Septic shock in man is characterized by microbial invasion of the bloodstream, hypotension, hyporeactivity to vasopressor agents, vascular leak and circulatory failure leading to multiple organ dysfunction and death [1]. A common cause of septic shock is Gram-negative bacterial infection, and administration of Gram-negative bacterial endotoxin in animals and man produces a shock-like syndrome [2,3]. 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), interleukin-6 (IL-6) and interferon-γ (IFN-γ). Indeed, administration of TNF-α and IL-1 in animals and man produces the systemic and pathological features of endotoxin-induced shock. Emerging evidence suggests that overproduction of NO may be the common mechanism by which endotoxin and cytokines bring about their deleterious action [4].

The L-arginine: NO pathway [5] is involved in a variety of physiological processes in the cardiovascular and nervous system where NO is generated via constitutive Ca<sup>2+</sup>-dependent NO syntheses [5,6]. In the vasculature, the NO synthase releases relatively small amounts of NO in response to receptor stimulation and shear stress and maintains a vasodilator tone [5,7]. An inducible Ca<sup>2+</sup>-independent NO synthase is expressed in phagocytic and other cells after activation by endotoxin and/or cytokines. Much larger amounts of NO are generated by this enzyme, which is responsible for the cytotoxicity of macrophages towards certain micro-organisms and tumour cells and may account for the phenomenon of non-specific immunity [5].

Endotoxin and/or cytokines also induce the expression of a Ca<sup>2+</sup>-independent NO synthase in vascular endothelial and smooth muscle cells and cardiac myocytes [4,8–10]. Isolated blood vessels treated with endotoxin show a time-dependent expression of the inducible NO synthase over a period of 8 h, which begins after a lag period of 2 h. This is accompanied by an increase in the levels of cyclic GMP in the tissue over the same time course. These observations, together with the finding that precontracted vessels exhibit a time-dependent relaxation which also begins after a lag period of approximately 2 h, indicate that endotoxin increases the synthesis of NO in these tissues ([4] and Figure 1). The relaxation over the 8 h period is attenuated by polymyxin B which binds and neutralizes endotoxin [11], confirming that endotoxin provides the stimulus for induction of the Ca<sup>2+</sup>-independent NO synthase. The expression of this enzyme is abolished by preincubation with cycloheximide, an inhibitor of protein synthesis, indicating that the increased amount of NO is dependent upon the synthesis of new enzyme. The Ca<sup>2+</sup>-dependent (constitutive) NO synthase activity is unchanged over this time course and is unaffected by preincubation with cycloheximide, indicating that the synthesis of NO by this enzyme does not contribute to the loss of tone [4].

The activation of the inducible NO synthase by endotoxin results in much larger quantities of NO being produced than that of the constitutive enzyme. The inducible NO synthase is expressed
Induction of a Ca\(^{2+}\)-independent (inducible) NO synthase (a) and loss of tone (b) in the rat aorta after addition of endotoxin (Salmonella typhosa, 100 ng/ml)

Abbreviation used: Phe, phenylephrine.

in both the endothelium and the smooth muscle layer, whereas the constitutive enzyme is present only in the endothelium. In addition, the increased synthesis of NO, the elevated cyclic GMP levels and the loss of vessel tone are largely independent of the presence of the endothelium. Therefore, the vascular smooth muscle layer is the major source of the increased NO and since the smooth muscle occupies a greater mass, it is likely that increased levels of enzyme result in increased NO production. The majority of the Ca\(^{2+}\)-independent NO synthase present in the vessel wall after administration of endotoxin in vivo is also located in the smooth muscle layer [12]. Prolonged exposure of rat aortae to endotoxin in vitro [13] and in vivo [14] also results in diminished contractility to vasoconstrictors [9,15]. The induction of NO synthase therefore provides an explanation for those observations, since the loss of vessel tone and the development of hyporesponsiveness to the vasoconstrictor phenylephrine is associated with the increased generation of NO.

Previous studies [15,16] have demonstrated that the Ca\(^{2+}\)-dependent NO synthase is not substrate-limited. By contrast, the Ca\(^{2+}\)-independent enzyme in the smooth muscle appears to be partially substrate-limited, since exogenous 1-arginine exhibits a time-dependent relaxation which occurs over an 8 h time course and is inhibited by cyclohexamide. Further work is required to clarify the substrate regulation of NO synthesis by these enzymes in both cells types and its functional consequences.

Several of the biological effects of endotoxin are mediated by induction of cytokines. In particular, IL-1 and TNF-\(\alpha\) have been implicated as important mediators involved in both systemic and local vasodilatation in response to endotoxin [1]. Although macrophages in the blood and tissue are the primary source of the cytokines, endotoxin has been shown to induce the release of IL-1 and TNF-\(\alpha\) from endothelial [17] and smooth muscle cells [18] in culture and fresh vascular tissue [13]. Indeed, TNF-\(\alpha\) and IL-1 lead to an increase in intracellular cyclic GMP in vascular tissue and to a hyporesponsiveness to vasoconstrictors which is enhanced by IFN-\(\gamma\) and inhibited by cycloheximide [19]. Furthermore, IL-1 induced prolonged 1-arginine-dependent cyclic GMP and nitrite production in rat vascular smooth muscle cells [20]. Thus the effect of endotoxin on vascular tone may also involve stimulation of cytokine synthesis which in turn stimulates the induction of the Ca\(^{2+}\)-independent NO synthase.

The glucocorticoid dexamethasone inhibits the time-dependent fall in vascular tone, the associated rise in cyclic GMP levels and the induction of the Ca\(^{2+}\)-independent NO synthase, without affecting the activity of the constitutive enzyme. However, dexamethasone cannot restore the tone of the blood vessels once the inducible enzyme is expressed, indicating that the steroid inhibits the synthesis of the enzyme rather than its activity. These findings suggest that inhibition of the expression of the inducible NO synthase by glucocorticoids contributes to the prevention of hypotension during endotoxin shock. Clinical data have shown that steroids are only effective if administered soon after the onset of shock (<4–6 h after onset [21]); therefore, these results explain why they are more effective at preventing rather than treating the condition. Glucocorticoids also inhibit the endotoxin-induced synthesis and release of IL-1 and TNF-\(\alpha\), and prevent the resultant hyporesponsiveness to vasoconstrictors [13] and their associated systemic and pathological features in shock.
Whether the inhibitory effect of dexamethasone is due not only to inhibition of the synthesis of cytokines but also to direct inhibition of the expression of this NO synthase remains to be established.

Intravenous administration of endotoxin to the conscious mouse stimulates the release of TNF-α and IL-6 within 0.5 h and 1 h respectively. After 2 h, a Ca²⁺-independent NO synthase is induced in the heart accompanied by an increase in plasma concentrations of nitrite/nitrate (NOx; the metabolic breakdown products of NO), a profound fall in blood pressure over a similar time course (Figure 2) and a markedly attenuated vasopressor response to norepinephrine. Thus, the data in vivo are consistent with the observations in vitro, indicating that an increase in the generation of NO from an inducible NO synthase appears to underlie the hypotension and hyporeactivity to vasoconstrictor agents in this experimental model of septic shock [22].

Inhibition of NO synthase may therefore be of benefit in septic shock by inhibiting the excessive release of NO. Indeed, the NO synthase inhibitor N⁶-monomethyl-L-arginine (L-NMMA; 546C88) restores the loss of vessel tone (Figure 3) and prevents the hyporesponsiveness to vasoconstrictor agents in endotoxin-treated isolated vessels in vitro [9,15,23], demonstrating that the increased production of NO functionality antagonizes the effect of phenylephrine (Figure 3). Furthermore, L-NMMA increases the blood pressure in dogs [24] and rats [25,26] and reverses the hyporesponsiveness to vasoconstrictor agents after endotoxin administration in vivo [27]. Although L-NMMA has been shown to restore blood pressure in animal models of endotoxin or septic shock, these studies have mainly used anesthetized animals and their overall effect on mortality has been inconclusive [24–26]. In a conscious mouse model of septic shock, L-NMMA has been shown to both reduce [28] and have no effect on mortality [29], whereas other animal models of shock have reported detrimental effects using L-NMMA [30,31]. However, these adverse responses occurred when either a high bolus dose of the compound was used (≥30 mg/kg) or when L-NMMA was administered at the same time as endotoxin. Recent studies in the conscious rat have shown that L-NMMA, when administered at the same time as endotoxin, enhances vascular leak. By contrast, administration of the compound several hours after endotoxin (i.e. when the inducible NO synthase is expressed) reverses the vascular leak [32]. Adverse effects of L-NMMA in these various animal models of septic shock may also relate to inadequate volume loading, a necessary requirement due to the vascular leak. Failure to do so leads to a hypodynamic state, leading to a low cardiac output and peripheral vasoconstriction. In this situation, treatment with L-NMMA would be inappropriate. Indeed, after appropriate fluid resuscitation similar to clinical practice, L-NMMA proved beneficial in a sheep model of...
septic shock [33]. All these data confirm that NO is both protective and damaging, depending on the amount of NO generated; in small quantities, NO maintains a physiological vasodilator tone and may be an early defence mechanism in shock. By contrast, when produced in excess, NO causes pathophysiological vasodilatation, vascular leak and circulatory failure. Our data, using the conscious mouse model of endotoxic shock, suggest that a continuous infusion of a low dose of L-NMMA over several hours during the shock phase affords optimal protection, and studies are ongoing to determine whether this dose regimen improves survival [22].

There is now substantial evidence that overproduction of NO underlies the hypotension and hyporeactivity to vasopressor therapy observed in septic shock in man [34–36]. Moreover, L-NMMA effectively restores blood pressure in patients with septic shock [36,37] and the vascular effects are similar in patients with sepsis due to either Gram-negative, Gram-positive or unidentified organisms [36,37]. Although their study was too small to detect changes in mortality, it suggests that L-NMMA has a potential advantage over therapies which are targeted at specific bacterial toxins or cytokines. Moreover, anti-endotoxin strategies suffer from the disadvantage that they will not help patients with non-Gram-negative infection and may in fact prove harmful in such patients [38]. L-NMMA (546C88) is now in phase II clinical development for the treatment of septic shock. By inhibiting the overproduction of NO and restoring cardiovascular homeostasis, L-NMMA represents a novel and promising therapeutic approach for this life-threatening condition.

Cell signalling events involved in mediating the induction of nitric oxide synthase in macrophages and vascular smooth muscle cells

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Introduction

The active component of endotoxin, lipopolysaccharide (LPS), elicits a number of well-recognized responses in several cell types including macrophages, hepatocytes and vascular smooth muscle cells [1]. In these cells, LPS stimulates the induction of the 130 kDa isoform of nitric oxide synthase (iNOS) and nitric oxide formation [2,3]. Increased NOS protein expression results from increased transcription of the iNOS gene and this can be regulated principally by the binding of transcription factors such as nuclear factor-κB and interferon regulatory factor-1 to promoters within the iNOS gene. Activation of such factors by components of intercellular signalling cascades is a key event in the initiation of iNOS gene transcription [3–5].

For the purpose of this paper, cell signalling pathways involved in the regulation of NOS induction will be discussed; however, it should be recognized that the activation of other transcription events such as induction of cyclooxygenase [6] and secretory phospholipase A₂ [7] are also a feature of LPS stimulation. Thus, defining the role for second messenger systems in the induction of NOS is likely to be of relevance to the other cellular effects observed in response to LPS.

Initiation of intracellular signals in response to LPS — activation of tyrosine kinase cascades

A large number of cellular signalling events have been proposed to play a role in the regulation of NOS induction; however, at present there is no consensus as to which pathways are involved. This is in part due to the lack of a defined receptor for LPS in a number of LPS-responsive cells. In cells of lymphoid origin however, LPS, bound to a serum-derived binding protein [8], is believed to interact with the surface differentiation molecule CD14, a phosphatidylinositol glycan-linked protein [9]. The structure of this receptor does not resemble those of either G-protein-coupled receptors or growth factor receptors that possess intrinsic tyrosine kinase activity. However, the signalling events initiated by LPS activation of CD14 do share similarities with classical antigen receptors since activation of tyrosine kinases of the Src family, p53/p69, p58/61 and p59/56, is observed [10]. Despite this, no evidence exists for the phosphorylation of antigen-recognition—activation motifs and interaction with effector molecules such as Grb2, an established feature of antigen receptor signalling [11], as CD14 lacks any form of intracellular domain. Although the precise mechanism by which initial transmembrane signalling occurs in response to CD14 remains to be elucidated, downstream kinase cascades are known to be activated. This includes the mitogen-activated protein kinases (p42 and p44 MAP kinase) [12,13] and also the recently defined stress-activated protein kinase (SAPkinase) cascade, consisting of p38 SAP kinase and the N-terminal Jun kinase p54 [14,15] (Figure 1). While the MAP

Abbreviations used: DAG, diacylglycerol; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; MAP, mitogen-activated protein; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PtdCho-PLC, phosphatidyocholine—phospholipase C; SAP, stress-activated protein.