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Association of microvascular leakage with induction of nitric oxide synthase: effects of nitric oxide synthase inhibitors in various organs

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Abstract

Endotoxin (*Escherichia coli* lipopolysaccharide 0111:B4, 3 mg/kg i.v.) induced the expression of a calcium-independent nitric oxide (NO) synthase, determined after 5 h in cardiac, hepatic, pulmonary and renal tissues, as assessed by the conversion of radiolabelled L-arginine to L-citrulline. This widespread induction of NO synthase in these conscious rats was associated with microvascular injury, as assessed by the vascular leakage of radiolabelled human serum albumin. Concurrent administration of the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME, 1–5 mg/kg s.c.) with endotoxin, provoked acute vascular leakage within 2 h in the various organs. By contrast, the delayed injection of L-NAME (1–5 mg/kg s.c.) or N^G-monomethyl-L-arginine (12.5–50 mg/kg s.c.) until 3 h after endotoxin challenge inhibited the subsequent microvascular leakage in these organs. These effects of NO synthase inhibitors were reversed by L-arginine (300 mg/kg s.c.) pretreatment. These results support a protective role of constitutive NO synthase in the early phase of endotoxin shock. Such actions contrast with the aggressive actions of the products of inducible NO synthase in the development of widespread microvascular injury in endotoxemic states.

Keywords: Nitric oxide (NO); Endotoxin; Microcirculation; N^G-Nitro-L-arginine methyl ester; N^G-Monomethyl-L-arginine; Multiple organ failure; Sepsis

1. Introduction

The most common origin of septic shock is the invasion of Gram-negative bacteria, frequently *Escherichia coli*, into the circulation (Root and Jacobs, 1991). The release of endotoxin lipopolysaccharides from the bacterial cell wall outer membrane is responsible for many of the pathological events including hypotension, microvascular injury and the consequent multiple organ failure, observed during Gram-negative sepsis (Parillo, 1990; Root and Jacobs, 1991; Bone, 1991). These effects may result from a direct injurious actions of lipopolysaccharides on the vascular endothelium (Harlan et al., 1983; Meyrick et al., 1986) or may reflect the formation of tissue-damaging vasoactive mediators (Parillo, 1990; Bone, 1991; Lefer and Lefer, 1993).

Under physiological circumstances, nitric oxide (NO), formed from L-arginine by the calcium-dependent constitutive NO synthase, plays a crucial role in the maintenance of vascular integrity (Moncada and Higgs, 1993). This vascular protective role of constitutive NO is also exerted under some pathological conditions, such as following the acute exposure to endotoxin (Hutcheson et al., 1990). However, following challenge with endotoxins or cytokines, the widespread expression of a calcium-independent inducible NO synthase can be detected after several hours in many organs (Salter et al., 1991; Knowles et al., 1990; Boughton-Smith et al., 1993; Mitchell et al., 1993; Szabo et al., 1994). Expression of the inducible isoform has also been determined in a number of distinct cell types including vascular endothelial cells, vascular smooth muscle, cardiac myocytes, parenchymal liver cells, intestinal mucosal cells, pulmonary alveolar macrophages, renal endothelial and juxtaglomerular cells (Radomski et al., 1990; Busse and Mülsch, 1990;

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Knowles et al., 1990; Brady et al., 1992; Schulz et al., 1992; Tepperman et al., 1993; Mitchell et al., 1993; Bandaletova et al., 1993; Tojo et al., 1994), as well as in inflammatory cells, such as neutrophils and macrophages (Marletta et al., 1988; McCall et al., 1989; Moncada, 1992; Bandaletova et al., 1993).

In comparison to the number of observations concerning the induction of NO synthase in response to endotoxin, much less evidence has accumulated concerning its relationship to the tissue injury or multiple organ failure, which commonly occurs in sepsis. We have, therefore, investigated the effects of *E. coli* lipopolysaccharide on microvascular endothelial damage, assessed by the vascular leakage of radiolabelled serum albumin, in the heart, lung, liver and kidney of the conscious rat and have correlated this with the induction of NO synthase over 5 h. In previous studies, the early administration of NO synthase inhibitors, N^G-nitro-L-arginine methyl ester (L-NAME) or N^G-monomethyl-L-arginine (L-NMMA) provoked acute plasma leakage into intestinal tissues following endotoxin challenge (Laszlo et al., 1994a). Therefore, to determine the role of NO, formed by the constitutive isoform in acute septic microvascular damage in various organs, L-NAME was injected concurrently with endotoxin. In further studies, L-NAME or L-NMMA was administered 3 h after endotoxin challenge, at a time of the known expression of the inducible calcium-independent isoform (Salter et al., 1991; Boughton-Smith et al., 1993, Bandaletova et al., 1993) to evaluate their effects on the plasma leakage associated with the induction of NO synthase.

2. Materials and methods

2.1. Nitric oxide synthase activity

Endotoxin (*E. coli* lipopolysaccharide 0111:B4; 3 mg/kg) was administered under transient halothane anaesthesia via a tail vein, to male Wistar rats (225–275 g) that had been fasted for 18 h. This dose of endotoxin was selected from previous studies as near-maximal for the induction of NO synthase (Knowles et al., 1990; Salter et al., 1991).

NO synthase activity in homogenates of liver, heart, lung and kidney was measured as the conversion of L-[^{14}C]arginine monohydrochloride to [^{14}C]citrulline, based on the method described by Salter et al. (1991) with minor modifications. Samples of hepatic, pulmonary, cardiac and renal tissues were taken 5 h after endotoxin administration, and homogenised (15 s) in ice-cold buffer (250 mg tissue/ml, 10 mM Hepes, 32 mM sucrose, 1 mM dithiothreitol, 0.1 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml of leupeptin, and 2 μg/ml of aprotonin) adjusted to pH 7.4 (8 M NaOH) followed by centrifugation for 20 min on 10000 $\times g$ at 4°C. A 40 μl sample of supernatant was incubated for 10 min at 37°C in 110 μl of reaction buffer comprising of (final concentrations) 50 mM KH_2P0_4, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 50 mM valine, 1 mM dithiothreitol, 15 nM L-arginine, 1 mM citrulline, 0.3 mM NADPH, 3 μM FAD, 3 μM FMN, and 157 pM of [^{14}C]L-arginine (110 dpm/ml). The reaction was arrested via the removal of the substrate L-arginine by the addition (0.5 ml) of a 1:1 suspension of Dowex (AG 50W-8) in water. The mixture was dispersed and diluted via the addition of 0.85 ml distilled water (total volume 1.5 ml). After allowing the resin to settle, the supernatant was removed for the estimation of the radiolabeled products by scintillation counting (2 ml Pico-Fluor). Sample protein content was estimated via spectrophotometric assay (Bio-Rad Protein Assay), allowing expression of NO synthase activity as pmol/min/mg protein.

NO synthase activity was confirmed by its inhibition by L-NMMA (300 μM) and the calcium dependency of this NO synthase activity under control conditions and following endotoxin administration (3 mg/kg i.v., for 5 h) was determined by incubation with 1 mM EGTA.

2.2. Plasma leakage

Plasma leakage of radiolabelled albumin was determined as previously described (Boughton-Smith et al., 1993). This [^{125}1]human serum albumin was administered intravenously via a tail vein (0.2 ml; 2 μCi/kg) immediately after endotoxin or isotonic saline in animals transiently anaesthetised with halothene, and the animals allowed to recover. Under terminal halothane anaesthesia, blood was collected from the abdominal aorta into syringes containing trisodium citrate (final concentration 0.318%) and centrifuged (10000 $\times g$, 10 min, 4°C). Segments of the liver, heart, lung parenchyma, kidney were removed, blotted dry and weighed and the total radiolabelled albumin content and that in the plasma (100 μl) was determined in a gamma spectrometer (Nuclear Enterprises NE 1600). Control values (from rats that had received saline) were subtracted from the treated values for each time point and the data were expressed as Δplasma leakage, μl plasma/g tissue, corrected for intravascular volume.

2.3. Intravascular volume

Changes in intravascular volume in hepatic, cardiac, pulmonary parenchymal and renal tissues were determined in additional groups of rats by administering radiolabelled albumin (2 μCi/kg) intravenously via the tail vein 2 min before tissue removal. The tissue and

plasma content of radiolabel was determined and the intravascular volume expressed as μl/g tissue.

2.4. Effect of early administration of L-NAME on endotoxin-induced plasma leakage

L-NAME (1–5 mg/kg s.c.) was administered concurrently with endotoxin (3 mg/kg i.v.) at the beginning of the experiment. For the determination of plasma leakage, the heart, lung and kidney were removed 2 h later.

2.5. Effect of delayed administration of L-NAME or L-NMMA on endotoxin-induced plasma leakage

L-NAME (1–5 mg/kg s.c.) was administered 3 h after endotoxin (3 mg/kg i.v.). Plasma leakage was determined in the heart, pulmonary parenchyma and kidney 2 h later, i.e. 5 h after endotoxin challenge. In additional experiments, the effect of the delayed administration (3 h) of L-NMMA (12.5–50 mg/kg s.c.) on plasma leakage following endotoxin challenge was also determined at 5 h in the heart, pulmonary parenchyma, kidney and liver.

2.6. L-Arginine reversal of the effects of L-NAME or L-NMMA

In separate groups of rats from both series of studies, where NO synthase inhibitors were administered either concurrently with endotoxin, or 3 h after endotoxin challenge, L-arginine (300 mg/kg s.c.) was injected 15 min before L-NAME (5 mg/kg s.c.) or L-NMMA (50 mg/kg s.c.) administration. Plasma leakage in the liver, heart, lung and kidney was determined 2 h after the administration of NO synthase inhibitors.

2.7. Drugs and materials

N^G-Monomethyl-L-arginine hydrochloride (L-NMMA) was synthesized in the Department of Medicinal Chemistry, Wellcome Research Laboratories. N^G-Nitro-L-arginine methyl ester (L-NAME) and *E. coli* lipopolysaccharide (0111:B4) were from Sigma Chemical Company (Poole, Dorset, UK). L-[U-^{14}C]Arginine monohydrochloride and ^{125}I-labelled human serum albumin were from Amersham International (UK). All other compounds and reagents were from Sigma.

2.8. Statistical analysis

For statistical comparisons, the Student's *t*-test for unpaired data or analysis of variance with the Bonferroni test were utilised, where $P < 0.05$ was taken as significant.

3. Results

3.1. Induction of nitric oxide synthase and plasma leakage by endotoxin

Basal NO synthase activity, that was abolished by incubation in vitro with L-NMMA (300 μM), was detected in the supernatants of homogenates of segments of the liver, heart, pulmonary parenchyma and kidney, being 3 ± 1, 12 ± 2, 8 ± 3 and 21 ± 2 pmol/min/mg protein ($n = 6$), respectively. The NO synthase activity in these supernatants was abolished by incubation in vitro with EGTA (1 mM), indicating it to be a calcium-dependent constitutive isoform.

Elevated NO synthase activity was detected 5 h after endotoxin administration in segments of the liver, heart, lung and kidney (Fig. 1). This increased NO synthase activity over basal levels, which was abolished by incubation with L-NMMA (300 μM), was not significantly inhibited by incubation with EGTA (1 mM), indicating it to be the calcium-independent inducible isoform.

Significant plasma leakage was found in the liver, heart, lung and kidney 5 h following endotoxin injection, as shown in Fig. 1.

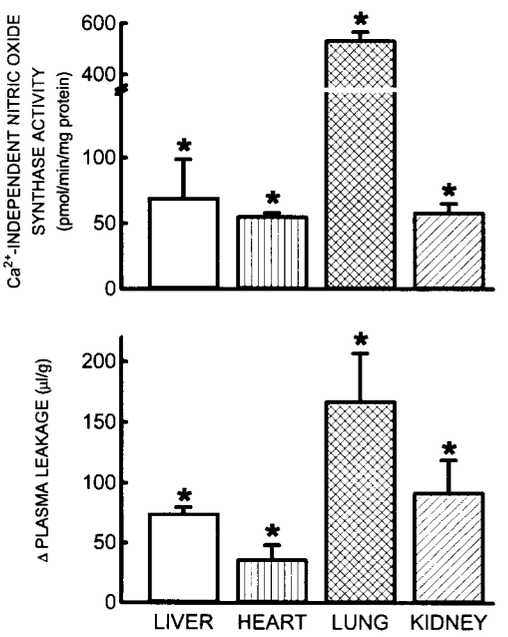

Fig. 1. Induction of calcium-independent nitric oxide synthase and vascular leakage of radiolabelled albumin by endotoxin (*E. coli* lipopolysaccharide; 3 mg/kg i.v.) in the liver, heart, lung and kidney of the conscious rats. Nitric oxide synthase activity (upper panel) and albumin leakage (expressed as Δplasma leakage, μl plasma/g tissue, lower panel) was determined 5 h after endotoxin challenge. Data are given as the mean ± S.E.M. of 4–6 rats per group; statistical significance is shown as * $P < 0.05$ compared to the control values (from rats that had received saline but not endotoxin).

3.2. Intravascular volume

The intravascular volumes did not change significantly 5 h after endotoxin (3 mg/kg i.v.) administration in the liver, heart, lung and kidney, being 131 ± 3 and 131 ± 4, 81 ± 4 and 82 ± 6, 203 ± 5 and 206 ± 16, and 105 ± 18 and 124 ± 13 μl/g tissue before and after endotoxin, respectively ($n = 3$ for each). Neither L-NAME (5 mg/kg s.c.) nor L-NMMA (50 mg/kg s.c.), administered either concurrently with endotoxin or 3 h after endotoxin challenge, affected intravascular volume in any of the tissues investigated ($n = 3$ for each; data not shown).

3.3. Effect of early administration of L-NAME on endotoxin-induced plasma leakage

Administration of endotoxin (3 mg/kg i.v.) or L-NAME (5 mg/kg s.c.) alone did not provoke significant plasma leakage in the heart (Δ0 ± 5 or Δ2 ± 4 μl/g tissue, $n = 6$ or 4, respectively), lung (Δ10 ± 8 or Δ7 ± 23 μl/g tissue, $n = 6$ or 4, respectively) and kidney (Δ5 ± 7 or Δ0 ± 3 μl/g tissue, $n = 6$ or 4, respectively) over a 2 h period. However, concurrent administration of L-NAME (1–5 mg/kg s.c.) with endotoxin (3 mg/kg i.v.) caused a dose-dependent elevation of plasma leakage in cardiac, pulmonary and renal tissues when determined 2 h later (Fig. 2).

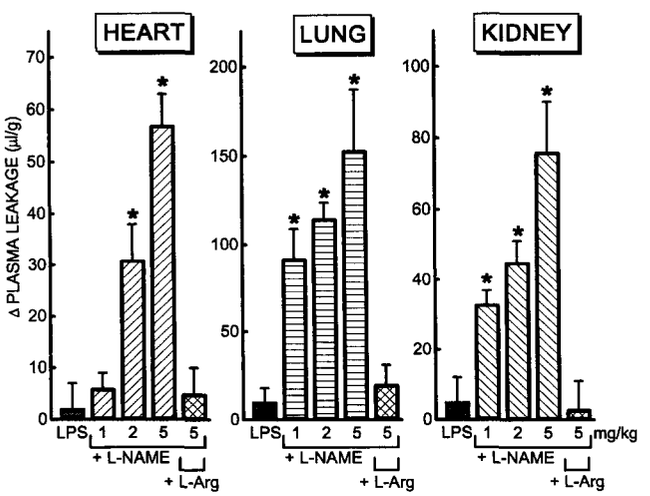

Fig. 2. Provocation of vascular leakage of radiolabelled albumin by the concurrent administration of endotoxin (LPS; 3 mg/kg i.v.) with N^G-nitro-L-arginine methyl ester (L-NAME; 1–5 mg/kg s.c.), and its reversal by the pretreatment with L-arginine (L-Arg; 300 mg/kg s.c.; 15 min before L-NAME) in the heart, lung and kidney of the conscious rat. Albumin leakage (expressed as Δplasma leakage, μl plasma/g tissue) was determined 2 h after challenge. Data are given as the mean ± S.E.M. of 6 rats per group; statistical significance is shown as * $P < 0.05$ compared to the LPS group.

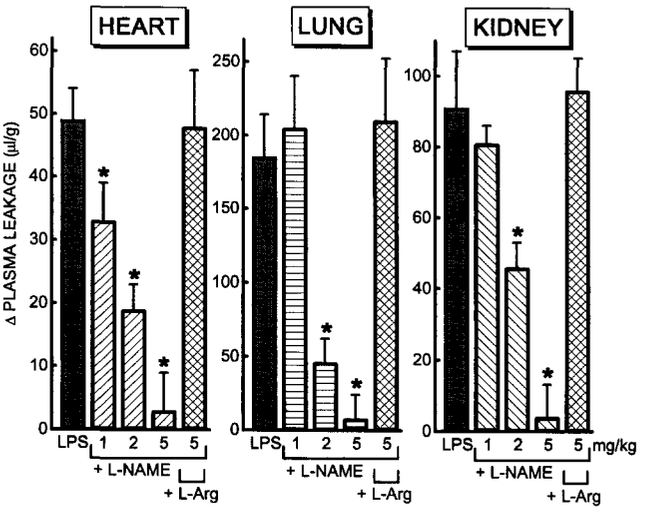

Fig. 3. Inhibition of endotoxin (LPS; 3 mg/kg i.v.)-induced vascular leakage of radiolabelled albumin by the delayed administration of N^G-nitro-L-arginine methyl ester (L-NAME; 1–5 mg/kg s.c., 3 h after endotoxin), and its reversal by L-arginine (L-Arg; 300 mg/kg s.c.; 15 min before L-NAME) in the heart, lung and kidney of the conscious rat. Albumin leakage (expressed as Δplasma leakage, μl plasma/g tissue) was determined 5 h after endotoxin challenge. Data are given as the mean ± S.E.M. of 6 rats per group; statistical significance is shown as * $P < 0.05$ compared to the LPS group.

3.4. Effect of delayed administration of L-NAME or L-NMMA on plasma leakage induced by endotoxin

By contrast, the delayed administration of L-NAME (1–5 mg/kg s.c.) 3 h after endotoxin challenge, dose-dependently inhibited plasma leakage in the heart, lung and kidney (with a maximal reduction of 94 ± 12%, 96 ± 9% and 96 ± 8%, respectively, $n = 6$, $P < 0.001$) determined 2 h later (Fig. 3).

Likewise, the delayed (3 h) administration of L-NMMA (12.5–50 mg/kg s.c.) reduced the endotoxin-induced plasma leakage into the cardiac, pulmonary, renal and hepatic tissues (by a maximal reduction of 92 ± 8%, 96 ± 9%, 88 ± 7% and 93 ± 4%, respectively, $n = 6$ for each, $P < 0.001$), determined 2 h later (i.e. 5 h after endotoxin challenge), as shown in Fig. 4.

3.5. L-Arginine reversal of the effects of the nitric oxide synthase inhibitors

Pretreatment with L-arginine (300 mg/kg s.c., 15 min before the NO synthase inhibitor) reversed the aggravation of plasma leakage, caused by the concurrent administration of L-NAME (5 mg/kg s.c.) with endotoxin (Fig. 2). Similarly, administration of L-arginine (300 mg/kg s.c., 15 min before the NO synthase inhibitors) abolished the protection caused by the delayed administration of L-NAME (5 mg/kg s.c.) or

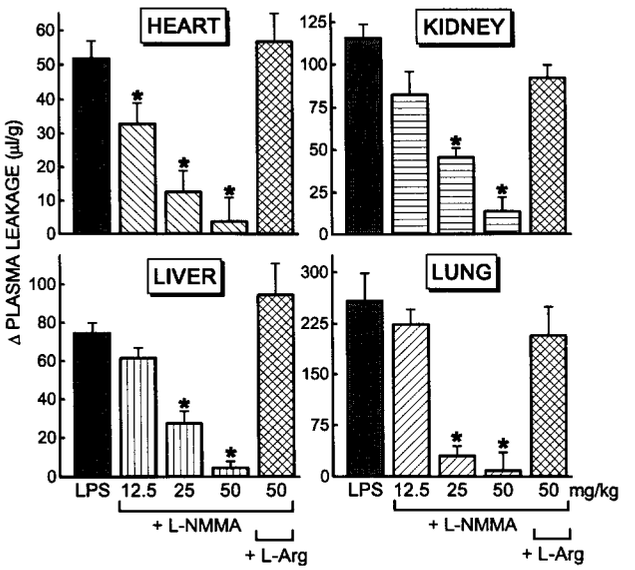

Fig. 4. Inhibition of endotoxin (LPS; 3 mg/kg i.v.)-induced vascular leakage of radiolabelled albumin by the delayed administration of N^G-monomethyl-L-arginine (L-NMMA; 12.5–50 mg/kg s.c., 3 h after endotoxin), and its reversal by L-arginine (L-Arg; 300 mg/kg s.c.; 15 min before L-NMMA) in the liver, heart, lung and kidney of the conscious rat. Albumin leakage (expressed as Δplasma leakage, μl plasma/g tissue) was determined 5 h after endotoxin challenge. Data are given as the mean ± S.E.M. of 6 rats per group; statistical significance is shown as * $P < 0.05$ compared to the LPS group.

L-NMMA (50 mg/kg s.c.) in all of the organs investigated (Fig. 3 and Fig. 4).

4. Discussion

Our study confirms previous observations that the administration of endotoxin leads to the induction of a calcium-independent NO synthase in the heart, lung, kidney and liver over a 5 h period (Knowles et al., 1990; Salter et al., 1991). The expression of the inducible enzyme in tissue following endotoxin challenge has also been demonstrated over this time period using immunohistochemical techniques (Bandaletova et al., 1993). Moreover, in the present study, we have now demonstrated the concurrent elevation of vascular albumin leakage into cardiac, pulmonary, renal and hepatic tissues, suggesting a correlation between these events. Additionally, the greatest degree of microvascular leakage was observed in the lung, which also exhibited the greatest expression of calcium-independent NO synthase activity, with total NO synthase activity increasing 60-fold. Vascular endothelial cell injury is known to have a key importance in the development of sepsis syndrome (Bone, 1991). Furthermore, incubation of endothelial cells with endotoxin and cytokines caused a time-and dose-dependent cell injury, which correlated with the induction of NO synthase (Palmer et al., 1992). Such cell injury derived from the sustained excessive generation of NO may involve the cytotoxic peroxynitrite moiety or other radicals (Beckman et al., 1990; Hogg et al., 1992; Lipton et al., 1993).

Administration of NO synthase inhibitors restored the reduced blood pressure in septic shock in various species including humans, indicating an important therapeutic utility (Kilbourn et al., 1990; Thiemermann and Vane, 1990; Petros et al., 1991; Nava et al., 1991). However, the beneficial effect of these non-selective inhibitors of the NO synthase isoforms during early studies in experimental sepsis appeared to depend on the dose used, with high doses being detrimental (Klabunde and Ritger, 1991; Wright et al., 1992; Nava et al., 1991; Rojas et al., 1993), and intermediate doses of NO synthase inhibitors improving the outcome in endotoxin shock (Nava et al., 1991). Such findings suggested that the simultaneous abolition of both constitutive and inducible NO synthase will be harmful in sepsis (Wright et al., 1992; Nava et al., 1991).

In the present study, we have shown that the concurrent administration of the NO synthase inhibitor, L-NAME with endotoxin provoked microvascular injury in the heart, lung and kidney over 2 h. Neither L-NAME nor endotoxin alone elevated plasma leakage after 2 h in any of the organs investigated. Previous studies have also supported the protective role of constitutive NO against endotoxin damage in other organs. Thus, in the small intestine, pretreatment with L-NMMA augmented acute mucosal injury following the injection of a high dose of endotoxin (Hutcheson et al., 1990). In the ileum and colon, concurrent administration of NO synthase inhibitors with a lower dose of endotoxin provoked microvascular permeability (Laszlo et al., 1994a,b). Furthermore, in the kidney, simultaneous administration of L-NAME with endotoxin caused renal cortical necrosis, while endotoxin or L-NAME alone did not cause this injury (Shulk and Raij, 1992). In the liver, pretreatment with NO synthase inhibitors potentiated hepatic parenchymal injury induced by endotoxin (Billiar et al., 1990; Frederick et al., 1993). These studies all support the protective role of constitutively-formed NO in acute shock and could explain the reported deleterious actions of NO synthase inhibitors when administered early in different experimental models of shock. These protective actions of NO may in part involve modulation of local organ blood flow, since in acute endotoxaemia in rats, pretreatment with NO synthase inhibitors reduced tissue perfusion in the heart, liver, intestine and kidney (Mulder et al., 1994).

In contrast to these findings, the delayed administration of L-NAME or L-NMMA at a time of known expression of the calcium-independent inducible NO synthase (Salter et al., 1991; Boughton-Smith et al.,

1993; Bandaletova et al., 1993) caused a dose-dependent reduction in vascular endothelial damage provoked by endotoxin over 5 h in the heart, lung, liver and kidney, effects that were reversed by L-arginine. The beneficial effect of the delayed administration of the NO synthase inhibitors on endotoxin-induced microvascular injury in the small and large intestine has also been demonstrated (Boughton-Smith et al., 1993; Laszlo et al., 1994a). Moreover, in another recent study, the delayed administration of L-NAME has been shown to normalize the endotoxin-induced hyperdynamic state, but did not impair oxygen consumption, indicating adequate tissue perfusion (Meyer et al., 1994).

Our current in vivo findings thus indicate that the early inhibition of NO synthase causes plasma leakage in the heart, lung and kidney in endotoxin-treated rats, clearly demonstrating the importance of the constitutive NO synthase in the maintenance of microvascular integrity in acute endotoxaemic states. Under these conditions, the formation of NO may thus promote vasodilatation and also offset the deleterious actions of acutely released cytotoxic mediators such as platelet-activating factor and thromboxane in the microcirculation (Laszlo et al., 1994b). These mediators may derive from neutrophils since pretreatment with anti-neutrophil serum attenuated the acute microvascular dysfunction (Laszlo et al., 1995). Such findings should therefore be considered when evaluating the outcome of studies in septic shock where NO synthase inhibitors are administered early in the experimental protocol. By contrast to these observations, the widespread tissue expression of the inducible NO synthase was associated with microvascular damage in cardiac, pulmonary, renal and hepatic tissues. These observations may reflect the initiating pathological events in the microcirculation, that lead to the multiple organ failure during sepsis. The correction of such microvascular leakage in these organs by the delayed administration of NO synthase inhibitors gives further support of the injurious actions of NO, synthesized by the inducible isoform, in the generalised microcirculatory dysfunction in endotoxaemia. Thus, inhibition of the inducible NO synthase may have potential therapeutic benefit in the management of multiple organ failure resulting from endotoxic shock and sepsis.

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