The effect of nitric oxide synthase inhibition on the plasma fibrinolytic system in septic shock in rats

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1 We have investigated the effect of pretreatment of rats with nitric oxide (NO) synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME) on the *E. coli* lipopolysaccharide (LPS)-induced changes in the plasma fibrinolytic system, platelet count, fibrinogen level, as well as in gross and microscopic pathophysiological changes indicative of disseminated intravascular coagulation (DIC) in rats.

2 *E. coli* LPS (6 mg kg⁻¹, i.p.) produced a decrease in the levels of plasma fibrinogen and a drop in the blood platelet count 6 h after administration. The decrease in fibrinogen but not the drop in platelet count was reversed by pretreatment with L-NAME (30 mg kg⁻¹, i.p., 24 h and 15 min before administration of LPS).

3 Pretreatment with L-NAME antagonized the LPS-induced activation of fibrinolysis as measured by changes in the euglobulin clot lysis time (ECLT) and enhanced the LPS-induced rise in the plasma level of plasminogen activator inhibitor (PAI). In animals pretreated with L-NAME there was also a marked reduction in the histological changes indicative of DIC.

4 We propose that L-NAME can act as a protective agent in LPS-induced DIC, and this protection is due to an increased generation of PAI following inhibition of NO synthase.

Keywords: Lipopolysaccharide; fibrinolysis; disseminated intravascular coagulation (DIC); plasminogen activator inhibitor (PAI); nitric oxide synthase inhibition

Introduction

Administration of lipopolysaccharide (LPS) *in vivo* induces septic shock, characterized by hypotension, vascular injury, and disseminated intravascular coagulation (DIC), which leads to a fatal dysfunction of various organs (Proctor, 1986). Both the early and late phase hypotension after LPS administration are associated with over-production of nitric oxide (NO) within the vasculature (Thiemermann & Vane, 1990; Nava *et al.*, 1991; Petros *et al.*, 1991; Szabo *et al.*, 1993) the latter stage being characterized by the induction of NO synthase in various organs (Salter *et al.*, 1991). Although inhibitors of NO synthase may be beneficial in clinical treatment of endotoxaemia (Petros *et al.*, 1991), it is not yet clear whether such treatment could affect LPS-induced DIC. Our previous studies (Korb *et al.*, 1990; Lidbury *et al.*, 1990) showed that NO was highly effective in protecting platelets against the release of plasminogen activator inhibitor (PAI), the level of which in plasma has been found to contribute to the pathogenesis of DIC in septic patients (Paramo *et al.*, 1988). We were, therefore, interested in whether the protective effects of NO synthase inhibitors were in part related to their ability to influence DIC formation. We have examined the effects of pretreatment with the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, Moore *et al.*, 1989) on the LPS-induced changes in euglobulin clot lysis time (ECLT), platelet count and fibrinogen and plasminogen activator inhibitor (PAI) levels in the rat.

Methods

Male Wistar rats (220–270 g) were injected with N^G-nitro-L-arginine methyl ester (30 mg kg⁻¹, i.p.) or vehicle, 24 h and 15 min before administration of *E. coli* LPS (6 mg kg⁻¹, i.p.) or vehicle (saline). Six h later, animals were anaesthetized (pentobarbitone, 40 mg kg⁻¹, i.p.) and blood samples taken from the carotid artery into tri-sodium citrate (3.15% w/v) at

a ratio of 9:1. Animals were then killed by exsanguination and examined *post-mortem* for gross and microscopic (following staining with haematoxylin-eosin) pathophysiological changes indicative of DIC. Plasma from the blood samples were prepared by centrifugation (14,900 g for 3 min, Biofuge A, Heraeus) and used to assess euglobulin clot lysis time (ECLT), platelet count, fibrinogen levels and plasminogen activator inhibitor (PAI) activity. ECLT was assayed according to the method of Von Kaulla & Schultz (1958), as described previously (Lidbury *et al.*, 1990). The levels of fibrinogen and PAI were measured with commercially available kits.

Materials

E. coli endotoxin (LPS serotype 0127:B8) and N^G-nitro-L-arginine methyl ester hydrochloride were obtained from Sigma. Assay kits for fibrinogen and PAI were purchased from MeDiTech, Inc. (U.S.A.) and Biopool (Sweden), respectively.

Statistics

Mean values ± s.e.mean were compared by ANOVA and *P* < 0.05 taken as significant.

Results

Effects on coagulation system

LPS treatment produced a decrease in the levels of plasma fibrinogen (Figure 1) and a drop in the blood platelet count by 64 ± 11% from 480 ± 30 × 10³/mm³ in controls to 172 ± 21 × 10³/mm³ (*n* = 15). The decrease in fibrinogen (Figure 1) but not the drop in platelet count was reversed by pretreatment with L-NAME. L-NAME had no effect on plasma fibrinogen levels in control animals (Figure 1).

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Administration of LPS significantly activated fibrinolysis, as measured by shortening of the ECLT (Figure 2), and significantly increased plasma PAI activity (Figure 3). Pretreatment with L-NAME antagonized the LPS-induced activation of fibrinolysis (Figure 2) and enhanced the LPS-induced rise in plasma PAI (Figure 3). L-NAME had no

Figure 1 Effect of N^G-nitro-L-arginine methyl ester (L-NAME, 30 mg kg⁻¹, i.p., 24 h and 15 min before lipopolysaccharide, LPS) on the decrease in plasma fibrinogen produced by *E. coli* LPS (6 mg kg⁻¹, i.p.). **P* < 0.05 as compared to corresponding control. Numbers in parentheses indicate number of experiments.

Figure 2 Effect of N^G-nitro-L-arginine methyl ester (L-NAME, 30 mg kg⁻¹, i.p., 24 h and 15 min before lipopolysaccharide, LPS) on the shortening of euglobulin clot lysis time (ECLT) produced by *E. coli* LPS (6 mg kg⁻¹, i.p.). ***P* < 0.01 as compared to corresponding control. Numbers in parentheses indicate number of experiments.

Figure 3 Effect of N^G-nitro-L-arginine methyl ester (L-NAME, 30 mg kg⁻¹, i.p., 24 h and 15 min before lipopolysaccharide, LPS) on the increase of plasminogen activator inhibitor (PAI) levels produced by *E. coli* LPS (6 mg kg⁻¹, i.p.). ***P* < 0.01 as compared to corresponding control. Numbers in parentheses indicate number of experiments.

effect on either the plasma fibrinolytic activity or PAI levels in control animals (Figures 2 and 3).

Histological effects

In animals treated with LPS there were pathological changes including diffuse haemorrhagic congestion of the small intestine and ecchymotic haemorrhages in visceral organs including the heart, lungs spleen and kidneys. Microscopic examination revealed numerous fibrin microthrombi and leukocytic and erythrocytic aggregates in small blood vessels of the lung, heart, spleen, kidneys and intestine (*n* = 7).

When animals were pretreated with L-NAME there was a marked reduction in the pathological changes seen following treatment with LPS. Very little haemorrhagic congestion of the intestine was seen and ecchymotic haemorrhages in the visceral organs were largely absent.

Discussion

Disseminated intravascular coagulation (DIC), apart from causing hypotension, is considered to be an important cause of circulatory shock and death evoked by endotoxin (Bick & Kunkel, 1992). DIC is characterized by an abnormal simultaneous activation of the blood coagulation and fibrinolytic systems with resultant formation of microthrombi, obstruction of blood flow as well as internal haemorrhage. Here, we show that L-NAME, a potent inhibitor of NOS (Moore *et al.*, 1990) prevents the reduction in plasma fibrinogen level produced by LPS and partially protects against the accompanying haemorrhagic congestion of the intestine and viscera. In addition, L-NAME significantly suppressed the activation of the plasma fibrinolytic system induced by LPS, as assessed by changes in the ECLT. This antifibrinolytic effect of L-NAME correlated well with the L-NAME-induced rise of the plasma level of PAI. It is worthy of note that L-NAME was effective at a moderate dose of 30 mg kg⁻¹, which is similar to the dose of N^G-monomethyl-L-arginine, another inhibitor of NO synthase, that is beneficial in combatting the fall in blood pressure produced by intravenous administration of LPS (Nava *et al.*, 1991). In these experiments a tenth of the dose of N^G-monomethyl-L-arginine was ineffective whereas a 10 times higher dose (300 mg kg⁻¹) accelerated and enhanced the fall in blood pressure. Interestingly, in our experiments L-NAME attenuated most of the symptoms of DIC induced by LPS, suggesting that thrombocytopenia and associated intravascular platelet aggregation may not be a crucial event in this disorder.

The mechanism by which LPS causes DIC has been a matter of considerable debate and a number of pathophysiological actions of LPS have been implicated as the primary cause (Bick & Kunkel, 1992). These include, stimulation of platelet aggregation, increased procoagulant activity of monocytes and macrophages and increased cellular release of platelet activating factor and thromboxane A₂, as well as the stimulation of fibrinolysis through activation of Factor XII and the release of plasminogen pro-activator (van Deventer *et al.*, 1990; Quax *et al.*, 1990). In addition, recent studies point to augmented plasma levels of PAI contributing to the pathogenesis of DIC in septicemic patients (van Deventer *et al.*, 1990). Thus, LPS-induced DIC is a very complex phenomenon. The results of our study demonstrate that the beneficial effects produced by inhibition of NO synthase on the symptoms of LPS-induced DIC correlate with an increased generation of PAI, which is in agreement with our previous observation that NO inhibits the release of PAI (Korbut *et al.*, 1990; Lidbury *et al.*, 1990). Possibly, the increased generation of PAI in septicemia is a compensatory mechanism against excessive activation of the fibrinolytic system produced by NO. This could well be of great importance, considering that the level of PAI is critical in regulating the fibrinolytic system. We would, therefore, sug-

gest that L-NAME can act as a protective agent in LPS-induced DIC, and this protection is due to an increased generation of PAI following inhibition of NO synthase.

Further studies on the beneficial effects of NO synthase inhibitors in other types of circulatory shock, including those produced by haemorrhage and trauma may provide new

insights into the understanding of the fibrinolytic system and its regulation in health and disease.

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