Nitric oxide in sepsis and endotoxaemia

James R. Parratt*

Department of Physiology and Pharmacology, University of Strathclyde, Royal College, 204 George Street, Glasgow G1 1XW, UK

Nitric oxide (NO) is one of many vasoactive substances released, from a variety of cells, under conditions of endotoxaemia and sepsis. Under physiological conditions it is produced by two constitutive calcium-dependent enzymes (nitric oxide synthase; NOS) in neurones (nNOS) and endothelial cells (eNOS) and has functions ranging from neurotransmission and vasodilatation to inhibition of platelet adhesion and aggregation. Following bacterial infection, especially with Gram-negative organisms, its formation from L-arginine is enhanced due to the cytokine-mediated induction of a NOS enzyme (iNOS) in cells (e.g. cardiac myocytes, vascular smooth muscle) that do not normally have the ability to synthesize NO. The result of this excessive NO production is enhanced bacterial lysis by activated macrophages, vasoplegia and myocardial depression. These cardiovascular effects can be alleviated by inhibitors of the L-arginine NO pathway, which results in elevated perfusion pressure, restored responsiveness to sympathetic nerve stimulation and to exogenous catecholamines, and to enhanced (endothelin-dependent) myocardial contractility. In patients in shock this approach also leads to detrimental effects (increased systemic vascular resistance, elevated pulmonary artery pressure, reduced cardiac output and oxygen delivery, increased platelet accumulation) and survival is not improved. Because some of these detrimental effects are due to inhibition of eNOS, attempts have been made to examine the effects of substances with a higher selectivity for the induced form of the enzyme. In experimental animals, one of these (L-canavanine) protects endothelial cells from damage, increases survival time and restores vascular responsiveness without increasing blood pressure or peripheral vascular resistance. However, whether even this approach will be of benefit to patients with sepsis remains in doubt since studies in iNOS knock-out mice do not support the concept that eliminating this particular source of NO improves ultimate survival.

Introduction

Many mediators which markedly influence cardiovascular function are generated in response to bacterial endotoxin release or administration. These vasoactive substances include catecholamines, histamine, 5-hydroxytryptamine, opioid peptides such as enkephalins and endorphins, purines, kinins, prostaglandins, thromboxanes, leukotrienes, angiotensin, various as yet uncharacterized ‘shock toxins’ and oxygen-derived free radicals. The particular relevance of any one of these mediators to the pathophysiology of sepsis is unclear. There have been numerous attempts to measure the concentration of mediators in blood, to determine their rate of synthesis and/or breakdown, and to interfere with their production, storage, destruction and effects on receptors. Such approaches are valid in that they attempt to simplify a complex situation. Where this approach fails is that it ignores the intimate interrelationships between so many of these circulating substances. A n examination of these complex interrelationships has been attempted recently and anyone with an interest in mediators of sepsis will find this review a good starting point.

Nitric oxide (NO) is perhaps the latest mediator to be scrutinized with reference to the pathophysiology of sepsis and has been shown to be particularly important both as a mediator of the loss of vascular tone (vasoplegia), which is characteristic of the early, hyperdynamic phase of sepsis, and as a participant in the myocardial depression which is...
also seen. Its potential importance lies in its role in governing the interaction between platelets, leucocytes and the vascular endothelium, and its critical place in local blood flow regulation. There are also important interactions between NO and oxygen derived-free radicals. Nitric oxide is thus both a physiological regulator and an important factor involved in cellular damage. This brief review will explain what NO is, how it is formed under physiological conditions and following the onset of sepsis, how it influences the function of the heart and blood vessels, what happens when its synthesis is inhibited and how it interacts with oxygen free radicals. Finally, it is discussed whether interfering with its generation, or its effects at cellular level, might result in strategies that could beneficially modify the development of shock in the septic patient.

Nitric oxide

Nitric oxide is a highly water-soluble gas, and is a pollutant found in vehicle exhaust fumes and in cigarette smoke with the capacity to form carcinogenic N-nitrosamines. It is not to be confused with nitrous oxide (N₂O or laughing gas). Nitric acid is an ideal local physiological messenger because of its small size, lipophilic nature and brief duration of action, having a half-life of <6 s. This highly reactive oxidation product of nitrogen is produced normally by many cell types, including endothelial cells, and has functions ranging from neurotransmission and vasodilatation to the cytotoxicity associated with activated macrophages. Nitric oxide is synthesized enzymatically from the amino acid L-arginine by different isoenzymes of nitric oxide synthase (NOS); these enzymes have been purified and the genes cloned from a number of species including man. Chromosomal mapping and DNA sequencing of the genes encoding NOS predicts three distinct gene products. Two of these NOS isoenzymes are expressed constitutively in vascular endothelial cells (eNOS or type III NOS) and in neurons (nNOS or type I NOS) whereas the expression of a third isoenzyme (iNOS or type II NOS) is inducible in a variety of cells (including macrophages, hepatocytes, vascular smooth muscle and cardiac myocytes) by cellular products of both Gram-negative (endotoxin) and Gram-positive bacteria. This induction is cytokine mediated. Constitutive isoforms (types I and III NOS) are calcium- and calmodulin-dependent enzymes and are activated following a rise in intracellular concentrations of free calcium. The formation of NO by iNOS (for example in cytokine-activated macrophages) is independent of calcium. This enzyme is an NADPH-, biopterin- and flavin-dependent enzyme, the expression of which requires de novo protein synthesis. A summary of the process of formation of NO from L-arginine in generator cells, such as the endothelium, is illustrated in Figure 1. Nitric oxide has a high binding affinity for haem iron (forming transitional metal complexes) and thus reacts with metalloproteins such as haemoglobin and soluble guanylate cyclase. The activation of this latter enzyme leads to the formation of cyclic guanosine 3’5’-monophosphate (cGMP). This has a variety of biochemical effects some of which, for example in vascular smooth muscle cells, lead to a decrease in intracellular calcium and to muscle relaxation. A potentially important mechanism for NO regulation under physiological conditions is that NO results in feedback inhibition of NOS. Inhibition of NOS certainly occurs following the administration of drugs (for

![Figure 1](image1.png)
example glyceryl trinitrate) which 'donate' nitric oxide to tissues. The formation of NO can also be inhibited, in a dose-dependent and enantiomer-specific manner, by various analogues of L-arginine (and also of L-ornithine) and these have been extensively used to assess the role of NO production under a variety of physiological and pathophysiological conditions. These analogues include N\(^6\)-monomethyl-L-arginine (L-NMMA), N\(_2\)-nitro-L-arginine methyl ester (L-NAME) and N-iminoethyl-L-ornithine (L-NIO).

Nitric oxide can be either cytotoxic or protective. Under physiological conditions it probably acts as an important free radical scavenger, limiting the toxicity associated with free radicals such as superoxide (O\(_2^−\)). Hence it inhibits platelet aggregation and leucocyte adhesion to endothelial cells and stabilizes cell membranes (resulting in reduced ischaemia-reperfusion damage). In macrophages the biological purpose of NO and of its degradation products, is to contain and eliminate invading organisms. However, NO also has direct cytotoxic effects. When it reacts with the superoxide radical formed, for example during sepsis, likely byproducts include peroxynitrite (O\(_2NO^-\); a potent and highly reactive oxidant) and nitrogen dioxide (NO\(_2\)), which in aqueous solution forms nitrous and nitric acids, and hence nitrite and nitrate, which are elevated in sepsis.\(^7\)

Peroxynitrite decomposes, albeit slowly, but especially at acid pH, to strong oxidants including the highly toxic hydroxyl anion (OH\(^−\)). The pathways of peroxynitrite-induced cellular damage include platelet aggregation, disseminated intravascular coagulation, stimulation of lipid peroxidation and inhibition of mitochondrial respiration.

### Nitric oxide in sepsis and endotoxaemia

The hyperdynamic phase of sepsis in patients, or following iv infusion of endotoxin in experimental animals, is characterized by low peripheral vascular resistance and a high cardiac output despite the early onset of impaired myocardial contractility. The low peripheral vascular resistance, or vasoplegia, presumably contributes to the unrelenting hypotension associated with sepsis, and to the resultant vascular hyporesponsiveness to administered catecholamines. This persistent defect in peripheral vascular tone, irrespective of the cardiac index, is a major cause of mortality.

Previous explanations for peripheral vasodilation in sepsis have included 'transmission failure' with the release of false transmitters from sympathetic nerves, down-regulation of receptors (particularly \(\alpha\)-adrenoceptors), the presence of potent circulating vasodilator substances and some defect in cell signalling. A though many vasodilator substances are released in sepsis, the present evidence suggests that the major factor involved in this loss of peripheral vascular tone is the generation of NO through the induction, by cytokines, of iNOS in vascular smooth muscle cells. The amounts of NO generated under these conditions are substantial. In the vessel wall, paralysis of vascular smooth muscle function is principally due to inhibition of responsiveness to noradrenaline, the predominant transmitter at sympathetic nerve endings. The two main pieces of evidence for this important concept can be summarized as follows:

1. The loss of responsiveness to sympathetic nerve stimulation (and to administration of catecholamines) persists even when blood vessels are removed from contact with circulatory vasodilator substances and studied in vitro, suggesting some defect in the contractile machinery of the cell. The interest generated by the discovery that NO is responsible for smooth muscle relaxation following activation of endothelial cells by a variety of agonists and its identification with endothelium-derived relaxing factor, together with the availability of inhibitors of the NO-generating pathway; prompted Jean-Claude Stoclet and his colleagues to determine whether NO had a role in this loss of responsiveness. The title of their presentation to the Physiological Society in December 1989 ('Impaired vascular reactivity in the rat following endotoxin treatment can be endothelium-independent yet involves the L-arginine pathway')\(^8\) is particularly instructive. First, it implicated NO in the reduced vascular responsiveness associated with endotoxin administration. Second, it suggested that the source of the NO was not endothelial cells, since this depression of responsiveness (for example to noradrenaline) persisted in vessels denuded of endothelium (Figure 2). The responsiveness to noradrenaline could be corrected by inhibition of the L-arginine NO pathway (Figure 2) and this was confirmed by estimations of the cGMP content of these isolated vessels. In vessels removed from rats given endotoxin there was an enormous rise in cGMP levels, in comparison with control vessels, and these levels were reduced to insignificant amounts by an inhibitor of the L-arginine NO pathway.\(^9\) Thus, endotoxin induced an extra-endothelial activation of the NO pathway; later studies showed that this production of NO occurred predominantly in vascular smooth muscle cells.\(^10\) In small vessels, which are those predominantly involved in the control of peripheral vascular resistance, the amount of NO is critically dependent upon circulating levels of L-arginine.\(^11\)

2. Inhibitors of the L-arginine NO pathway, or substances that inhibit the effect of NO on soluble guanylyl cyclase (such as methylene blue), restore responsiveness to noradrenaline and to sympathetic nerve stimulation in experimental animals given endotoxin, or in which sepsis has been induced by a combination of caecal ligation and puncture. Figure 3 illustrates that pressor responses to noradrenaline are reduced following a continuous infusion of Escherichia coli endotoxin, that this loss of responsiveness is reversed by an inhibitor of the L-arginine NO pathway but is accentuated by administration of the NO
precursor, L-arginine. This competition is stereospecific, i.e. it does not occur with D-isomers of arginine analogues. There seems to be a general loss of vascular responsiveness under conditions of endotoxaemia and sepsis. This applies to vasodilator, as well as to vasoconstrictor, responses. This is probably because endotoxin damages vascular endothelium and it is endothelium-dependent vasodilator responses that are particularly modified in endotoxaemia and sepsis. This is important because of the role of endothelium-generated NO in the normal regulation of tissue blood flow. This endothelial dysfunction is probably mediated through the generation of oxygen-derived free radicals. An example of how endotoxin interferes with physiologically important vasodilator responses

Figure 2. Contraction responses to noradrenaline in aortic rings with endothelium (○, ●) and without endothelium (□, ▲) from control (○, □) and lipopolysaccharide treated rats (●, ▲) in the presence of (a) saline or (b) L-NMMA (30 μM) dissolved in saline. Bars indicate standard error. The inhibitor of the L-arginine NO pathway restored responsiveness to noradrenaline in rats administered endotoxin. Since this occurred in endothelium-denuded vessels as well as vessels with an intact endothelium, it suggests that, under these conditions the NO is generated from other cell types in the vessel wall, e.g. vascular smooth muscle cells.

Figure 3. Pressor responses to noradrenaline 1 μg/kg given at a time point (●) in anaesthetized rats before and during an infusion over 70 min, of (a) bacterial lipopolysaccharide (hatched bar) derived from Escherichia coli (5 mg/kg, h⁻¹) or (b) saline (open bar). Responses to noradrenaline were depressed 50 min after commencing the endotoxin infusion. These responses were restored to normal (middle panel) following the administration of L-NMMA (30 mg/kg), which itself increased systemic arterial pressure. Administration of the NOS substrate (L-arginine 100 mg/kg) allowed the depressed responses to noradrenaline to be regained. In control rats administered saline the pressor responses to noradrenaline were unaffected by either L-NMMA or L-arginine. From Julou-Schaeffer et al.²³
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is shown in Figure 4. When a major blood vessel is occluded and then released there is an increase in blood flow (post-occlusion hyperaemia) which is mediated largely by NO in many vascular beds. Early after the administration of endotoxin to experimental animals this post-occlusion hyperaemia is almost completely obliterated. It is clear then that endotoxaemia and, probably also sepsis, interferes with the regulation of blood vessel tone, which normally results from mediators formed and released from endothelial cells, or by substances circulating in the blood stream. This derangement of blood vessel tone has important consequences for blood flow regulation under these conditions.

Nitrergic oxide and myocardial depression in sepsis and endotoxaemia

Although it has been known for many years that myocardial responses are depressed following the administration of endotoxin to experimental animals this is often masked, particularly in septic patients, by markedly elevated cardiac outputs (often around twice normal) during the hyperdynamic phase. In patients with sepsis, there is a decreased ejection fraction despite increased cardiac output; this is evidence of myocardial depression. A vasoplegia of sepsis outlined above there are many possible explanations for this reduction in myocardial contractility. They include reduced responsiveness to cardiac sympathetic nerve stimulation and the presence of circulating myocardial depressant factors, although this is still an open question. There is certainly a contribution from cytokine-mediated NO (reviewed by Kumar & Parillo and Brady & Poole-Wilson). NO can be produced in cardiac tissue, as in the vasculature, by two distinct pathways. A constitutive NOS appears to be involved in the physiological regulation of myocardial contractility (reviewed by Shah & Henderson) through interaction between endothelial cells and cardiac myocytes. The NO produced can also be stimulated by cytokines such as tumour necrosis factor (TNF), interleukin 1 (IL-1) and possibly IL-2, and by activated macrophages. The inflammatory stimulus of sepsis is sustained at a higher capacity through iNOS, which is responsible for the larger part of the prolonged myocardial depression in sepsis. This may be secondary to elevated cGMP levels leading to a decreased influx of calcium through L-type channels (both in the sarcoplasmic reticulum and in the sarcolemma) and perhaps, by a decreased myofilament response to calcium. There appears to be some relationship between levels of serum depressant activity (e.g. cytokines) and mortality in patients with sepsis.

The effects of inhibiting nitric oxide production (or action) under conditions of endotoxaemia and sepsis

As outlined above, NO formed by endothelial cNOS is a most important regulator of tissue blood flow and plays an essential role in cardiovascular function. In addition, NO has other cytoprotective roles including inactivation of oxygen free radicals, prevention of microvascular thrombosis, inhibition of platelet aggregation and leucocyte adhesion, bronchodilation (hence the usefulness of inspired NO in modulating pulmonary hypertension and the consequences of the adult respiratory stress syndrome) modulation of sympathetic nervous activity (including central control) and protection of the myocardium against ischaemia. This latter effect is illustrated in Figure 5; the administration of endotoxin (or the non-toxic monophosphoryl derivative of lipid A) suppresses the severity of arrhythmias resulting from coronary artery occlusion and reduces infarct size. NO nitric oxide has also been implicated in the antiarrhythmic effects of ischaemic preconditioning in certain animal models (reviewed by Parratt & Vegh). Clearly, if the generation of NO through the l-arginine NO pathway was inhibited then these protective effects of NO would be markedly impaired. This might have detrimental effects on cardiovascular function. If NO is such an important physiological mediator, does it make sense to attempt to reverse the consequences of NO generation in septic patients by inhibiting the formation of NO through all forms of NOS, the constitutive as well as the induced? It is this question which has dominated recent
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attempts to apply to the clinical situation the sometimes dramatic results seen experimentally when the pathway is inhibited. Endogenous NO is both beneficial and harmful, to the patient. The same is true for the inhibition of NO production. Should then inhibitors of the pathway be given to patients with sepsis? What are the benefits and what possible harm could result?

The potential benefits of giving inhibitors of the **L**-arginine nitric oxide pathway are illustrated in Figure 3. There is elevation of systemic arterial (perfusion) pressure and a restored responsiveness both to noradrenaline released from sympathetic nerve endings and to iv catecholamines administered to support the circulation. These effects are also seen in patients. On the other hand, inhibition of NO under these conditions leads to enhanced jejunal damage characterized by hyperaemia, vasocongestion, increased vascular permeability and haemorrhage into the lumen. Increased platelet adhesion to endothelial cells and platelet accumulation in the lungs occurs together with increased pulmonary vascular resistance. There is reduced cardiac output and oxygen delivery to tissues, marked vasoconstriction in almost all vascular beds and, since cardiac output is reduced, a reduction in organ perfusion.

There is conflicting evidence as to whether inhibiting NOS leads to lower mortality in animals given endotoxin but the consensus seems to be that mortality is not reduced although there may be an increase in survival time. Attempts to apply to the clinical situation the sometimes dramatic results seen experimentally when the pathway is inhibited. Endogenous NO is both beneficial and harmful, to the patient. The same is true for the inhibition of NO production. Should then inhibitors of the pathway be given to patients with sepsis? What are the benefits and what possible harm could result?

The study by Petros et al. described the effects of administering **L**-NMMA iv to two patients in whom mean blood measure was maintained at about 65 mmHg by a combination of fluids, dopamine and noradrenaline. In these patients there was a rapid, short lived increase in arterial pressure following **L**-NMMA administration and a marked increase in systemic vascular resistance. One of these patients ultimately died of a combination of recurrent abdominal sepsis, adult respiratory distress syndrome and disseminated intravascular coagulation. Since this original report a number of, generally small, studies of methylene blue or non-selective inhibitors of the **L**-arginine NO pathway have been published. The results of these clinical studies are summarized in the Table. These uncontrolled studies have been reviewed and extensively discussed but it is difficult to come to any firm conclusion. A more recent, as yet unpublished, study with **L**-NMMA in over 200 patients also failed to show an improvement in mortality. The detrimental effects of such procedures include markedly increased systemic vascular resistance (with associated tissue blood flow reductions), increased central venous pressure and pulmonary arterial (occlusion) pressure and resistance, decreases in cardiac output and oxygen delivery and in platelet count. One site of platelet accumulation appears to be the lungs.

Table. Mortality in septic shock patients given inhibitors of NOS and of guanylyl cyclase

<table>
<thead>
<tr>
<th>Drug</th>
<th>Treated</th>
<th>Controls</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L</strong>-NMMA</td>
<td>1/2</td>
<td>ND</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>3/5</td>
<td>5/6</td>
<td>43</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>2/2</td>
<td>ND</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>5/6</td>
<td>ND</td>
<td>Gachot J. et al. (unpublished observations)</td>
</tr>
<tr>
<td></td>
<td>11/14</td>
<td>ND</td>
<td>45</td>
</tr>
<tr>
<td>Total</td>
<td>76%</td>
<td>50% (assumed)</td>
<td></td>
</tr>
</tbody>
</table>

**a** ND, not done.
Future approaches to modulating nitric oxide generation in patients with sepsis

All the above studies, both in experimental animals and in patients, have used non-selective inhibitors of the L-arginine NO pathway. As should be clear from the foregoing discussion this would prevent the formation of NO by both cNOS (for example in endothelial cells) and iNOS (e.g. in cardiac myocytes and vascular smooth muscle cells). A nother approach would be to attempt to inhibit selectively the NO formed by iNOS whilst maintaining ‘physiological’ NO formed by cNOS in endothelial cells.

This approach assumes that the formation of NO by iNOS has only detrimental effects; this assumption can be questioned. For example, perhaps iNOS is induced under these conditions in order to replace an essential physiological mediator, the formation of which has been impaired by endotoxin-induced endothelial dysfunction. This endothelial dysfunction under conditions of sepsis is probably an early event; there is certainly early impairment of vasodilator responses to mediators which depend upon an intact endothelium as well as to shear stress and to vessel occlusion (see data presented in Figure 4). If the formation of NO by iNOS is to ‘replace’ that which is lost as a consequence of early endothelial dysfunction then the situation would be similar to the role of catecholamines in shock. Catecholamine release in various shock states is essential for survival. However, the excessive and prolonged generation of noradrenaline ultimately has detrimental effects on nutritional blood flow (reviewed by Ledingham & Parratt). The situation may be similar for NO; early generation by iNOS would be important to replace that lost from endothelial cells. However, later in the inflammatory process excessive amounts of NO are produced with detrimental effects on organ function. It is at this stage that inhibition of NO formation is appropriate.

A novel way of investigating the role of cytokine-inducible iNOS in the pathology of sepsis and endotoxaemia is to examine such responses in animals (mice) lacking the iNOS gene, the so-called iNOS knockout mice. When the macrophages of these mice are stimulated they produce no detectable iNOS and the mice show reduced non-specific inflammatory responses. They are also more resistant to endotoxin; the fall in blood pressure resulting from endotoxin administration is greatly attenuated and survival is increased. Mutant (iNOS knockout) mice survived a dose of 12.5 mg/kg with only minimal and transient weight loss and malaise; in contrast, there was a 50% mortality in control, wild-type mice 24 h after a similar endotoxin dose.

There are now several examples of substances that have a greater affinity for inhibiting iNOS than cNOS. The first such substance was aminoguanidine and there are also some interesting studies with L-canavanine. Unlike non-selective inhibitors of NOS, both aminoguanidine and L-canavanine restore vascular (and possibly cardiac) responsiveness to noradrenaline (and possibly sympathetic nerve stimulation) without themselves increasing blood pressure or systemic vascular resistance. Of particular interest is the finding that L-canavanine, while significantly reducing endotoxin-induced hypotension, reduces the detrimental effects of endotoxin on vascular endothelium, at least in kidneys and lungs. There is some evidence that this endothelial dysfunction results from excessive NO production by iNOS since it can also be reduced by glucocorticoids.

More recently Thiemann and his colleagues have examined the effects of more selective inhibitors of iNOS, including other guanidine derivatives. The 1-amino-2-hydroxy derivative is a particularly potent inhibitor of iNOS. Isothiourea derivatives also demonstrate some selective activity against iNOS; amino ethyl-thiourea and S-methylisothioure a (SMT) being particularly selective. These selective inhibitors also appear to attenuate endotoxin-induced multiple organ dysfunction. Clinical studies are awaited with interest. It needs to be determined precisely at what point during sepsis such selective inhibitors should be administered and their relative ability to selectively inhibit iNOS rather than cNOS. One advantage of selective inhibition would be that any detrimental effects should be readily overcome by the administration of L-arginine.

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