula line. Following recovery from surgery, blood pressure was measured over the following 24 h period. Only animals showing a mean blood pressure within the normal range (90–110 mmHg) over this period were entered into the study. Endotoxin (*E. coli*, 026:B6, 12.5 or 6 mg kg^{-1}, i.v.) was administered to the mice via the femoral vein and the blood pressure monitored over an 18 h period. Blood samples (100 μl) were obtained via the femoral artery to a maximum of 4 samples (4×100 μl) per mouse over the study period. Alternatively, when larger blood samples were required, endotoxin was administered via the tail vein and blood samples were obtained at the relevant time point by exsanguination via the carotid artery following terminal gaseous anaesthesia with isofluorane (2%).

Assay for plasma concentrations of TNF-α, IL-6, nitrite and nitrate

The concentration of the cytokines TNF-α and IL-6 in the plasma were measured by ELISA as described previously (Deakin *et al*., 1995). The plasma nitrite concentration was determined by first reducing the nitrate enzymatically, by use of nitrate reductase from aspergillus species. Briefly, plasma samples were diluted 1:4 or 1:10 with Milli-Q distilled water (ro reduce the interference of proteins with the assay) and incubated with assay buffer (KH_2PO_4 50 mM, NADPH 0.6 mM, FAD 5 mM, nitrate reductase 20 mu; pH 7.5) for 1 h at 37°C. The resultant nitrite concentrations were determined by chemiluminescence as described previously (Rees *et al*., 1995) and expressed as the amount of total plasma nitrite/nitrate in μM.

Assay for NO synthase activity

Nitric oxide synthase was assayed by use of the conversion of L-[U-^{14}C]-arginine to L-[U-^{14}C]-citrulline as described previously (Rees *et al*., 1995). Briefly, the mice were terminally anaesthetized with isofluorane (2%) and the hearts removed and washed in Krebs buffer (containing in mM: NaCl 118, KCl 4.8, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24 and glucose 11), gassed with 95% O_2/5% CO_2. The hearts were then freeze-clamped in liquid nitrogen and stored at −80°C until the day of assay.

Blood pressure measurements

Effect of endotoxin on blood pressure Endotoxin (6 or 12.5 mg kg^{-1}, i.v.) or saline was administered as a bolus over 30 s and the arterial blood pressure monitored continuously over an 18 h period.

Effect of noradrenaline on the fall in blood pressure following administration of endotoxin Noradrenaline (0.5–4 μg kg^{-1}, i.v.) was administered as a continuous infusion (10 min per incremental dose) to untreated mice or to mice 7 h after endotoxin (12.5 mg kg^{-1}, i.v.) treatment. The maximum increase in blood pressure was measured between successive doses of noradrenaline.

Effect of L-NMMA on blood pressure following administration of endotoxin L-NMMA (1–1000 mg kg^{-1}, i.v.) was administered as a bolus over 30 s to untreated mice or to mice 7 h after endotoxin (12.5 mg kg^{-1}, i.v.) treatment. The maximum increase in blood pressure was measured over 20 min between successive doses of L-NMMA. In a separate series of experiments, designed to maintain a constant blood concentration, L-NMMA (10 mg $kg^{-1}h^{-1}$ i.v.) $kg^{-1}h^{-1}$ was administered as a continuous infusion to mice, 4 h after treatment with a lower dose of endotoxin (6 mg kg^{-1}, i.v.).

Effect of endotoxin on blood pressure in mice lacking the inducible NO synthase Endotoxin (3 mg kg^{-1}, i.v.) or saline was administered as a bolus over 30 s and the arterial blood pressure monitored continuously over an 18 h period in wild type (MF1) and homozygous mutant mice (MF1) lacking the inducible nitric oxide synthase. The method of homologous recombination in embryonic stem cells to disrupt the inducible NO synthase gene and the mice generated from these cells that lack the inducible NO synthase have been described previously (Wei *et al*., 1995).

Chemicals

L-[U-^{14}C]-arginine (Amersham), protein reagent, bovine serum albumin (Bio-Rad); endotoxin (*E. Coli*, 026:B6, Difco); foetal calf serum (FCS, Gibco), Picofluor (Packard Chemical supplies); biopterin (Dr. B. Schircks Laboratories); *2,2′-azino-bis[3-ethylbenzythiazoline-6-sulphonic acid]*, AG50-X8 Dowex resin, NADPH, L-arginine HCl, NO_3^- reductase (from *Aspergillus* species), Tris, sucrose, EDTA, cysteine, dithiothreitol, leupeptin, aprotinin, soybean trypsin inhibitor, p-nitrophenyl phosphate (Sigma Chemicals); antibodies (goat anti-rabbit IgG-alkaline phosphatase conjugate), avidin-peroxidase conjugate, recombinant murine TNF-α (R and D Systems Europe Ltd.); antibody (MP5-20F3), biotinylated antibody (MP5-32C11) and recombinant murine IL-6 standard (Pharminogen) were obtained as indicated. Polyclonal antibody to murine TNF-α was provided by Dr J. Tite

(Wellcome Research Laboratories). L-NMMA (N^G-monomethyl-L-arginine HCl, 546C88) and N-iminoethyl-L-ornithine (L-NIO) were synthesized at Wellcome by Dr H. Hodson, as described previously (Scannell *et al*., 1972; Patthy *et al*., 1977).

Statistics

Results are expressed as mean ± s.e.mean for n separate experiments. Within-group analyses were compared by one way analysis of variance (ANOVA) for repeated measures, followed by the Friedman's test. Differences between groups were analysed by two-way ANOVA for repeated measures, followed by the Mann–Whitney U test or the Kruskal–Wallace test, as appropriate. Student's unpaired t test was used for all *in vitro* studies. $P<0.05$ was taken as statistically significant.

Results

Induction of endotoxin shock

Following administration of endotoxin (3, 6 or 12.5 mg kg^{-1}, i.v. bolus), the mice showed characteristic 'shock-like' symptoms (i.e. piloerection and lethargy) within a few hours. Following the higher dose of endotoxin (12.5 mg kg^{-1}, i.v. bolus) deaths began to occur after 12 h and by 18 h only 3 out of 9 animals survived. However, when the animals were treated with a lower dose (3 or 6 mg kg^{-1}, i.v. bolus) there were no deaths during the 18 h of observation. No deaths occurred in the animals treated with saline only (sham-treated group).

TNF-α and IL-6 measurements

TNF-α and IL-6 were not detectable in the plasma from the control group (limit of detection 1.6 u ml^{-1} and 0.16 ng ml^{-1}, respectively). Administration of endotoxin (12.5 mg kg^{-1}, i.v.) to the conscious mouse elevated the concentration of TNF-α in the plasma within 0.5 h. A maximum was reached at 2 h (116 ± 21 u ml^{-1}, $n=3-8$, $P<0.05$) and by 3 h the concentration had returned to below the limit of detection ($n=3-8$, Figure 1). The concentration of IL-6 in the plasma increased within 1 h, reaching a maximum at 3 h (300 ± 27 ng ml^{-1}, $n=3-8$, $P<0.05$) and declining thereafter, although it remained higher than the concentration before administration of endotoxin ($n=3-8$, Figure 1, $P<0.05$). For comparison, 1 u ml^{-1} TNF-α ≃ 0.16 ng ml^{-1} TNF-α.

NO synthase activity

Administration of endotoxin (12.5 mg kg^{-1}, i.v.) induced the expression of a Ca^{2+}-independent NO synthase in the heart within 4 h, reaching a maximum at 12 h (2.86 ± 0.34 pmol-min^{-1} mg^{-1} protein, $n=6$, Figure 2, $P<0.05$).

Nitrite and nitrate measurements

The basal concentration of nitrite/nitrate in the plasma was 16 ± 4 μM ($n=11$). Endotoxin (12.5 mg kg^{-1}, i.v.) elevated the concentration of nitrite/nitrate in the plasma within 2 h,

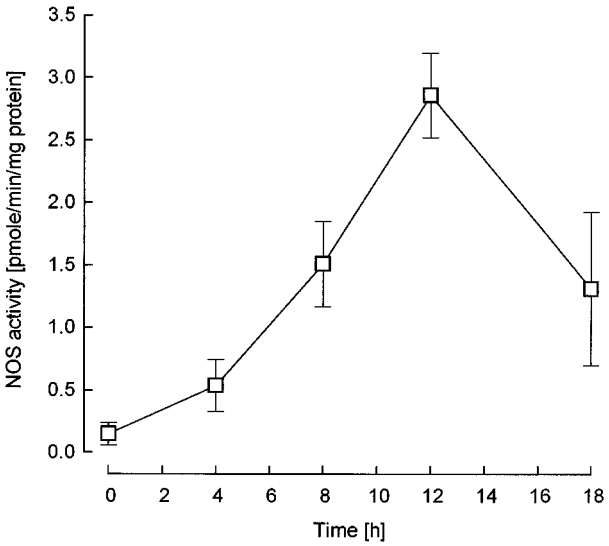

Figure 2 Effect of endotoxin (*E. coli*, 026:B6, 12.5 mg kg^{-1}, i.v.) on the Ca^{2+}-independent (inducible) NO synthase activity in the mouse heart. Each point is the mean of 6–13 animals; vertical lines show s.e.mean.

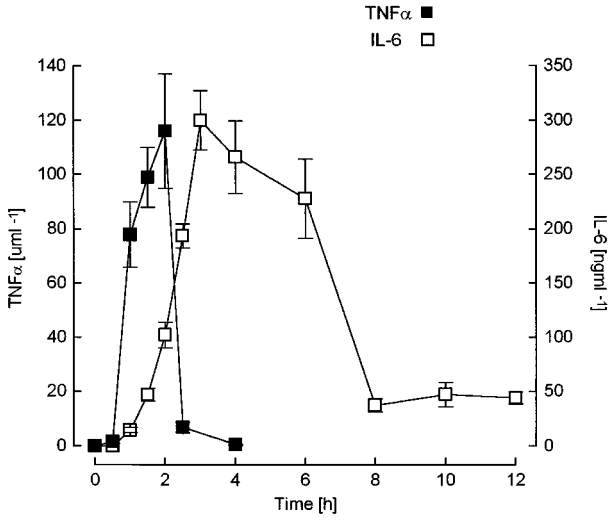

Figure 1 Effect of endotoxin (*E. coli*, 026:B6, 12.5 mg kg^{-1}, i.v.) on the concentrations of TNF-α and IL-6 in the plasma of the mouse. Each point is the mean of 3–8 animals; vertical lines show s.e.mean.

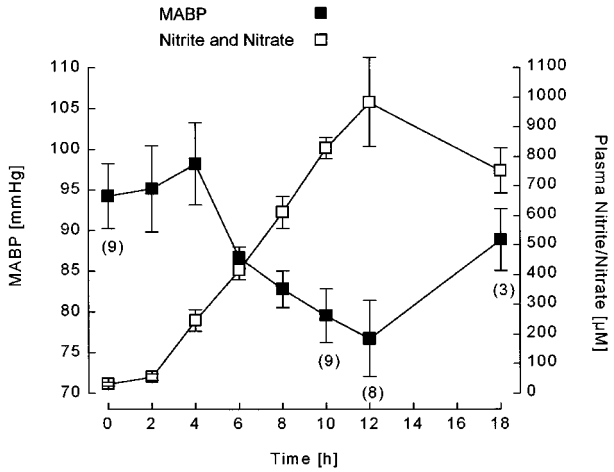

Figure 3 Effect of endotoxin (*E. coli*, 026:B6, 12.5 mg kg^{-1}, i.v.) on mean arterial blood pressure (MABP) and on concentrations of plasma nitrite and nitrate in the conscious mouse. Each point is the mean of 3–9 animals, where (n) represents numbers of survivors; vertical lines show s.e.mean.

reaching a maximum at 12 h (984±150 μM, $n=9$, Figure 3, $P<0.05$).

Blood pressure measurements

After recovery from surgery (16–24 h), basal mean arterial blood pressure (MABP) of untreated animals (saline only) remained constant throughout the following 18 h of observation (98±3 mmHg, 95±2 mmHg and 94±2 mmHg at 0, 12 and 18 h, respectively, $n=4$). Administration of endotoxin (12.5 mg kg^{-1}, i.v.) caused a progressive fall in blood pressure, after a delay of approximately 4 h, from 94±4 mmHg at 0 h to 76±4 mmHg ($n=8$, Figure 3, $P<0.05$) at 12 h returning to 88±4 mmHg ($n=3$, Figure 3) at 18 h.

Effect of noradrenaline on the fall in blood pressure following administration of endotoxin

Noradrenaline (0.5–4 μg kg^{-1} min^{-1}, i.v.) administered to untreated animals as a continuous infusion (10 min per incremental dose) elevated blood pressure in a dose-dependent manner from a basal MABP of 91±2 mmHg to 111±4 mmHg ($n=8$, Figure 4, $P<0.05$) at 4 μg kg^{-1} min^{-1}, i.v. In a separate group of mice, administration of endotoxin (12.5 mg kg^{-1}, i.v.) caused a progressive fall in blood pressure over a 7 h period, from a MABP of 103±3 mmHg to 83±3 mmHg ($n=7$, $P<0.05$) at 7 h. When noradrenaline (0.5–4 μg kg^{-1} min^{-1}, i.v.) was administered as a continuous infusion at the 7 h time point, the pressor response over the 10 min period was significantly reduced ($n=7$, Figure 4, $P<0.05$).

Effect of L-NMMA on blood pressure following administration of endotoxin

In untreated mice, L-NMMA (1–1000 mg kg^{-1}, i.v.) administered as a bolus over 30 s elevated blood pressure in a dose-dependent manner from a basal MABP of 93±2 mmHg to 153±7 mmHg ($n=6$, Figure 5a, $P<0.05$) at 1000 mg kg^{-1}, i.v. In a separate group of mice, administration of endotoxin (12.5 mg kg^{-1}, i.v.) caused a progressive fall in blood pressure over a 7 h period, from a basal MABP of 99±3 mmHg to 84±5 mmHg ($n=4$, Figure 5b, $P<0.05$) at 7 h. L-NMMA (1–10 mg kg^{-1}, i.v.) administered as a bolus over 30 s reversed the fall in blood pressure in a dose-dependent manner to 97±7 mmHg ($n=4$, Figure 5b, $P<0.05$) at 10 mg kg^{-1}, i.v. Higher doses of L-NMMA (100–1000 mg kg^{-1}, i.v.) increased the blood pressure further ($n=4$, Figure 5b, $P<0.05$).

Effect of a continuous infusion of L-NMMA on blood pressure, nitrite and nitrate following administration of a lower dose of endotoxin

Administration of a lower dose of endotoxin (6 mg kg^{-1}, i.v.) caused a progressive fall in blood pressure after a delay of approximately 4 h, from a basal MABP of 94±4 mmHg to 67±6 mmHg ($n=6$, Figure 6a, $P<0.05$) at 18 h. The concentration of nitrite/nitrate in the plasma was elevated

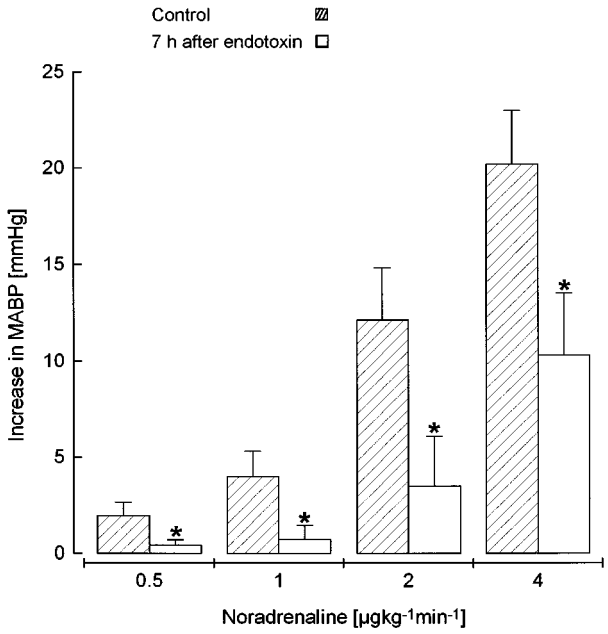

Figure 4 The increase in mean arterial blood pressure (MABP) by a 10 min continuous infusion of noradrenaline (0.5–4 μg kg^{-1} min^{-1}) is significantly reduced 7 h after endotoxin (*E. coli*, 026:B6, 12.5 mg kg^{-1}, i.v.) administration in the conscious mouse. The maximum increase in blood pressure was measured between successive doses of noradrenaline. Each point is the mean of 7–8 animals; vertical lines show s.e.mean. *$P<0.05$.

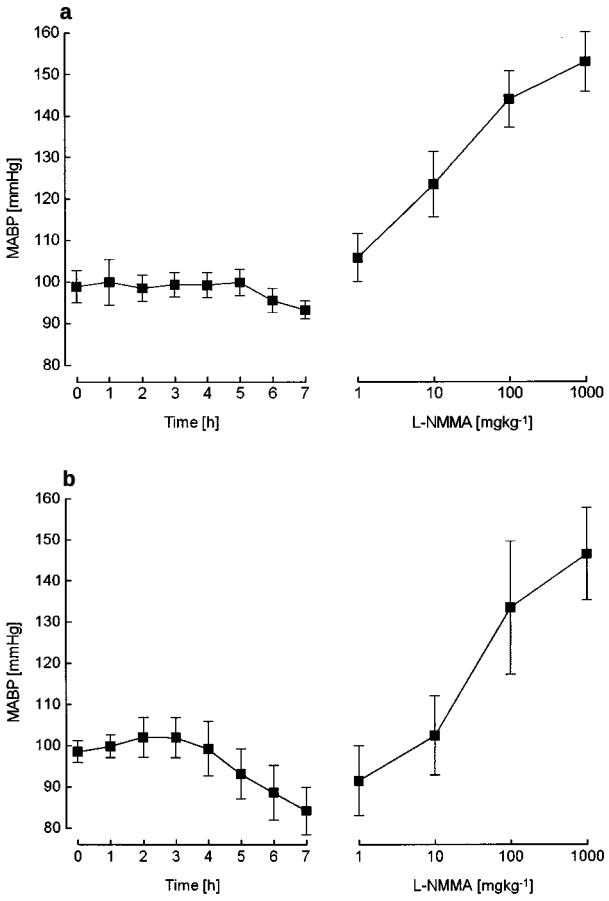

Figure 5 (a) Basal mean arterial blood pressure (MABP) over a 7 h period prior to L-NMMA administration in the conscious mouse and effect of L-NMMA (1–1000 mg kg^{-1}, i.v.) on MABP. (b) The progressive fall in MABP over a 7 h period following endotoxin (*E. coli*, 026:B6, 12.5 mg kg^{-1}, i.v.) administration and the effect of L-NMMA (1–1000 mg kg^{-1}, i.v.) on MABP, 7 h after endotoxin (*E. coli*, 026:B6, 12.5 mg kg^{-1}, i.v.) administration. The maximum increase in MABP was measured over the 20 min period between successive doses of L-NMMA. Each point is the mean of 4–6 animals, vertical lines show s.e.mean.

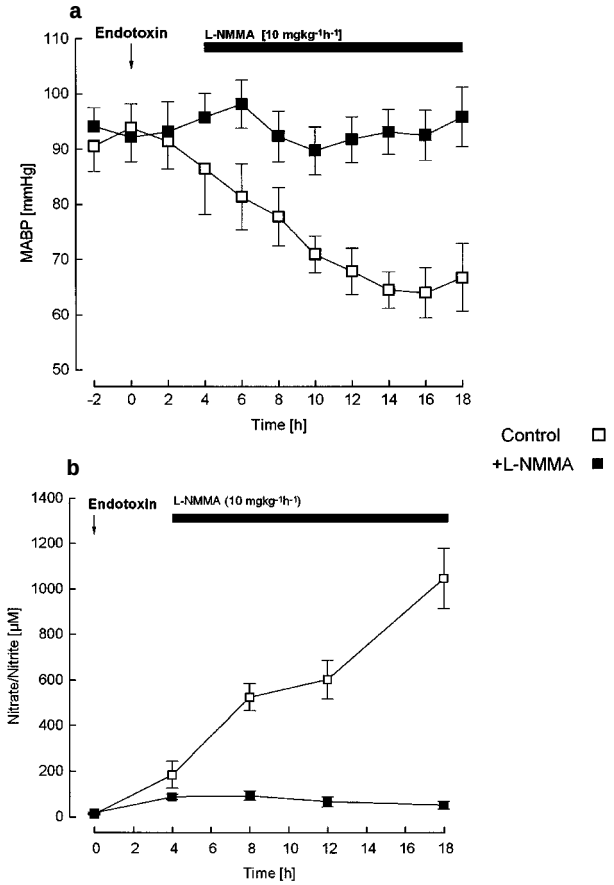

Figure 6 (a) Effect of L-NMMA (10 mg $kg^{-1}h^{-1}$ $kg^{-1}h^{-1}$ $kg^{-1}h^{-1}$i.v.) administered as a continuous infusion on (a) mean arterial blood pressure (MABP) and (b) concentrations of plasma nitrite and nitrate in the conscious mouse following endotoxin (*E. coli*, 026:B6, 6 mg kg^{-1}, i.v.) treatment. Each point is the mean of 4–6 animals; vertical lines show s.e.mean.

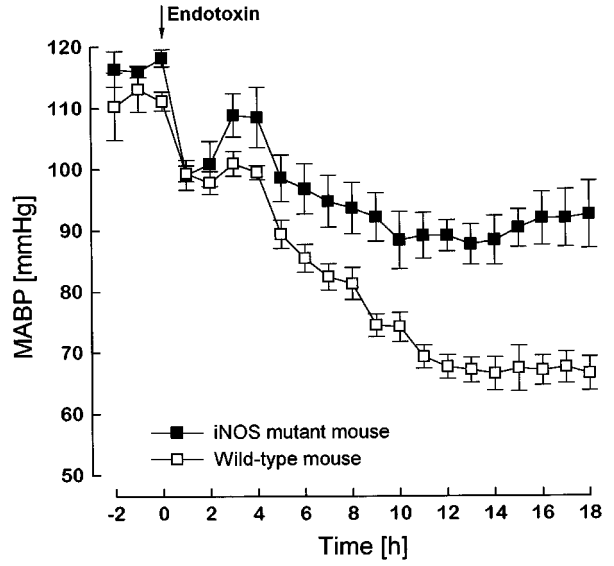

Figure 7 Effect of endotoxin (*E. coli*, 026:B6, 3 mg kg^{-1}, i.v.) on mean arterial blood pressure (MABP) in conscious wild-type mice ($n=5$) and mice lacking inducible NO synthase (iNOS, $n=4$). Each point is the mean of 4–5 animals; vertical lines show s.e.mean.

within 4 h, reaching a maximum at 18 h (1046 ± 133 μM, $n=5$, Figure 6b, $P<0.05$). In a separate group of mice, L-NMMA (10 mg kg^{-1} h^{-1}) prevented the endotoxin-induced fall in blood pressure so that it went from a basal MABP of 98 ± 8 mmHg to 106 ± 5 mmHg ($n=4$, Figure 6a, $P<0.05$) at 18 h. This was accompanied by a significant inhibition of the elevation in plasma nitrite/nitrate throughout the 18 h of study ($n=5-11$, Figure 6b, $P<0.05$).

Effect of endotoxin on blood pressure in mice lacking the inducible NO synthase

Administration of a lower dose of endotoxin (3 mg kg^{-1}, i.v.) in wild type (MF1) mice caused a progressive fall in blood pressure after a delay of approximately 4 h, from a basal MABP of 110 ± 6 mmHg to 66 ± 3 mmHg ($n=5$, Figure 7, $P<0.05$) at 18 h. The fall in blood pressure was significantly reduced in mice (MF1) lacking the inducible nitric oxide synthase from a basal MABP of 116 ± 3 mmHg to 93 ± 5 mmHg ($n=4$, Figure 7, $P<0.05$) at 18 h.

Discussion

Over the last decade cytokines, including TNF-α, IL-1, IL-6 and IFN-γ, have been implicated as key mediators in the pathogenesis of septic shock. In this study, endotoxin stimulated the release of TNF-α and IL-6 within 30 and 60 min, respectively. The increase in plasma concentration of TNF-α was rapid, reaching a maximum at 2 h and returning to control concentrations by 4 h. By contrast, IL-6 increased at a slower rate, reaching a maximum at 3 h and declining thereafter, although it did remain elevated throughout the 12 h study period. A similar cytokine profile is observed in patients with septic shock (Damas *et al*., 1992), and in one study following administration of endotoxin in man (Taveira da Silva *et al*., 1993) the concentrations of TNF-α and IL-6 released were quantitatively similar to those measured in this study. The transient increase in the plasma concentration of TNF-α is in accordance with other findings (Sheehan *et al*., 1989; Silva *et al*., 1990) and its initiating role in the signs and symptoms of septic shock is well established. Whether IL-6 represents a negative feedback mechanism that downregulates TNF-α production, as has been suggested by results *in vitro* and *in vivo* (Aderka *et al*., 1989), remains to be elucidated.

The combination of cytokines and microbial products required to activate the inducible NO synthase and the subsequent time course of expression varies according to the type of cell, the experimental conditions and the species used (Rees *et al*., 1990a; 1995; Salter *et al*., 1991). In this study, administration of endotoxin with the subsequent generation of several cytokines resulted in the induction of a Ca^{2+}-independent NO synthase (iNOS) in the heart within 4 h, which reached a maximum at 12 h. This was accompanied by an increase in the plasma concentrations of nitrite/nitrate over a similar time course and was closely associated with a progressive fall in blood pressure and a significantly reduced vasopressor response to noradrenaline, suggesting a similar induction profile in the heart and blood vessels. After 12 h a number of deaths occurred; the survivors at 18 h showed a declining iNOS activity with the blood pressure returning towards normal. Thus, in the mouse, as has been indicated for other species (Ochoa *et al*., 1991; Wright *et al*., 1992; Evans *et al*., 1993; Wei *et al*., 1995; MacMicking *et al*., 1995), the increased generation of NO from an inducible NO synthase underlies the hypotension and hyporesponsiveness to vaso-

constrictor agents in endotoxin shock. This was further confirmed by our observations with mice lacking the inducible NO synthase, in which endotoxin treatment results in a greatly reduced fall in blood pressure compared with wild-type animals. Although there is significant induction of NO synthase in wild-type animals at 4 h, the blood pressure does not begin to fall until after that time. This early dissociation may be due to the elevated circulating concentrations of endogenous catecholamines that follow endotoxin administration; these may limit the hypotension and contribute to the initial hyporesponsiveness to exogenous catecholamines (Benedict & Grahame-Smith, 1978; Jones & Romano, 1989).

In conscious untreated mice, L-NMMA caused a dose-dependent increase in mean arterial blood pressure, as it does in other species including man (Rees *et al*., 1989; 1990b; Haynes *et al*., 1993). L-NMMA administered as a bolus also reversed in a dose-dependent manner the endotoxin-induced fall in blood pressure. In an attempt to maintain a steady plasma concentration during the shock phase, L-NMMA was also administered by continuous infusion 4 h after endotoxin treatment. Such treatment inhibited the fall in blood pressure and the associated increase in plasma nitrite/nitrate concentrations.

This L-NMMA-induced increase in blood pressure, which has also been observed in man (Petros *et al*., 1991; 1994), may be important for the successful management of septic shock. In a Phase II study of 312 patients with septic shock, L-NMMA caused a significant reversal of shock (defined by a mean arterial pressure $\geqslant 70$ mmHg at 72 h without conventional vasopressors). This endpoint correlated with survival at 28 days in both the treatment and placebo groups. Although more patients achieved resolution of shock in the treatment than the placebo group the number of patients used in this study was not sufficiently large enough to demonstrate a survival benefit (Grover *et al*., unpublished observations).

Some important questions remain; the first is whether or not inhibiting overproduction of NO has any additional benefit over conventional vasopressor therapy? The possible advantages include limiting the increase in microvascular permeability (Laszlo *et al*., 1995; Whittle, 1995) and the impaired oxygen utilization and acidosis (Brown *et al*., 1995; Fink, 1996) that occur at high concentrations of NO. Thus, inhibition of excessive NO generation, in addition to restoring cardiovascular homeostasis, may also reverse the metabolic abnormalities of septic shock. The second question is whether full restoration of blood pressure will be required to achieve a therapeutic benefit? Studies in our laboratory are now underway to investigate this. Our hypothesis is that full restoration of pressure may not be necessary and may even be harmful since it could drastically reduce flow in some important vascular beds. A further question is when should treatment with NO synthase inhibitors be initiated and for how long should such treatment be maintained? High levels of NO may trigger processes such as oedema formation and tissue damage that are themselves injurious, thus early intervention with NO synthase inhibitors may be the best course of action to reduce these downstream consequences. Whether the fall in blood pressure should be considered to be an early indicator of the overproduction of NO and a suitable marker for the initiation of L-NMMA treatment remains to be determined. It is likely that the duration of treatment with NO synthase inhibitors will need to be titrated to the blood pressure of each individual patient until vasoconstrictor therapy can safely be withdrawn.

The combination of inhibition of NO synthase and NO donation may prove beneficial in septic shock (Wright *et al*., 1992). For instance, in those patients with a high pulmonary artery pressure, inhalation of NO gas (Rossaint *et al*., 1993) or of an NO donor in addition to treatment with an NO synthase inhibitor, may be the optimum therapy.

In summary, we have developed a conscious, long-term model of endotoxin shock, which exhibits many of the characteristics of septic shock in man. The model allows the study of potential therapeutic agents, both as a prophylactic and, more importantly, as a treatment. In this respect, we have demonstrated that overproduction of NO is responsible for the haemodynamic disturbances associated with endotoxin shock and that inhibition of NO synthase represents a promising approach for the treatment of septic shock in man.

The authors thank Dr Angela Deakin for assistance with this work and Annie Higgs for critical appraisal of the manuscript

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(Received January 16, 1998
Accepted February 6, 1998)