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The Role of Nitric Oxide for the Regulation of Vascular Tone in Health and Disease

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INTRODUCTION

Since the discovery that EDRF is identical to the gaseous mediator nitric oxide (NO) in 1987, we have learned that NO serves as a ubiquitous signalling molecule in the cardiovascular, central nervous and immune system. For instance, NO regulates blood pressure and organ blood flow distribution and prevents the adhesion of blood-borne cells to the endothelium. There is, however, also evidence that an enhanced formation of NO (particularly following the induction of iNOS) contributes to the haemodynamic abnormalities associated with shock of various aetiologies. This article reviews the biochemistry and physiology of the L-arginine-NO system in the cardiovascular system and discusses the effects and side effects of pharmacological approaches aimed at reducing (NO synthase inhibitors) the formation and/or availability of NO in circulatory shock.

BIOSYNTHESIS OF NITRIC OXIDE

Nitric oxide (NO) is generated from L-arginine by a family of enzymes collectively called NO synthases (NOS). The oxidation of one of the guanidino nitrogen atoms of this semi-essential amino acid L-arginine by NOS generates NO as well as L-citrulline. The haeme-iron-dependent oxidation of L-arginine is coupled to the reductive activation of molecular oxygen and requires input of reducing equivalents shuttled from the electron donor NADPH to the haeme through the flavins FAD and FMN. In addition to haeme, flavins and NADPH, the NOS also requires the presence of tetrahydrobiopterin (BH_4), which appears to act both as allosteric effector and redox-active co-factor of the oxidation of L-arginine. Thus, NOS contains an oxygenase domain (containing the catalytic center) and a reductase domain. The oxygenase domain appears to contain binding sites for BH_4, haeme and L-arginine. The synthesis of NO from L-arginine and molecular oxygen involves the generation of N^G-hydroxy-L-arginine and water (first step) and subsequently the oxidation of N^G-hydroxy-L-arginine in the presence of molecular oxygen to form NO, L-citrulline and water. When generated, NO diffuses to adjacent cells where it activates soluble guanylate cyclase, resulting in the formation of cGMP, which in turn mediates many of the effects of NO. NO is generated by many mammalian cells by at least three different isoforms of NOS. Thus, it is not surprising that NO has many biological functions in the cardiovascular, nervous and immune systems (see Moncada & Higgs, 1993). The NOS in endothelial cells (eNOS or NOS III) and neuronal cells (nNOS or NOS I) are expressed constitutively, and both enzymes require an increase in intracellular calcium

(Ca^{++}) for activation. Activation of macrophages and many other cells with pro-inflammatory cytokines or endotoxin results in the expression of a distinct isoform of NOS (inducible NOS; iNOS or NOS II), the activity of which is functionally independent of changes in intracellular Ca^{++} (see Nathan, 1992; Dinerman et al., 1993; Moncada & Higgs, 1993; Morris & Billiar, 1993; Thiemermann, 1994; Szabo & Thiemermann, 1995 for review).

PHYSIOLOGICAL ROLE OF NITRIC OXIDE PRODUCED BY ENDOTHELIAL CELLS

Localisation of eNOS within endothelial cells

Due to N-terminal myristoylation and thiopalmitoylation, eNOS is targeted to the particulate subcellular fraction, where the enzyme is localised in plasmalemmal calveolae. Claveolae are specialised invaginations of the plasma membrane, and may serve as sites for the sequestration of diverse signalling proteins (e.g G-proteins, calcium channels, protein kinases and cell surface receptors. In endothelial cells of large vessels, the majority of eNOS is associated with the Golgi complex and inhibition of N-myristoylation causes the diffuse, cytosolic localisation of the enzyme. Indeed, mutation of the glycine-2 of eNOS inhibits N-myristoylation of eNOS and converts the enzyme from a membrane-bound to a cytosolic form.

Activation of eNOS by shear stress

Although eNOS has been classified as a Ca^{++}/calmodulin-dependent enzyme (see above), eNOS is able to generate NO at "basal" levels of intracellular Ca^{2+}. Although it is not disputed that a rise in intracellular Ca^{2+} e.g. in response to stimulation of endothelial cells with certain agonists (acetylcholine, bradykinin etc) results in an increase in NO formation, there is also good evidence that shear stress is able to modulate the generation of NO by eNOS in a Ca^{2+}/calmodulin-independent fashion. *Professor Rudi Busse (Frankfurt) and colleagues* have recently proposed that the signal transduction events mediating the enhanced formation of NO by endothelial cells exposed to shear stress involves the tyrosine phosphorylation of cytoskeletal proteins as well as eNOS. These findings form the basis for the hypothesis that the redistribution of tyrosine phosphorylated proteins and eNOS to the integrin-rich cytoskeletal fraction results in the formation of a shear stress sensor/NOS complex. In addition to activating eNOS activity (see above), shear stress also enhances the expression of eNOS in native and cultured endothelial cells, possibly by causing the tyrosine phosphorylation of MAP kinases, enhancing the expression of c-Fos and binding of AP-1 to the eNOS promotor.

Physiological effects NO produced by eNOS

Activation of eNOS by shear stress results in a continuous release of NO (active vasodilatation) which regulates blood pressure and organ blood flow. NO also reduces the adhesion of platelets and polymorphonuclear leukocytes (PMNs) to the endothelium. The latter effect of NO is, at least in part, due to the prevention by NO of the expression of the adhesion molecules P-selectin and intercellular adhesion molecule (ICAM-1) on the surface of endothelial cells. Interestingly, the enhanced expression of eNOS mRNA (e.g. following exposure to shear stress) is associated with a decrease in the transcription of the genes for GAPDH, E-selectin and MCP-1.

Effects of NO on platelets

In addition to preventing the adhesion of platelets to endothelial cells, NO also directly attenuates the activation of platelets. These effects of NO are associated with and/or due to prevention of (i) the expression of P-selectin (on platelets), (ii) secretion of platelet granules, (iii) intracellular calcium flux, as well as (iv) binding of glycoprotein IIb/IIIa to fibrinogen. It should be noted that both platelets and megakaryocytes are able to generate NO, as both

cells contain a constitutive NOS (homologous to eNOS or NOS III, but with a molecular weight of 85 kDa), and megakaryocytes also contain iNOS (NOS II).

Effects of NO on leukocytes

NO can, in principle, inhibit the activation of PMNs (direct effect) e.g. to release radicals and enzymes. More importantly, NO attenuates the expression of the adhesion molecules P-selectin, E-selectin and possibly VCAM-1 (see above) and, hence, may interfere with rolling and attachment of PMNs to the endothelium. Inhibition of the endogenous formation of NO in the mesenteric vascular bed results in a rapid increase in the number of PMNs which role on and attach to the endothelium. This effect of the NOS inhibition may, however, be indirect, as inhibition of the formation of NO by tissue mast cells results in a burst like release of histamine, which subsequently triggers the adhesion of PMNs to the endothelium (see Granger et al., 1995).

The above studies clearly demonstrate that the formation of NO by eNOS importantly contributes to the regulation of vascular tone and also to the maintenance of blood vessel patency. A reduced formation of NO by eNOS (endothelial dysfunction) may contribute to the pathophysiology associated with atherosclerosis, diabetes mellitus and myocardial ischaemia reperfusion injury. Efforts are currently being made to evaluate therapeutic strategies aimed at enhancing the formation of NO from eNOS. In addition, it is possible to enhance the availability of NO by giving drugs which either enzymatically or non-enzymatically release NO (e.g. NO-donors, organic nitrates).

THE ROLE OF NITRIC OXIDE IN CIRCULATORY SHOCK

Pathophysiology of Circulatory Shock

In contrast to the Oxford Dictionary, which defines "Shock" as a *"violent collision, concussion or impact"*, the medical syndrome of shock can be defined as a *"progressive failure of the circulation to provide blood and oxygen to vital organs of our body"*. In clinical practice, the key symptom of shock is a severe fall in blood pressure which often results in the dysfunction or failure of several important organs including lung, kidney, liver and brain. The most common causes of shock is the contamination of blood with bacteria resulting in systemic infection and ultimately shock (septic shock). Despite improvements in intensive care medicine, the mortality of septic shock remains very high. Thus, there is still a great need for new approaches to improve therapy and outcome of patients with shock. Septic shock, regardless of its aetiology, is defined as sepsis (systemic reponse to infection) with hypotension despite adequate fluid replacement resulting in impaired tissue perfusion and oxygen extraction (Parillo, 1990). The definition of septic shock is independent of the presence or absence of a multiple organ dysfunction syndrome (MODS), which is defined as impaired organ function such that homeostasis cannot be maintained without intervention (Baue, 1993). Primary MODS is a direct result of a well-defined insult to a specific organ. Secondary MODS occurs as a consequence of an exaggerated host response, termed systemic inflammatory response syndrome (SIRS). SIRS may occur in response to infection, multiple trauma, haemorrhage, ischaemia and immune-mediated organ injury (Baue, 1993). Current therapeutic approaches for septic shock include antimicrobial chemotherapy, volume replacement, inotropic and vasopressor support, oxygen therapy and mechanical ventilation as well as haemodialysis and haemofiltration. These have, however, failed to make a substantial impact on the high mortality associated with septic shock (Nathanson et al., 1994) and, hence, septic shock remains the major cause of death in non-coronary intensive care units with an estimated mortality ranging between 50 and 80%. As shock is also by far the most common cause of prolonged admission

to an intensive care unit, the clinical and socio-economical importance of this illness is substantial. Thus, there is still a great need to explore the pathophysiological events leading to circulatory failure, tissue ischaemia and MODS in septic shock.

Nitric oxide in the pathophysiology of circulatory shock

In 1990, several groups independently discovered that an enhanced formation of endogenous NO contributes to (i) hypotension (Thiemermann & Vane, 1990) and vascular hyporesponsiveness to vasoconstrictor agents (Julou-Schaeffer et al., 1990; Rees et al., 1990) in rodents with endotoxic shock (ii) hypotension caused by cytokines and endotoxin in dogs (Kilbourn et al., 1990a & 1990b), (iii) the reduction in liver protein synthesis (Curran et al., 1991); and (iv) protection of liver integrity in rodents with sepsis (Billiar et al., 1990). We know today that circulatory shock is associated with an enhanced formation of NO due to the early activation of eNOS and the delayed induction of iNOS activity in e.g. macrophages, vascular smooth muscle, hepatocytes, cardiac myocytes etc. (Szabo et al., 1993). This overproduction of NO may contribute to circulatory failure (hypotension, vascular hyporeactivity to vasopressor agents, increase in shunts, maldistribution of blood flow), myocardial dysfunction, organ injury and ultimately MODS. The formation of NO also exerts beneficial effects in endotoxic shock including vasodilatation, prevention of platelet and leukocyte adhesion, improvement of microcirculatory blood flow and augmentation of host defence. Thus, it is not surprising that many colleagues have advocated the use of contrasting therapeutic approaches including (i) inhibition of NOS activity, (ii) enhancement of the availability of NO (NO-donors, NO-inhalation) or (iii) a combination of both approaches.

Hypotension

The circulatory failure associated with shock of various aetiologies is characterised by severe hypotension (peripheral vasodilatation), hyporeactivity of the vasculature to vasoconstrictor agents, myocardial dysfunction, maldistribution of organ blood flow and reduced tissue oxygen extraction. There is now good evidence that an enhanced formation of NO contributes to several of these pathophysiological features of septic shock. For instance, an enhanced formation of NO due to activation of eNOS (acute phase of shock) and particularly following the induction of iNOS in the vascular wall (late phase of shock) importantly contributes to the hypotension in animals (rat, dog, pig, sheep) and man with septic shock. Similarly, an enhanced formation of NO by iNOS also contributes to ① the delayed hypotension in animals and patients exposed to immunotherapy with interleukin (IL)-2, ② the delayed vascular decompensation (excessive peripheral vasodilatation) in haemorrhagic shock; and ③ possibly to the hyperdynamic circulatory failure associated with liver cirrhosis. Although the circulatory failure associated with anaphylactic shock, traumatic shock or burns also results in an increase in the plasma levels of nitrite, it is unclear whether this is merely a surrogate markers of these disorders or cause of the associated haemodynamic alterations (see Thiemermann, 1994 & 1995; Szabo & Thiemermann, 1994 for review).

Vascular hyporeactivity to vasoconstrictor agents ("vasoplegia")

The peripheral vascular failure in animals and man with septic shock also reslts in a progressive attenuation of the pressor effects afforded by norepinephrine and other vasoconstrictor agents (epinephrine, vsopressin, angiotensin II, serotonin, histamine, calcium, potassium). This phenomenon, which has also been termed "vasoplegia" also contributes to the therapy-refractory hypotension in septic shock. Clearly, the hyporeactivity of blood vessels obtained from animals exposed to endotoxic or hemorrhagic shock (for several hours) to catecholamines is largely -but not exclusively- due to an enhanced formation of NO secondary to the

induction of iNOS. In endotoxemia, an NO-mediated vascular hyporeactivity occurs in conductance, resistance as well as venous vessles (see Parratt & Stoclet, 1995 for review). An enhanced formation of NO by eNOS (early phase) and iNOS (late phase) also contributes to the hyporeactivity to catecholamines in rats with haemorrhagic shock (Thiemermann et al., 1993).

Myocardial dysfunction

The question as to whether an enhanced formation of NO contributes to the myocardial dysfunction associated with shock is still controversial (see Kumar & Parrillo, 1995, Lefer, 1995). Large amounts of exogenous NO (authentic NO or NO-donors) reduces cardiac contractility by a cGMP-dependent mechanism it is unclear whether the amounts of NO generated under physiological or even pathophysiological conditions are sufficient to exert this effect. Clearly, isolated (rat) cardiac myocytes or papillary muscle exposed to TNFα or IL-1 express iNOS; and NOS inhibitors attenuate the impairment of contractility in isolated myocytes obtained from animals with endotoxic shock. Although the iNOS activity in the heart of animals exposed to endotoxaemia (for 3 to 6 h) is relatively small when compared with the activity found in other organs, longer periods of septic shock may well result in a more pronounced expression of iNOS protein and activity. It has been difficult to demonstrate that NOS inhibition in animals with septic shock results in an improvement in cardiac contractility or cardiac output, as most studies have employed non-selective NOS inhibitors of even eNOS-selective inhibitors (L-NAME) which cause a reduction in crdiac output by reducing myocardial blood flow (eNOS inhibition). Thus, further studies are warranted aimed at evaluating the effects of iNOS-selective NOS inhibitors on cardiac function in septic shock (Thiemermann, 1994 & 1995).

Reduced tissue oxygen extraction and direct cytotoxic effects

Circulatory shock often results in a marked defect in tissue oxygen extraction resulting in tissue hypoxia and an increased venous oxygen concentration. As the local generation of large amounts of NO e.g. by activated macrophages serves to kill bacteria or tumour cells as part of the host defence, it is not surprising that the generation of NO by iNOS in other cells is cytotoxic (suicide mechanism). Indeed, large amounts of NO cause an autoinhibition of mitochondrial respiration by inhibiting several key enzymes in the mitochondrial respiratory chain (NADH-ubiquinone reductase, succinate-ubiquinone oxidoreductase) or in the Krebs' cycle (e.g. cis-acconitase) resulting in a shift in glucose metabolism from aerobic to anaerobic pathways (Morris & Billiar, 1994; Thiemermann, 1995). NO (like other radicals and oxidants) also causes DNA strand breakage which triggers a futile, energy-consuming repair cycle by activating the nuclear enzyme poly(ADP)ribosyltransferase (PARS). Activation of PARS results in the rapid depletion of the intracellular concentration of NAD^+ (its substrate) slowing the rate of glycolysis, electron transfer and ATP formation which ultimately results in cell death ("PARS suicide hypothesis") (Schraufstetter et al., 1986). NO and superoxide anion generate peroxynitrite anions (Beckmann et al., 1990), which also cause DNA strand breaks and activate PARS (Zingarelli et al., 1996). There is preliminary evidence that endotoxic and hemorrhagic shock results in the formation of peroxynitrite. Most notably, inhibitors of PARS activity (e.g. 3-aminobenzamide, nicotinamide) attenuate the inhibition of cellular respiration caused by peroxynitrite (Zingarelli et al., 1996). Thus, the generation of large amounts of NO by iNOS may cntribute to the defect in oxygen extraction and ultimately cell hypoxia and death by causing (i) maldistribution of regional blood flow (reduced oxygen supply), (ii) formation of a diffusion barrier for oxygen within the vascular wall

(reduced oxygen transport), (iii) inhibition of the generation of ATP (reduced oxygen utilisation), and (iv) excessive and futile consumption of ATP. In concert with the severe hypotension (reduced perfusion pressure), these effects of the local overproduction of NO may importantly contribute to the organ injury and dysfunction associated with septic shock. Prolonged periods of septic shock also result in the development of an endothelial dysfunction, which is characterised by the impairment of "endothelium-dependent vasodilatation" and therefore presumably eNOS activity. The mechanism(s) of this endothelial dysfunction may include the down-regulation of the expression of the eNOS gene by proinflammatory cytokines such as TNFα, endothelial cell damage due to cytotoxc effects of NO, peroxynitrite or oxygen-derived radicals, and (to a lesser extent) the inactivation of NO by oxygen radicals (see Thiemermann, 1994 & 1995).

Host defence (iNOS "knock-out" studies)

The recent generation of mice deficient in iNOS (iNOS mutant or "iNOS knock-out" mice) has helped to shed a further light into the physiological and/or pathophysiological importance of the generation of NO by iNOS (Wei et al., 1995; MacMicking et al., 1995). For instance MacMicking and colleagues demonstrate that iNOS-deficient mice failed to restrain the replication of Listeria monocytogenes in vivo or lymphoma cells in vitro. Moreover, the hypotension and early mortality caused by endotoxic shock was reduced in iNOS deficient mice, while the degree of liver injury was unaltered (MacMicking et al., 1995). In a separate study, Wei and colleagues show that iNOS deficient mice were resistant to the mortality caused by endotoxin and exhibited reduced non-specific inflammatory responses to carageenin. Moreover, iNOS-deficient mice (but not wild-type or heterozygous mice) were highly susceptible to infection with the protozoa parasite Leishmania major (Wei et al., 1995). Taken together, these studies support the view that an enhanced formation of NO by iNOS (in macrophages) defends the host against infectious agents and tumour cells, while an excessive induction of iNOS in other tissues (e.g. vasculature) may cause shock and tissue destruction.

Nitric oxide in humans with septic shock

Although our understanding of the role of NO in animal models of circulatory shock has improved substantially over the past years, our knowledge regarding the biosynthesis and importance of NO in the pathophysiology of patients with shock (of various aetiologies) is still very limited. Indeed, a Medline search covering the time period from 1987 to November 1995 revealed that only 8 to 14% of all of the publications which included the key word 'nitric oxide' also included the key word 'human' (Preiser & Vincent, 1996). What, then, is the evidence that septic shock in man is associated with an enhanced formation of NO? Elevated plasma and urine levels of nitrite/nitrate have been reported in adults and children with severe septic shock as well as in patients with burns injuries who subsequently developed sepsis. Moreover, elevated plasma levels of nitrite/nitrate occur in patients receiving IL-2 chemotherapy. In contrast, there is also evidence that the plasma levels of nitrite/nitrate are lower in patients after trauma, surgery and in patients with HIV infections (see Preiser & Vincent, 1996). Interestingly, the increase in iNOS activity in leukocytes obtained from patients with sepsis appears to correlate with the number of failing organs, but not with blood pressure. Taken toether, these studies support the conclusion that septic shock in man is associated with an enhanced formation of NO. It should, however, be stressed that the increase in the plasma levels of nitrite/nitrate elicited by endotxin, cytokines or bacteria in rodents (10-fold) is substantially higher than the observed increases in the plasma levels of these metabolites of NO in other animal species (pig, sheep etc) or humans. Moreover, our understanding of (i) the biosynthesis of NO, (ii) the regulation of and the mechansm involved in the expression of iNOS, and (iii) the role

of NO in MODS in shock are largely based on animal experiments of endotoxic shock in rodents. In contrast, we know relatively little about the role of NO in patients with septic and other forms of circulatory shock.

There is evidence that endotoxin and cytokines (when given in combination) causes the expression of iNOS as well as the formation of NO in various human cells (primary or cell lines) including hepatocytes, mesangial cells, retinal pigmented epithelial cells and lung epithelial cells (Morris & Billiar, 1994; Preiser & Vincent, 1996). Interestingly, IL-1 causes the hyporeactivity of human hand veins *(in situ)* to the constrictor effects elicited by exogenous or endogenous norepinephrine, and this vascular hyporeactivity is largely attenuated by L-NMMA suggesting that it is mediated by NO (P.Vallance, personal communication). Early reports of beneficial haemodynamic effects of L-NMMA in humans with septic shock (Petros et al., 1994) stimulated a phase I, multi-center, open-label, dose-escalation (1, 2.5, 5, 10 or 20 mg/kg/h for upto 8 hours) study using L-NMMA (546C88) in 32 patients with septic shock. In this study, L-NMMA sustained blood pressure and enabled a reduction in vasopressor (norepinephrine) support. The cardiac index fell (possibly due to an increase in peripheral vascular resistance) and left ventricular function was well maintained. Moreover, L-NMMA increased oxygen extraction, while pulmonary shunt was not worsened (Watson et al., 1995). Although the development of thrombocytopenia has been documented in septic patients treated with L-NMMA, it is unclear whether this effect is due to the NOS inhibitor or caused by the underlying illness. A multi-center clinical trial evaluating the effects of L-NMMA on morbidity and mortality in patients with septic shock is ongoing, and the results of this trial are awaited with interest.

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