Differential induction of nitric oxide synthase in various organs of the mouse during endotoxaemia: role of TNF-α and IL-1-β

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SUMMARY

BALB/c mice injected intraperitoneally with bacterial lipopolysaccharide (LPS) developed lethal septic shock. This was accompanied by significantly elevated concentrations of nitrite and nitrate in the plasma and expression of high levels of nitric oxide (NO) synthase activity in the lungs, heart, spleen and peritoneal macrophages. Mice pretreated with anti-tumour necrosis factor-α (TNF-α) monoclonal antibody or anti-interleukin-1β (IL-1β) polyclonal antibody were protected, in a dose-dependent manner, from endotoxin-induced mortality. This effect was accompanied by a significant reduction in plasma levels of nitrite and nitrate. Antibody treatment also reduced the level of NO synthase activity in peritoneal macrophages, spleen and heart but had no effect on enzyme expression in the lung. These results demonstrate that TNF-α and IL-1β play an important role in the induction of NO following administration of LPS and in the development of endotoxin-induced shock. In addition, NO synthase activity is differentially expressed in various organs and this may not always require TNF-α and IL-1β.

INTRODUCTION

Septic shock associated with Gram-negative infection is characterized by hypotension, hyporeactivity to vasoconstrictor agents, inadequate tissue perfusion, vascular damage and disseminated intravascular coagulation leading to multiple organ failure and death.[1] Many of the pathological consequences of Gram-negative shock are attributable to the bacterial membrane component lipopolysaccharide (LPS).[2] This induces the production of host inflammatory mediators such as tumour necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) which are involved in the pathophysiological events of septic shock.[3] Administration of LPS has been shown to increase the serum concentration of TNF-α and IL-1β.[4,5] Conversely, administration of antibodies against TNF-α[6,7] or receptor antagonists for TNF-α[8,9] or IL-1β[10,11] improved survival of animals following lethal LPS administration. Similar findings were obtained in primates pretreated with anti-TNF-α antibody and injected with live *Escherichia coli*.[12]

Recent studies have linked the production of nitric oxide (NO) to LPS-induced hypotension, vascular hyporesponsiveness and death, suggesting that excess generation of NO plays an important role in the development of septic shock.[13-15] NO is derived from the oxidation of the terminal guanidino nitrogen atom of L-arginine by NO synthase, of which two general types have been identified (reviewed in ref. 16). One is constitutive,

Ca^{2+}/calmodulin- and NADPH-dependent. The other, inducible by LPS and cytokines, is also NADPH-dependent but Ca^{2+}-independent. The constitutive enzyme is present mainly in vascular endothelium, brain and platelets. The Ca^{2+}-independent enzyme can be induced in many cells including macrophages, neutrophils, Kupffer cells, hepatocytes, vascular smooth muscle and endothelial cells. Once expressed, it catalyses the generation of large quantities of NO which is cytostatic/cytotoxic to pathogens and tumour cells.[17]

Administration of LPS to animals has been shown to result in an increase in the level of serum nitrate, a metabolite of NO.[18] This was inhibited by L-N^{G}monomethyl arginine (L-NMMA), a specific inhibitor of NO synthase. L-NMMA also inhibited TNF-α-induced hypotension.[19] Here, we show that administration of anti-TNF-α and anti-IL-1β antibodies markedly reduces LPS-induced shock and NO synthesis *in vivo*. Furthermore, we demonstrate that there is a differential expression of NO synthase in various organs, which may not be mediated primarily by TNF-α or IL-1β.

MATERIALS AND METHODS

Mice

Female BALB/c mice were purchased from Harlan Olac Ltd (Bicester, U.K.). They were housed in temperature-controlled rooms and received water and food *ad libitum*.

Materials

LPS from *E. coli* (026:B6) was obtained from Difco (West Molesley, U.K.). A monoclonal anti-TNF-α IgG1 antibody was

purified from culture supernatants of the XT22.11 hybridoma line (kindly provided by Dr R. L. Coffman, DNAX Institute, CA). A control IgG1 antibody against acyclovir was obtained from the Wellcome Research Laboratories. A polyclonal antibody against IL-1β was raised in sheep. A control polyclonal antibody against digoxin was also obtained from the Wellcome Research Laboratories. Nitrate reductase was purchased from Boehringer Mannheim (Lewes, U.K.). All other reagents were obtained from Sigma (Poole, U.K.).

Induction of endotoxin shock

Mice were injected i.p. with 10 mg/kg of LPS. Nitrite and nitrate concentrations in the plasma and NO synthase activity in the lung, spleen and heart were determined from groups of three mice 6, 12, 18 and 24 hr later. In a second series of experiments, some mice were pretreated with anti-TNF-α or anti-IL-1β antibody alone, anti-TNF-α plus anti-IL-1β, or control antibodies. Antibodies were injected intravenously 10 hr before LPS administration. Twelve hours after LPS administration, nitrite and nitrate concentrations in the plasma and NO synthase activity in peritoneal macrophages, heart, spleen and lungs were determined. Mortality following LPS treatment was also monitored at regular intervals.

Assays for nitrite and nitrate

Animals were anaesthetized and the peritoneal macrophages harvested for NO synthase determination using 5 ml cold RPMI. The peritoneal cavity was then opened, a blood sample obtained by cutting the splenic artery and plasma collected by centrifugation. The nitrite concentration was determined using a chemiluminometer as previously described.[20] The plasma nitrate concentration was determined by reducing nitrate enzymatically using the enzyme nitrate reductase, as described elsewhere.[21] Briefly, plasma samples were diluted 1:5 in water and 50 μl incubated with the same volume of reductase buffer (0·1 M KH_2PO_4, pH 7·5; 1 mM NADPH; 10 mM FAD and 4 U/ml nitrate reductase) for 2 hr at 37°. A standard curve of nitrate was constructed by incubating sodium nitrate (10–500 μM) with the reductase buffer. The total amount of nitrite was then determined by chemiluminescence. Results are expressed as the total amount of nitrate plus nitrite per ml of plasma.

Assay for NO synthase activity

Immediately after collecting the blood sample, 20 ml of phosphate-buffered saline (PBS) was injected into the abdominal vein and the remaining blood washed out through the splenic artery. Immediately after perfusion, the heart, spleen, and lungs were collected and frozen at −80° until processed for NO synthase activity as previously described.[22] Briefly, tissues were homogenized in a glass homogenizer and the homogenates centrifuged at 100,000 *g* for 10 min at 4°. Supernatants were assayed for NO synthase activity by measuring the conversion of L-[U-^{14}C]arginine to [U-^{14}C]citrulline as described previously,[22] except that the incubation was carried out at room temperature for 60 min. Protein content of the supernatants was determined by the Coomassie blue binding method according to the manufacturer's recommendations (Pierce Chemical, Rockford, IL). NO synthase activity was expressed as pmol NO/mg protein/hr.
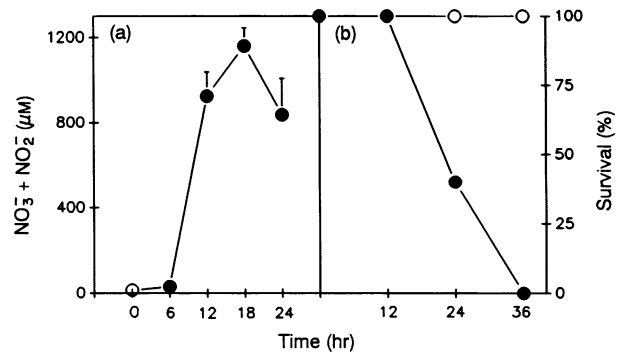

Figure 1. Effect of LPS on (a) plasma levels of NO_2^- and NO_3^- and (b) on mortality. BALB/c mice were injected i.p. with 10 mg/kg LPS (●) or with PBS (○). Plasma samples were harvested as described in Materials and Methods. For mortality, groups of 10 BALB/c mice were injected with 10 mg/kg LPS (●) or PBS (○) and the per cent survival recorded for 36 hr. Vertical bars represent 1 SEM ($n = 3$). Results are representative of two experiments.

Statistical analysis

Statistical significance ($P < 0.05$) was analysed by Student's *t*-test. Results are expressed as means ± SEM ($n = 3$–6).

RESULTS

Plasma from mice injected i.p. with 10 mg/kg of LPS contained high concentrations of nitrite and nitrate 12 hr after treatment (Fig. 1a). Control mice, not treated with LPS, had no detectable nitrite or nitrate (Fig. 1a). Mortality following injection of LPS was also monitored. Over half the animals given LPS were dead 24 hr later (Fig. 1b) and all had died by 36 hr. No deaths were observed in the control, untreated group (Fig. 1b). A similar time–course was observed for the expression of NO synthase activity in the lungs, spleen and heart (Fig. 2). Significantly higher levels of NO synthase activity were detected in the lungs compared to those in the heart and spleen (Fig. 2; $P < 0.05$).

Mice pretreated with a monoclonal anti-TNF-α antibody were protected from LPS-induced death (Fig. 3a). This protection was dose-dependent and was accompanied by a significant reduction in the concentration of nitrite and nitrate detected in the plasma of these mice (Fig. 4). Animals treated with the
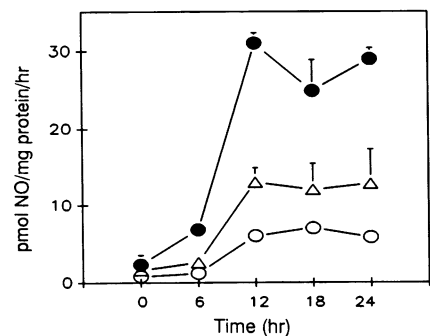

Figure 2. Effect of LPS on NO synthase levels in the lung (●), spleen (○) and heart (△). Mice were injected i.p. with 10 mg/kg LPS and organs harvested as described in Materials and Methods at regular intervals following LPS. Vertical bars represent 1 SEM ($n = 3$). Results are representative of two experiments.
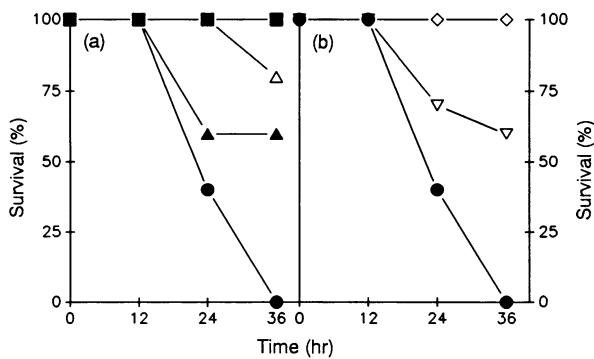
Figure 3. Effect of anti-TNF-α or anti-IL-1β antibodies on LPS-induced mortality. Groups of five BALB/c mice were injected i.v. with (a) anti-TNF-α monoclonal antibody at 0·1 mg (▲), 0·3 mg (△), 1·0 mg (■) or 2·0 mg (□) per mouse or (b) anti-IL-1β polyclonal antibody at 0·08 ml (▽) or 0·25 ml (◇) per mouse. LPS (10 mg/kg) was injected i.p. 10 hr later. Control mice were injected with an unrelated antibody followed by LPS (●). Mortality was recorded at the times indicated and expressed as per cent survival. Results are pooled from two experiments.

control monoclonal antibody were unprotected (Fig. 3a) and their plasma nitrite and nitrate concentrations were not significantly altered compared to animals given only LPS (Fig. 4). A polyclonal anti-IL-1β antibody also protected mice from LPS-induced death (Fig. 3b) and significantly reduced the plasma concentrations of nitrite and nitrate induced by LPS (Fig 4). Again, the control antibody had no protective effect and did not significantly change the plasma nitrite or nitrate concentrations (Figs 3b and 4). Mice treated with both antibodies also had significantly reduced concentrations of plasma nitrite and nitrate compared to untreated controls. However, the effect was no different from that of either antibody alone (Fig. 4).
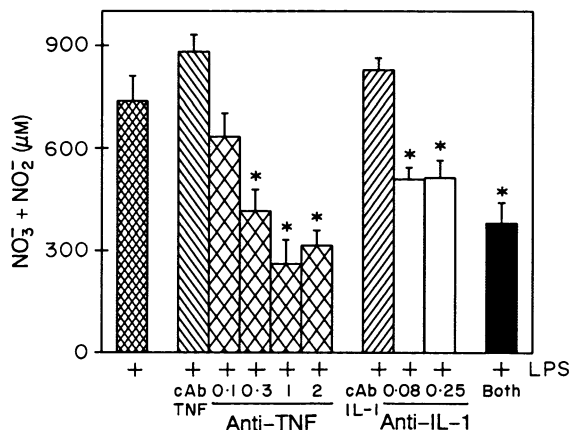

Figure 4. Effect of anti-TNF-α or anti-IL-1β antibodies on the plasma concentrations of NO_2^- and NO_3^- following LPS. Mice were injected i.v. with the indicated amounts of anti-TNF-α (mg/mouse) or anti-IL-1β (ml/mouse) antibodies. Some animals received both anti-TNF-α (0·3 mg/mouse) and anti-IL-1β (0·08 ml/mouse) antibodies. Control animals (cAb) received an unrelated antibody (1 mg/mouse for the TNF control or 0·25 ml for the IL-1 control). Mice were injected i.p. with LPS (10 mg/kg) 10 hr later. Plasma samples were obtained as described 12 hr after LPS administration. The concentration of NO_2^- plus NO_3^- in animals injected with LPS alone is shown (in the first bar). Vertical bars represent 1 SEM ($n = 3$). Results are representative of two experiments. *$P < 0.05$ compared with control animals.

Pretreatment of mice with the anti-TNF-α antibody significantly inhibited, in a dose-dependent manner, the expression of NO synthase activity in peritoneal macrophages, spleen and heart (Fig. 5). However, it had no effect on NO synthase activity in the lung. Anti-IL-1β also inhibited the induction of NO synthase in the spleen and heart (Fig. 5), but had no effect on enzyme induction in macrophages or in the lung. Treatment of mice with both antibodies had an additive inhibitory effect on enzyme expression in the spleen, but not in the heart and had no inhibitory effect on NO synthase activity in the lung (Fig. 5).

DISCUSSION

NO has been implicated in endotoxin shock in several animal models[13-15,18,19,22] and in man.[23] Although it has been shown that constitutive NO may be beneficial in endotoxin shock, possibly by maintaining the vasodilator tone necessary for organ perfusion, the large amounts of NO induced by cytokines and LPS are detrimental and contribute to the observed mortality.[15] Although the involvement of TNF-α and IL-1β in endotoxin shock has been reported,[4,6,19] the course of events in the induction of LPS shock is not clear.

The data reported here suggest that *in vivo* administration of LPS to mice results in the production of TNF-α and IL-1β which can then induce the activation of NO synthase in a variety of organs, resulting in the production of NO and the development of shock. It is at present unclear which cytokine, TNF-α or IL-1β, is induced first following LPS treatment, or if both are produced simultaneously. Indeed, IL-1 can stimulate the release of TNF-α and vice versa.[1,24] However, the involvement of other cytokines in this cascade can not be excluded. Although high concentrations of anti-TNF-α or anti-IL-1β antibody prevented the development of LPS-induced lethal shock, the elevated plasma concentrations of NO and expression of NO synthase in the tissues were not completely inhibited. There was also a lack of linear correlation between the nitrite and nitrate levels in the serum and LPS-induced mortality when lower concentrations of anti-TNF-α and anti-IL-1β antibodies were used. Therefore, NO synthesis during LPS-induced shock may be activated by a number of factors. These other factors, in particular interferon-γ and LPS, may synergize with TNF-α and/or IL-1β to induce high levels of NO, as has been shown in other systems.[25-28]

Treatment of mice with LPS induced different levels of NO synthase activity in macrophages, spleen, heart and lung, consistent with previous findings in the rat.[13,22] The variable levels of NO synthase activity in each organ may reflect different percentages of cells capable of expressing NO synthase within each organ. Treatment of these animals with anti-TNF-α antibody resulted in a decrease in NO synthase activity in all tissues tested except the lung. Anti-IL-1β antibody reduced NO synthase activity in the spleen and heart, but not in peritoneal macrophages or the lung. The lack of effect of anti-TNF-α and anti-IL-1β antibodies in reducing NO synthase activity in the lung suggests that NO is induced in this organ by an immunological stimulus, or stimuli, other than TNF-α and IL-1β. In this context, it has been reported recently that a neutralizing antibody to TNF-α protected mice from endotoxin-induced shock, but failed to reduce the level of hepatic NO synthase activity.[29]

Data presented here demonstrate that the induction of lethal LPS shock in mice is accompanied by a significant increase in
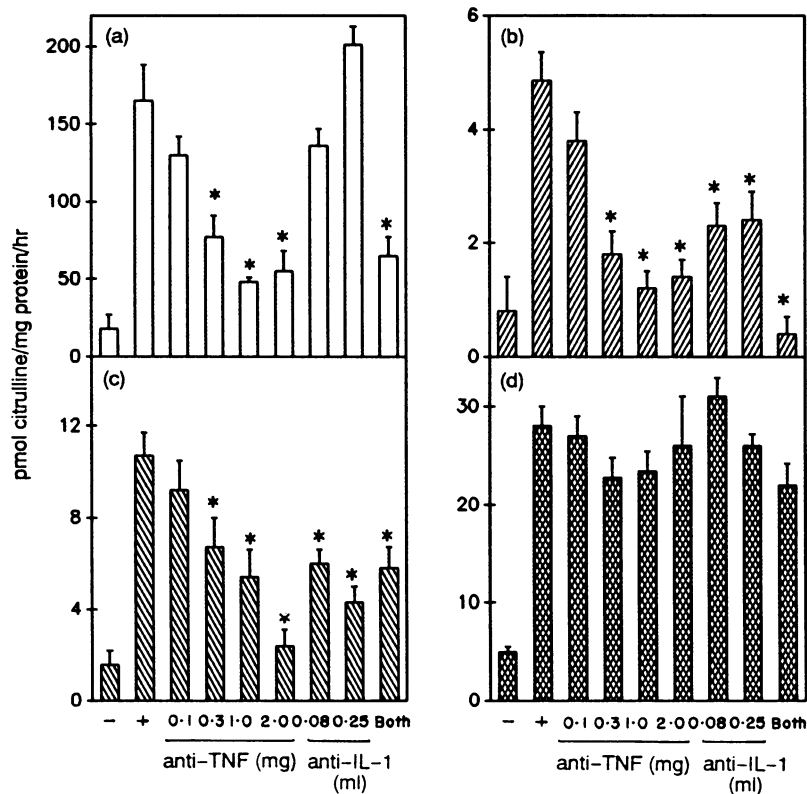
Figure 5. Effect of anti-TNF-α or anti-IL-1β antibodies on the induction of NO synthase by LPS in peritoneal macrophages (a), spleen (b), heart (c) and lung (d). Animals were pretreated with anti-TNF-α or anti-IL-1β antibodies, a combination of both or with unrelated control antibodies as in Fig. 4. Animals received LPS (10 mg/kg) 10 hr later and the organs were collected as described in Materials and Methods 12 hr after LPS. Control animals were either not injected with LPS ($-$) or received LPS alone ($+$). Vertical bars represent 1 SEM ($n=3$). Results are representative of two experiments. $*P < 0.05$ compared with animals given LPS alone.

plasma concentrations of nitrite and nitrate and in NO synthase activity in the heart, spleen and lungs. However, animals pretreated with antibody against TNF-α or IL-1β are protected from LPS-induced shock and this is accompanied by a significant decrease in NO concentration in the plasma and in the level of NO synthase activity. Thus, our results demonstrate the importance of TNF-α and IL-1β in endotoxin-induced NO synthesis. Furthermore, there is a differential induction of NO synthase activity in various organs during endotoxin-induced shock.

ACKNOWLEDGMENTS

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