Nitric oxide in septic shock

An experimental and clinical study

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Stikstofoxide in septische shock
Een experimentele en klinische studie

Proefschrift

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On the cover: Electron micrograph of *E. coli* bacteria in blood (EM x 20.000).

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Chapter 1

General Introduction

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1.1 Sepsis and septic shock

Sepsis and its sequelae are the leading cause of mortality in todays medical and surgical intensive care.[1-5] The incidence of sepsis continues to increase. Mortality of septic shock ranges from 30 to 80% depending on the severity of disease and the presence of organ failure. Despite recent progress in antibiotic therapy and intensive care support mortality from septic shock has remained high over the last years.[1,2]

Sepsis can be defined as the systemic response to severe infection.[5,6] When certain micro-organisms or their toxic products invade the bloodstream, this may result in a wide variety of symptoms that are characteristic of sepsis and its sequelae (Table 1). When hypotension develops unresponsive to fluid therapy and signs of inadequate organ perfusion are present, the condition is often referred to as septic shock.[5] Shock develops in approximately 40 % of septic patients. The initial cardiovascular changes during hyperdynamic sepsis are characterized by massive vasodilatation with a normal to high cardiac output, low peripheral vascular resistance and severe hypotension.[6-9] In a large number of patients the initial hypotension is unresponsive to treatment with fluid substitution or vasopressors. Unresponsive hypotension is present in 50% of patients that die of septic shock.[10] In the first week unresponsive hypotension is the primary cause of mortality of septic patients. In the later stages of sepsis, the hyperdynamic circulatory state may turn into hypodynamic septic shock with failure to guarantee adequate oxygen supply to tissues and irreversible organ damage. In a retrospective study with one hundred intensive care patients with sepsis 80% of mortality in the first week was caused by severe hypotension.[11]

Although manifestations of sepsis can be seen with systemic infections with all classes of micro-organisms, septic shock is most often caused by gram negative bacteria (40 to 60% of cases). Staphylococci, pneumococci, streptococci and other gram positive organisms are slightly less frequent causes (30 to 50% of cases), but their relative incidence has increased over the last few years.[4,12] Mixed pathogens are found in 10 to 20% of septic patients. Other micro-organisms are opportunistic fungi (2 to 5 %), and rarely mycobacteria, certain viruses and protozoa. Gram negative sepsis is seen primarily in hospitalised patients, most of whom have underlying diseases or procedures which render their blood stream susceptible to invasion

by micro-organisms. Gram positive sepsis may be community acquired, with the exception of staphylococcal sepsis that mostly arises from indwelling intravenous catheters. Other predisposing factors include the use of corticosteroid or immunosuppressive therapy for organ transplantation or inflammatory diseases, cytotoxic chemotherapy causing neutropenia, diabetes

Table 1. Characteristics of sepsis and septic shock

fever or hypothermia	release of pro-inflammatory mediators:
chills	TNF-α, IL-1β, IL-2, IL-6, IL-8, IL-15, IFN-γ, MDF, thromboxane, PAF, prostacyclin, prostaglandins, phospholipase A_2, free radicals, NO
tachycardia	
tachypnea	
altered mental state	
skin lesions	release of anti-inflammatory mediators:
leucocytosis or leucopenia	IL-1ra, IL-4, IL-10, IL-13, transforming growth factor-β, soluble TNF-α receptors, LPS binding protein, NO (?)
thrombocytopenia	
hypotension	
low systemic vascular resistance	
high cardiac output	coagulation abnormalities
left and right ventricular dilatation	complement system activation
myocardial depression	endothelial dysfunction
oliguria and renal failure (ATN)	increased microvascular permeability
hypoxemia, respiratory failure and ARDS	bacterial translocation
elevated lactate and acidosis	maldistribution of blood flow
organ failure (single or multiple)	microcirculatory oxygen deficit

From references [1,2,5-11,13-23]

mellitus, a wide variety of surgical and invasive medical procedures, mechanical ventilation and prolonged antibiotic therapy. Blood cultures are only positive in approximately 40 to 50% of patients with manifestations of sepsis.

Most important in the pathophysiology of sepsis are macro- and microvascular disturbances in combination with myocardial depression resulting from the complex interaction between bacterial cell products like lipopolysaccharides (LPS) and/or toxins and host defense mechanisms leading to the release of several pro-inflammatory mediators such as cytokines [e.g., tumor necrosis factor-alpha (TNF-α), interleukines (IL-1β, IL-2, IL-6, IL-8 and IL-15), interferon-gamma (IFN-γ)], myocardial depressant factor (MDF), platelet activating factor

(PAF), kinins, arachidonic acid metabolites and several others (Figure 1).[3,6,9,13-19] High levels of pro-inflammatory cytokines are found in septic patients and these levels correlate with organ failure and survival.[16-19] More recently it has been found that quite rapidly after the first pro-inflammatory mediators are released, the body mounts a compensatory anti-inflammatory response.[20] The goal of this anti-inflammatory reaction is to downregulate synthesis of pro-inflammatory mediators and to modulate their effects, thereby restoring homeostasis. However, during sepsis, the balance between pro- and anti-inflammatory mediators is upset by a variety of forces, resulting in disturbed homeostasis. At macro-circulatory level the disturbance of homeostasis leads to increased cardiac output, reduced vascular resistance and hypotension.[6-9] At microcirculatory level the disturbances lead to increased permeability of capillaries and maldistribution of blood flow causing inadequate tissue perfusion and tissue damage.[21-23] The maldistribution of blood flow during sepsis may result from defective vascular autoregulation.[24,25] In addition to these changes myocardial depression is present in septic patients.[26] The combination of cardiovascular derangements will eventually lead to inadequate oxygen transport to tissue, causing multiple organ failure and death.

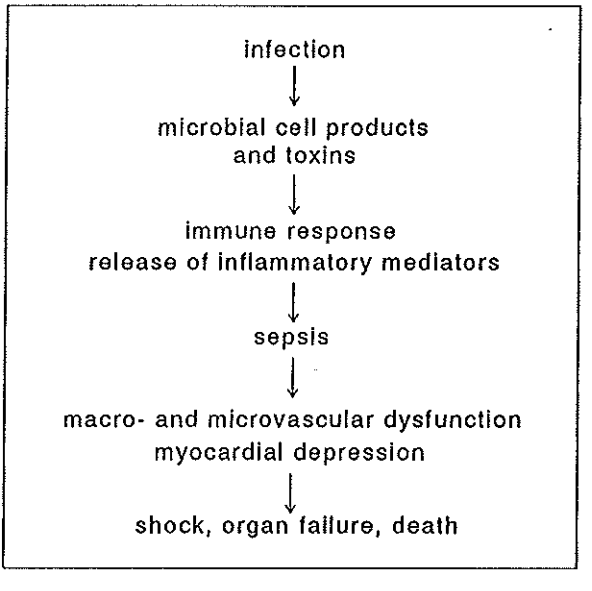

Figure 1. Sequelae of sepsis.

1.2 Nitric oxide, mechanism of action

In 1987 it was demonstrated that the NO radical was the same molecule as the earlier discovered but never isolated Endothelium Derived Relaxing Factor (EDRF).[27,28] We can say that this was the beginning of a whole new area of cardiovascular research. Nitric Oxide (NO) released from the endothelium is an important endogenous vasodilator and plays an important role in the regulation of tissue perfusion.[29,30] Other biological roles of NO include a role as neurotransmitter in the brain and peripheral nerves,[31] a role in host defense by being cytotoxic to bacteria,[32-34] and tumour cells,[35] and a role in preventing platelet aggregation.[36] Nitric oxide is released from the amino acid L-arginine, but not D-arginine, by the enzyme NO synthase, that is located in a number of different cell types. The NO radical is synthesized from the terminal guanidino nitrogen atom of L-arginine and forms L-citrulline as a co-product. Evidence from studies using $^{18}O_2$ and mass spectrometry indicates that in this reaction molecular oxygen is incorporated into both NO and citrulline by NO synthase which shows that the enzyme is a dioxygenase.[37] Endothelial cells react to physical and chemical stimuli like shear stress, hypoxia or endothelial dependent vasodilators such as bradykinine, acetylcholine, serotonin and adenosinediphosphate (ADP) which can elicit Ca^{2+} release from intracellular stores, with the release of NO in small amounts and for short periods.[38-40] The enzyme responsible for NO production in endothelial cells and neural tissue is called constitutive NO synthase (c-NOS). NO synthase is a heme containing enzyme which belongs to the P-450 class of enzymes.[41] The constitutive NO synthase is activated by Ca^{2+}, which binds to calmodulin, forming a complex that is a crucial cofactor for enzyme activity.[42] Furthermore nicotinamide adenine dinucleotide phosphate (NADPH), flavine mononucleotide (FMN), flavin adenine dinucleotide (FAD), and tetrahydrobiopterin (BH_4) are required as cofactors.[32,43] NO is a small lipophilic molecule that travels freely through cell membranes and can, therefore, act on neighbouring target cells. NO released from endothelial cells diffuses readily to the vascular smooth muscle cells where, by binding to iron in the heme at the active site of soluble guanylate-cyclase, the enzyme is activated to convert guanyl-tri-phosphate (GTP) to cyclic-3'5'guanyl-mono-phosphate (c-GMP). The intracellular rise of c-GMP will lead to relaxation of the vascular smooth muscle cell which causes vasodilation.[44] NO can be considered as an endoge-

nous nitrovasodilator since the same mechanism is responsible for vasodilation upon infusion of nitrovasodilator drugs as glyceryltrinitrate or nitroprusside that act as exogenous donors of NO.[45] Organic nitrovasodilators, such as glyceryltrinitrate, undergo intermediate metabolism, an enzymatic step requiring thiol groups whereas the inorganic nitrovasodilators, such as sodiumnitroprusside, release NO readily when exposed to vascular tissue by a mechanism that is not fully understood.

Since NO is soluble in water and lipid, it is freely diffusible in the environment of the cell. The half-life of the NO radical is only a couple of seconds because following production the NO radical reacts easily with sulphydryl groups in amino acids or proteins, metal ions in redox reactions, hemoglobin in the blood, oxygen and radicals like superoxide. Nitrate and

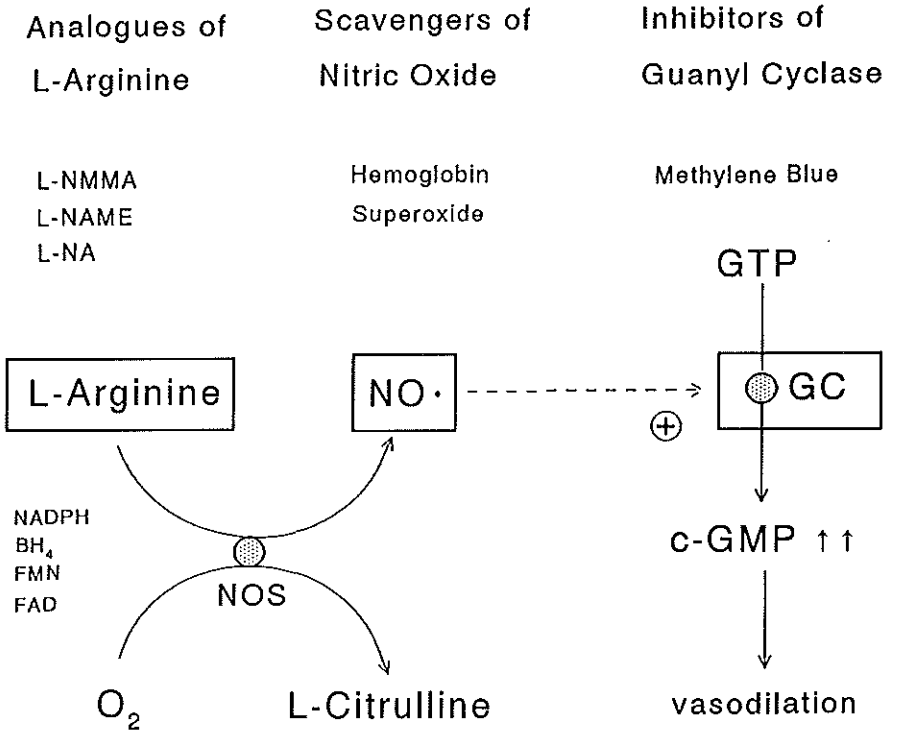

Figure 2. Schematic representation of the chemical reaction leading to formation of NO and c-GMP and the different pharmacological agents that intervene with the arginine/NO/c-GMP pathway at their specific sites. L-NMMA = N^G-monomethyl-L-arginine, L-NAME = N^G-nitro-L-arginine methylester, L-NA = N^G-nitro-L-arginine, NO = nitric oxide, GC = guanylate-cyclase, GTP = guanyltriphosphate, c-GMP = cyclic-guanylmonophosphate, BH_4 = tetrahydrobiopterin, NADPH = nicotinamide adenine dinucleotide phosphate, FMN = flavine mononucleotide, FAD = flavin adenine dinucleotide.

nitrite are the inactive and stable endproducts of NO production which are further eliminated in urine.[46,47] Although cultured endothelial cells depend on the presence of L-arginine in their medium for production of NO for longer periods, intracellular concentrations of L-arginine *in vitro* and *in vivo* are under normal conditions not rate limiting in the production of NO so that administration of L-arginine will not lead to vasodilation through increased NO release.[39,48]

1.3 Inhibitors of nitric oxide synthesis

There are several ways to intervene with the L-arginine/NO/c-GMP pathway (Figure 2). Nitro-substituted or methylated analogues of L-arginine are able to competitively and stereospecific inhibit the production of NO by the constitutive and inducible NO synthase. Analogues that are frequently used are N^G-monomethyl-L-arginine (L-NMMA),[49] N^G-nitro-L-arginine (L-NA or NNLA)[50] and N^G-nitro-L-arginine methylester (L-NAME).[51] The structural formulas of these analogues are displayed in Figure 3. These analogues all inhibit NO

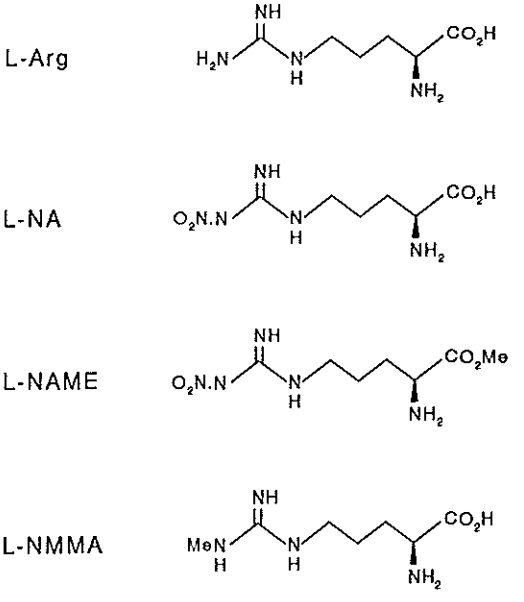

Figure 3. Structural formulas of L-arginine and analogues that inhibit NO formation. L-arg = L-arginine, L-NA = N^G-nitro-L-arginine, L-NAME = N^G-nitro-L-arginine methyl ester, L-NMMA = N^G-monomethyl-L-arginine.

synthesis by competition with L-arginine, but differ in their specific actions. The order of potency in inhibiting endothelial derived NO synthesis seems to be L-NA > L-NAME > L-NMMA whereas in macrophages their order of potency is L-NMMA > L-NAME > L-NA.[51,52] The pharmacokinetics of these NO inhibitors are largely unknown, although some differences have been shown in uptake and degradation. L-NMMA is converted to L-citrulline by endothelial cells whereas L-NA is not.[53] In dogs and rabbits L-NAME is rapidly converted to

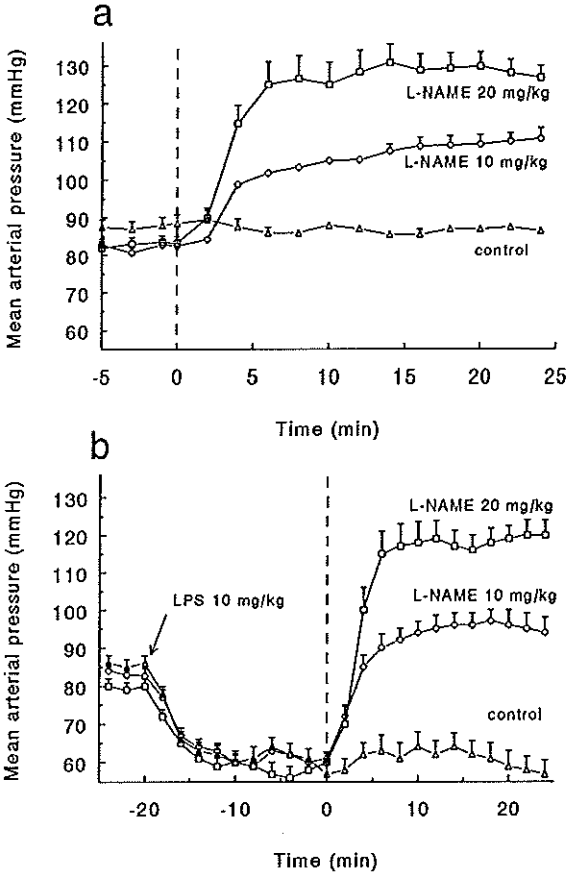

Figure 4. Effect of L-NAME on mean arterial blood pressure (±SEM) in anaesthetized male Wistar rats without (A) and with endotoxemia (B). Following anaesthesia blood pressure was measured by inserting a canula in the carotid artery. In the non-endotoxemic group, rats were injected with a bolus injection (0.3 ml) of either saline (n=3), L-NAME 10 mg/kg (n=4) or L-NAME 20 mg/kg (n=4) at T=0. In the endotoxemic group the procedure was preceded by intravenous injection of lipopolysaccharide (LPS 10 mg/kg) at T=-20 min. Note that following injection of a single bolus L-NAME mean arterial blood pressure remains elevated for at least 30 min.

L-NA which seems to be the final degradation product.[54,55] L-NAME however easily enters the intracellular compartment whereas L-NA does not. These three analogues of L-arginine are orally active. *In vivo* these analogues of L-arginine can raise blood pressure by inhibition of NO synthesis. Figure 4 shows the dose-dependent rise of blood pressure during inhibition of NO synthesis with a bolus injection of L-NAME in the anaesthetized rat. In patients with chronical renal failure endogenous inhibitors of NO synthase are present in the serum.[56] It is not clear whether these endogenous inhibitors of NO synthesis play a role in the hypertension seen in these patients.

Scavengers of NO production can be used to inactivate the NO radical itself preventing interaction of NO with the ferrous heme-containing receptor site on soluble guanylate cyclase.[57] Two potent scavengers of NO are the superoxide anion (O_2^-) and free hemoglobin.[58] The superoxide anion can inactivate NO/EDRF in the vascular endothelium thus causing local vasoconstriction and selective loss of endothelium dependent vasodilation.[59] The chemical reaction that could be involved is that the superoxide anion reacts with NO to form peroxynitrite ($ONOO^-$).[60,61] Peroxynitrite is a powerful and very toxic oxidant that can react with biological molecules such as structural proteins and may result in DNA strand breakage and tyrosine nitration. Nitrotyrosine measurements can be used to demonstrate peroxynitrite free radical-mediated cellular and organ damage.[61] Nitrotyrosine residues are found in human lung biopsy and autopsy samples with sepsis or ARDS.[62,63] Control lung samples show relatively little nitration. Other cytotoxic effects of nitric oxide that are possibly mediated by peroxynitrite formation include inhibition of cellular DNA synthesis, complex formation with iron-containing compounds and enzymes, and inactivation of complexes I and II of the mitochondrial electron-transport chain.[29] The enzyme superoxide dismutase (SOD) specifically catalyses the reaction $2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$, and thus decreases the local concentration of the superoxide anion. Since with SOD less O_2^- is present to form toxic peroxynitrite and inactivate NO, the half-life of NO is increased by SOD. This may explain the vasodilating effect of superoxide dismutase in the microcirculation.

Hemoglobin is a scavenger that directly binds NO.[58,64] However, the interaction of NO with hemoglobin is complex. Depending on the degree of oxidation of hemoglobin, the oxidation state of the heme and other factors, NO reacts differently with hemoglobin.[65]

Deoxyhemoglobin (Hb) reacts with NO producing nitrosyl-hemoglobin (HbNO). Oxyhemoglobin (HbO_2) and NO react quantitatively forming oxidized or methemoglobin (MetHb) and nitrate. MetHb binds NO and is slowly reduced to Hb, which, in the presence of more NO can form HbNO. Since hemoglobin is a large protein that does not enter the intracellular space, the binding of NO is confined to the extracellular space. However, because NO readily crosses cell membranes, binding by hemoglobin in the extracellular space may increase the concentration gradient across the cell membranes, increase the net rate of diffusion out of the cells and thereby reduce the magnitude of vascular smooth muscle relaxation. Hemoglobin from lysed erythrocytes has been implicated in the profound cerebral vasospasm which often follows cerebral hemorrhage,[66] and macrophage cytotoxicity can be inhibited by the presence of free hemoglobin or erythrocytes.[67] The NO scavenging properties of hemoglobin could play a role in this.

Another way to interfere with NO metabolism is to inhibit the effector enzyme of NO synthesis with methylene blue (MB) which inhibits the enzyme soluble guanylate cyclase (GC) in the vascular smooth muscle cell.[58] Methylene blue easily enters cells and inhibits soluble guanylate cyclase probably by oxidizing the ferrous form of the hemoprotein component of the enzyme to the ferric form. However, other mechanisms of action by MB, independent of guanylate cyclase inhibition, have also been suggested. MB can act as a direct inhibitor of NOS and by generation of a superoxide anion which directly inactivates NO.[68,69]

1.4 Nitric oxide in experimental sepsis

Recently much interest has been focused on the role of nitric oxide in septic shock. Following discovery of the NO radical it was clear that NO could play a role in the vascular relaxation and hypotension during sepsis and endotoxemia. *In vitro* studies showed that isolated macrophages produced increased amounts of nitrate and nitrite upon stimulation with lipopolysaccharides (LPS=endotoxin).[32-34] This was shown to be caused by the increased production of NO that depended on the presence of L-arginine and could be inhibited by analogues of L-arginine.[70]

The enzyme responsible for production of NO in macrophages differs from the c-NOS

in endothelial cells and neural tissue (Table 2). The c-NOS is calcium and calmodulin dependent and releases small amounts of NO whereas the enzyme in activated macrophages is calcium and calmodulin independent and releases high amounts of NO for a prolonged

Table 2. Comparison of the constitutive and inducible NO synthase

	c-NOS	i-NOS
Substrate	L-arginine	
Cofactors	NADPH, FAD, FMN, BH_4	
Heme group	Protoporphyrin IX	
Inhibitors	Analogues of L-arginine	
Expression	always present	must be induced
Activation/induction	activators: thrombin, serotonin, histamine, ADP, acetylcholine, calcium ionophores, pressure, shear stress	inducers: LPS, IFN-γ, TNF-α, IL-1β inhibition by corticosteroids
Rate limiting	calcium/calmodulin binding	substrate/cofactor availability
NO formation rate	small (picomoles) intermittent	large (nanomoles) continuous
Cellular sources	endothelial cells certain brain cells peripheral neurons myocardial cells	macrophages Kupffer cells vascular smooth muscle myocardial cells
Human chromosomal location	ecNOS: 7, ncNOS: 12	17
Pathophysiologic role	down-regulated in hypertension and atherosclerosis	up-regulated in inflammation, sepsis, hemorrhagic shock, dilated cardiomyopathy, type I diabetes, immune-mediated arthritis, inflammatory bowel disease, allograft rejection

From references [29-32,40-43,70-75,80-93]

period of time and independent of any stimuli. The same enzyme shown in macrophages could also be found in neutrophils, hepatocytes, myocardial cells and vascular smooth muscle after stimulation with LPS or cytokines and is called inducible NO synthase (i-NOS).[29,30,71,72] In humans, each enzyme is a product of a unique gene, and they share approximately 50% aminoacid homology.[73-75] The constitutive enzyme is located on chromosome 7 (endothelial type) and chromosome 12 (neural type), and the inducible enzyme is located on chromosome 17. The i-NOS is expressed under influence of LPS and several cytokines like TNF-α, interleukin-1 (IL-1) and interferon-γ.[72,76-79] The expression of i-NOS takes a lag-phase of several hours and can be inhibited by pretreatment with glucocorticoids and inhibitors of protein synthesis.[80,81] Once the i-NOS is expressed glucocorticoids are without effect. Both c-NOS and i-NOS depend on the presence of NADPH and tetrahydrobiopterin as a cofactor.[82] There is some evidence that when i-NOS is present the availability of the substrate L-arginine can be a limiting factor of NO production.[83]

A schematic representation of the different types of NO synthase and their role in the circulation are displayed in Figure 5. In general the constitutive NO synthase plays a physiological role in maintaining organ perfusion, whereas the expression of the inducible form, as in sepsis and endotoxemia, plays a more pathological role leading to the production of excessive amounts of NO resulting in vascular relaxation and tissue damage.[84] The expression of i-NOS has been implicated in several other disease processes such as type I diabetes mellitus,[85,86] immune-mediated arthritis,[87] inflammatory bowel disease,[88] and allograft rejection.[89] Furthermore downregulation of the constitutive NO synthase has been implicated in atherosclerosis,[90,91] and hypertension.[92,93]

Studies with isolated vessels of septic rats show that the contractile response to noradrenaline is reduced.[94] The same is seen in vessels of endotoxemic animals and in vessels of healthy animals incubated with LPS.[95,96] The reduced response to noradrenaline is accompanied by a rise in c-GMP content of the vessel wall.[97] Inhibitors of NO synthesis such as L-NMMA, L-NAME or L-NA and methylene blue, an inhibitor of soluble guanyl cyclase, can normalise the vasodilatation and the reduced response to noradrenaline.[95-98] Furthermore it was found that following endotoxin injection the i-NOS is present primarily in the vascular smooth muscle cells and not in the vascular endothelium.[96,99] These *in vitro* studies are

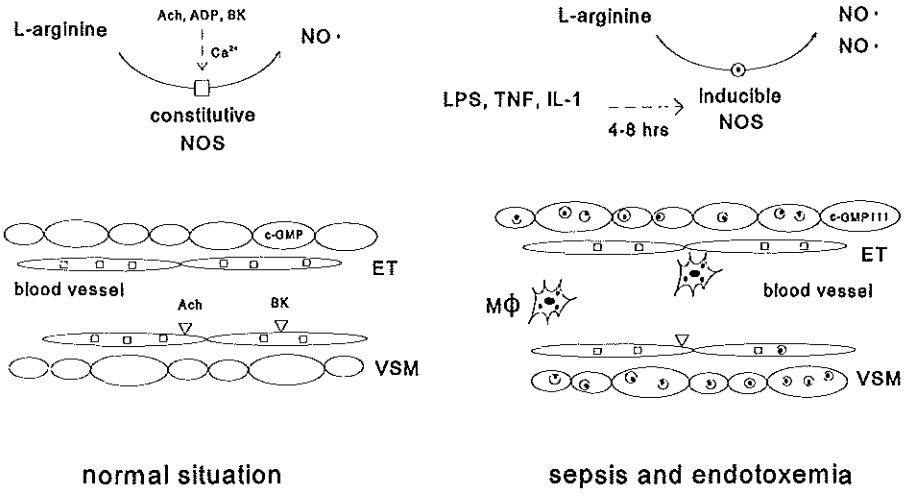

Figure 5. Schematic representation of blood vessels with the different types of NO synthase in the normal situation (*left*) and during sepsis and endotoxemia (*right*). In the normal situation only constitutive NO synthase is present in endothelial cells (ET) that can be stimulated by specific agonists such as acetylcholine (Ach), adenosinediphosphate (ADP), or bradykinine (BK) and/or flow induced shear stress. During sepsis and endotoxemia a second type of NO synthase is induced in macrophages (MΦ) and vascular smooth muscle cells (VSM) by stimulation with cytokines e.g., interleukin-1 (IL-1) and tumor necrosis factor (TNF), and endotoxin (LPS) with a lag-phase of 4-8 hours. This non-regulated inducible NO synthase produces large amounts of NO for long periods of time resulting in massive vasodilation and tissue damage.

underscored by *in vivo* experiments. Rats injected with LPS have reduced blood pressure and a diminished response to noradrenaline together with increased serum levels of nitrate and nitrite. Inhibition of NO synthesis in these endotoxemic rats increases blood pressure and restores the response to noradrenaline.[100-103] Similar findings were done in dogs where the NO inhibitor L-NMMA could prevent the fall in blood pressure during LPS and cytokine induced shock.[77-79] In i-NOS knockout mice, that lack the inducible NO synthase, some protection has been reported against bacterial infection and endotoxin shock.[104,105] Furthermore inhibition of NO synthesis may improve survival in animal models of sepsis although much depends on the specific model used.[106,107] Figure 4 shows the dose-dependent rise of blood pressure during inhibition of NO synthesis with a bolus injection of L-NAME in the anaesthetized endotoxemic

rat. However, this model and many other models of endotoxemia,[77-79,100-103] result in an instant reduction in blood pressure which is not likely related to induction of i-NOS.[108] Continuous infusion of endotoxin for 24 hours in conscious sheep resulted in a hyperdynamic circulation with systemic vasodilatation, hypotension and increased cardiac output.[109] This model resembles the cardiovascular findings of human sepsis. Infusion of the NO inhibitor L-NAME normalised the hemodynamic changes despite continuation of endotoxin infusion. Although oxygen delivery was reduced following L-NAME by reduction of cardiac output, total oxygen consumption was unaffected. It was concluded that L-NAME caused reduced perfusion of tissues with low metabolic demand. In the same model the effect of L-NAME on blood perfusion of the separate organs was studied.[110] Twenty-four hours following endotoxin infusion increased blood flow to brain, heart, intestines, liver and kidneys was seen. L-NAME reduced all regional flows. No detrimental effects of inhibition of NO synthesis were seen in this model of endotoxemia. However, a direct negative inotropic effect on the myocardium causing the reduction in cardiac output could not be excluded.

The findings from these animal studies suggest that NO may play an important role in the pathophysiology of sepsis and inhibition of NO synthesis may offer a novel therapeutic target for treatment of hypotension in patients with septic shock.

1.5 Nitric Oxide in the myocardium

In the coronary vessels NO plays an important role in the regulation of myocardial blood flow. In the isolated perfused rabbit heart there is a basal release of NO and increased release of NO accounts for the vasodilatation induced by acetylcholine and bradykinine.[111,112] In the same heart preparation inhibition of NO synthesis with L-NMMA increases coronary resistance and prevents the vasodilator response to acetylcholine.[113] In the awake dog L-NMMA causes a dose-dependent reduction in epicardial coronary diameter.[114] In humans intracoronary infusion of L-NMMA causes a reduction in epicardial calibre and coronary blood flow.[115]

Common features of sepsis are myocardial depression with ventricular dilatation, decreased stroke work and reduced left ventricular and right ventricular ejection

fractions.[6,9,10,116] Similar changes are seen in healthy volunteers when given endotoxin,[117] and in animal models of sepsis and endotoxemia.[118-120] Some have related these changes to the depressant action of circulating factors such as myocardial depressant factor (MDF) and cytokines, especially TNF-α and IL-1β, on cardiomyocytes.[9,10,116] More recently myocardial depression has been suggested to result directly from increased NO production within cardiac myocytes. The myocardial depression probably does not depend on the presence of myocardial ischemia.[121] The human coronary circulation during sepsis is often characterized by inappropriately high coronary flow with decreased oxygen extraction, closely mimicking the peripheral circulatory anomalies of vascular relaxation.[122,123] Although defects in coronary autoregulation have been suggested, this has never been shown directly. The pattern of disordered coronary flow regulation could also be seen in models of hyperdynamic sepsis in the rat both *in vivo* and *in vitro* and was suggested to result from the release of a putative vasodilator substance.[124,125] In the isolated rabbit heart preparation the coronary vasodilation induced by endotoxin was found to be nitric oxide dependent and could be inhibited by dexamethasone suggesting that i-NOS plays a role.[126] In the isolated rat heart endotoxin rapidly stimulates endothelium dependent vasodilatation which suggests that c-NOS can also play a role.[127] Both isoforms of NO synthase may therefore contribute to the coronary vasodilatation during sepsis. Not only the coronary vessels express NO synthase, but also the endocardium and myocardium itself have the capacity to express the constitutive and inducible NO synthase in both animal and human. The constitutive form of NO synthase has been shown to be present in cultured endocardial cells of the pig and in normal rat myocardium.[128,129] The inducible NO synthase is expressed following endotoxemia and isolated rat myocytes express the inducible NO synthase after stimulation with cytokines which can be inhibited with dexamethasone.[129] The increased release of NO by i-NOS within cardiomyocytes itself could play a role in myocardial depression during sepsis. In the isolated papillary muscle of pig and ferret, endocardial NO release reduces the duration of contraction and accelerates relaxation, an effect that is mediated by c-GMP.[130,131] Decreased influx of calcium from the sacroplasmatic reticulum through specific calcium channels and decreased myofilament response to calcium induced by c-GMP results in loss of cardiac myocyte contractility. Isolated ventricular myocytes of the guinea pig heart following endotoxemia show reduced contractility to electrical stimulation.[71] The depres-

sion in myocyte contraction can be corrected with L-NAME, L-NMMA, MB or dexamethasone pretreatment which suggests a causative role for i-NOS. Myocytes exposed to MB show signs characteristic of calcium overload with hypercontractility and fibrillation. This suggests that MB has direct toxic effects on cardiomyocytes.[132] Human ventricular tissue of patients with dilated cardiomyopathy shows a significant activity of the inducible NO synthase and increased levels of c-GMP with low activity of the constitutive enzyme.[133] This could suggest that the inducible enzyme plays a pathological role in the human myocardium, whereas the constitutive enzyme has a more physiological role. Increased production of nitric oxide in the myocardium may be directly responsible for myocardial depression during sepsis.

In theory inhibition of NO synthesis during sepsis or endotoxemia can normalise the pattern of disordered coronary flow regulation and correct the contractile dysfunction caused by overproduction of NO. However, in contrast to this, many investigators have reported reductions in heart frequency and cardiac output following inhibition of NO production during sepsis and endotoxemia *in vivo*.[84,109,110,134,135] Combined with the increased vascular resistance seen after inhibition of NO formation such reductions in cardiac output may compromise myocardial and tissue perfusion. It is not clear whether the fall in cardiac output seen after administration of inhibitors of NO synthesis during sepsis and endotoxemia is only secondary to increased blood pressure and increased afterload or a direct effect of NO synthase inhibition in the heart. A direct negative effect on isolated cardiomyocytes was shown not to be present using the NO inhibitor L-NMMA and L-NAME.[71,136] Decreased coronary flow causing myocardial ischemia has been hypothesized. In the isolated perfused rat heart L-NMMA caused a dose dependent reduction in coronary flow together with a reduction in cardiac performance and increased release of lactate in the effluent which could be suggestive for anaerobic metabolism and myocardial ischemia.[136] Administration of L-NMMA *in vivo* has been reported to exacerbate global myocardial ischemia in a rabbit model of endotoxemia as indicated by changes on the electrocardiogram.[84] However, in this model of endotoxemia, ischemic changes were already present before administration of L-NMMA which suggest that a hypodynamic circulation was present with reduced cardiac output and reduced coronary flow rates. In Chapter 2 and 3 of this thesis we studied the pathophysiological role of nitric oxide in coronary flow regulation and myocardial metabolism in a rat model of hyperdynamic endotoxemia.

1.6 Nitric Oxide in human sepsis

Plasma levels of nitrate (NO_3^-) and nitrite (NO_2^-) represent the stable endproducts of NO synthesis and can be used as a marker of NO synthase activity.[27,137] In a study by Ochoa et al.[138] in 39 intensive care patients it was found that septic patients had increased levels of nitrate and nitrite whereas trauma patients had not. Systemic vascular resistance showed an inverse correlation to the levels of nitrate and nitrite in the plasma. High plasma levels of c-GMP during sepsis correlate with low systemic vascular resistance.[139] In pediatric sepsis increased plasma nitrite and nitrate concentrations are associated with the development of multiple organ failure.[140] In another clinical study plasma levels of nitrate were evaluated in 12 patients treated with IL-2 as antitumour therapy.[141] An important complication of this therapy is the presence of a hyperdynamic circulation with hypotension and low systemic vascular resistance. Patients under treatment showed an eight-fold raise in plasma nitrate levels and urine nitrate excretion. This increased production of nitrate was derived from the guanidino nitrogen atom of L-arginine. There was a significant negative correlation between mean arterial pressure and maximum increase in plasma nitrate levels. These results suggest that sepsis and cytokine-induced hypotension in humans result from increased NO release.

Another way to indirectly estimate NO production is to determine consumption of L-arginine that delivers the nitrogen atom in the NO radical.[142,143] Freund et al. showed that arginine levels in the plasma of septic patients were reduced 24% as compared to normal. Non-survivors had lower serum levels of arginine than survivors although a causative relation is difficult to establish.

NO plays a physiological role in human microcirculation.[30] Intra-arterial infusion of L-NMMA to healthy volunteers resulted in a rise in vascular resistance in the forearm and a reduced response to acetylcholine.[144] Local intracoronary infusion of L-NMMA showed a reduction in coronary diameter without the presence of myocardial ischemia and prevented the response to acetylcholine.[115] This shows that in humans NO has a physiological role in both the coronary vasculature and the peripheral arterial system. However, under normal conditions endothelial derived NO is not necessarily vital for adequate tissue perfusion. Possibly NO gives the vasculature some reserve in conditions of increased demand as during exercise.

In addition, NO has a role as a physiologic mediator in the lung.[145,146] NO synthase is present in lung epithelium and other pulmonary cells and has been suggested to be a mediator of nerve-dependent bronchodilatation.[147] Furthermore hypoxic pulmonary vasoconstriction may result from reduced NO release.[148,149] Inspired NO gas acts locally without systemic vasodilatation or hypotension in experimental models and healthy volunteers.[149,150] NO is absorbed from ventilated alveoli, induces local vasodilatation, and is rapidly inactivated in the blood by binding to hemoglobin. Since NO exerts its effects selectively in aerated lung regions, the matching of lung perfusion with ventilation is enhanced, thereby reducing the right to left shunt and improving gas exchange. Inhalation of NO improves oxygenation in patients with acute respiratory distress syndrome (ARDS),[151] and reduces pulmonary artery pressure in pulmonary hypertension.[152,153] Unfortunately, in ARDS patients NO inhalation may only be effective in a subgroup of responders,[154] and in recent clinical trials NO inhalation during ARDS failed to improve outcome despite the improvement of oxygenation.[155,156]

1.7 Inhibitors of NO synthesis in human sepsis

Inhibitors of NO synthesis have been used in the treatment of hypotension in patients with septic shock or to investigate the pathophysiological role of NO in human sepsis. Most studies were pilot studies and had only a limited amount of patients included.

Several studies have used methylene blue (MB), an inhibitor of soluble guanylate-cyclase, the effector enzyme of nitric oxide, in patients with septic shock.[157-160] MB has been used for years in the treatment of patients with nitrous oxide and nitrate poisoning.[161] Furthermore the oxidizing properties of MB have been used clinically to induce methemoglobinemia in the treatment of cyanide poisoning.[162] Infusion of MB in a dose range of 1-3 mg/kg causes an early rise in blood pressure and systemic vascular resistance without reduction in cardiac output. Although *in vitro* studies have shown that MB has direct toxic effects on cardiomyocytes,[71,132] MB seems to improve cardiac function in human sepsis. Except for a blue coloration of the urine and skin, no negative side effects were noted in these studies. Despite the hemodynamic improvements most patients died because of shock and multiple organ failure.

Petros et al.[163] was the first to describe the use of the NO synthase inhibitors L-NMMA and L-NAME in two patients with unresponsive septic shock. In the first patient L-NMMA (0.3-1.0 mg/kg) increased blood pressure and systemic vascular resistance. In the second patient L-NAME was given in a bolus of 0.15 mg/kg and continued with continuous infusion of 5 μg/kg/min. Bolus injection increased mean arterial pressure from 84 to 102 mmHg. This effect was present for 10-15 min and was caused by increased systemic vascular resistance since cardiac output and pulmonary wedge pressure were unchanged. During continuous L-NAME administration the noradrenaline infusion could be reduced from 0.6 to 0.2 μg/kg/min. Within 48 hours the infusion of noradrenaline and L-NAME could be stopped and blood pressure and systemic vascular resistance returned to normal. The first patient eventually became independent of intensive care support. The second patient died two days later because of the combination of repeated intra-abdominal sepsis, adult respiratory distress syndrome (ARDS), and disseminated intravascular coagulation (DIC).

Schilling et al.[164] used L-NMMA in the treatment of unresponsive hypotension in a septic patient that had two episodes of severe hypotension within a period of four weeks. Treatment with this inhibitor of NO synthesis resulted in an increase in mean arterial blood pressure, a reduction in cardiac output and an increase in arterial oxygen pressure. During infusion of L-NMMA catecholamines were reduced. The initial response to a bolus of L-NMMA lasted only 5-10 min so that bolus injection had to be repeated several times. In a similar case report by Lin et al.[165] a single gift of L-NMMA 50 mg increased blood pressure and systemic vascular resistance in a patient with severe septic shock.

In a double blind, placebo controlled study of Petros et al.[166] the effects of L-NMMA were investigated in 11 patients with severe sepsis. L-NMMA (0.3 and 1.0 mg/kg) resulted in a dose-dependent increase in mean arterial pressure, systemic vascular resistance, pulmonary vascular resistance, central venous pressure, and pulmonary artery occlusion pressure, and a decrease in cardiac output and heart rate. Continuous infusion of L-NMMA (1 mg/kg/h) produced sustained hemodynamic changes. No negative side effects were noted. In the L-NMMA group 2/5 patients survived the 28 day study period whereas in the control group 1/6 patients survived which was not significantly different.

In a prospective intervention study in septic patients by Lorente et al.[167] the effect of

a bolus injection of L-NA was evaluated followed by a bolus injection of L-arginine. Injection of L-NA in a dose of 20 mg/kg resulted in a generalised vasoconstriction of the systemic and pulmonary circulation with hypertension and a strong reduction of cardiac output. These effects could be reversed by administration of L-arginine. Administration of L-arginine in a separate group of septic patients resulted in transient hypotension and an increase in cardiac index together with an increase in oxygen consumption. This could be due to increased formation of NO, however, both L-arginine and D-arginine can cause vasodilation when administered into the brachial artery of healthy volunteers which suggests that this effect is not mediated by NO.[168] Compared to the studies mentioned above the dose of L-NA used was very high. Furthermore in vitro studies have shown that L-NA is a more potent inhibitor of constitutive NO synthase than L-NMMA and L-NAME. This could explain the generalised vasoconstriction seen in this study.

Recently the scavenging properties of cell-free hemoglobin that has been cross-linked to avoid renal toxicity, have been used to control NO biosynthesis.[56] The administration of hemoglobin to vasopressor dependent patients resulted in a reduction of the catecholamine requirements and an improvement in hemodynamics, similar to that observed with NO synthase inhibitors.[169,170]

From these previous studies one can conclude that inhibition of NO synthesis in human sepsis leads to increased blood pressure and systemic vascular resistance. Furthermore, a reduction in the vasopressor requirements was observed, suggesting that catecholamine sensitivity was restored. Inhibition of NO synthesis could therefore be of help in patients with hyperdynamic septic shock where hypotension does not respond to conventional therapy. However, the exact role of nitric oxide in human septic shock remains to be determined. Most previous reports on inhibition of NO synthesis only describe initial changes and not much is known about prolonged NO synthase inhibition. The effects NO synthase inhibitors on organ function, myocardial performance, pulmonary gas exchange and metabolism in human sepsis are not totally clear. Furthermore inhibition of NO synthesis may have immunological consequences,[32-34] and NO may interact with other vasoactive mediators as has been described in recent animal studies.[171,172] In this thesis we investigated some of the unanswered questions regarding NO and inhibition of NO synthesis in experimental and human septic shock.

1.8 Aims of the studies

Sepsis is the systemic response to infection and characterised by macro- and microcirculatory changes resulting from the release of several mediators. Nitric oxide, probably the smallest of these mediators in diameter but certainly not in action, has been suggested to be largely responsible for the cardiovascular derangements of sepsis and septic shock. We investigated the pathophysiological role of nitric oxide and the effect of nitric oxide inhibitors in experimental and clinical sepsis.

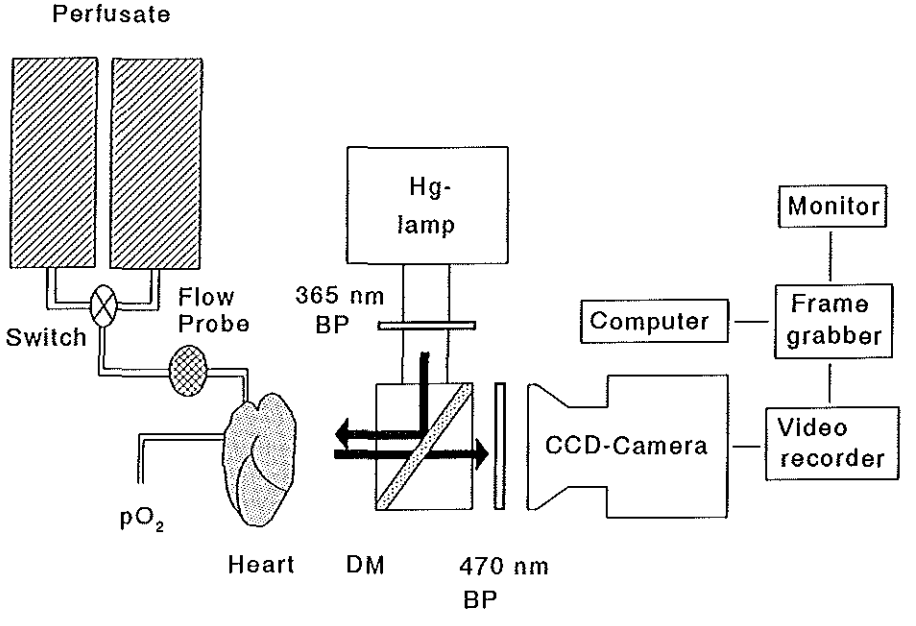

Figure 6. Experimental design of the set-up used for measurements in the isolated perfused rat heart. Hearts are perfused according to Langendorff with controlled perfusion pressure and two reservoirs of perfusate. Coronary flow rates are measured with an electromagnetic flow probe and Clark-type oxygen electrodes are used for measurement of influent and effluent pO_2. Mitochondrial energy state is evaluated using NADH autofluorescence. NADH is a key intermediate in the transfer of reducing equivalents from metabolic substrates in the cytosol to oxygen in the mitochondria. During hypoxia less NADH is oxidized to NAD^+ leading to accumulation of NADH. When excited with light at 360 nm the NADH, unlike NAD^+, emits fluorescence light at wavelengths centred around 460 nm. Hypoxic regions therefore will appear as areas of high fluorescence on the surface of the heart. A Hg arc lamp provides the 360 nm light needed for NADH excitation and a CCD video camera detects 460 nm NADH fluorescence. The dichroic mirror (DM) separates excitation and emission light at appropriate wavelengths.

In **chapter 2** we studied the role of nitric oxide in during endotoxemia in the rat. Intraperitoneal injection of lipopolysaccharides (LPS) was used as a model for hyperdynamic sepsis.[173,174] The isolated perfused rat heart was studied during retrograde perfusion in a Langendorff set-up (Figure 6). This set-up was used because it enabled us to control coronary perfusion pressure, flow and oxygen supply. Furthermore the effect of nitric oxide synthase inhibition on redox state of the myocardium was studied by using the fluorescent properties of reduced nicotinamide adenine dinucleotide (NADH). This technique is able to detect local areas of myocardial ischemia.[175]

The pattern of massive vasodilatation together with maldistribution of blood flow, as has been reported in human sepsis, could reflect disturbances of vascular autoregulation. In **chapter 3** the isolated rat heart model was used to study coronary autoregulation following endotoxemia. Autoregulation was studied by analyzing flow-pressure relations and the pattern of reactive hyperemia after coronary occlusion. Furthermore the role of nitric oxide in autoregulatory disturbances was evaluated by studying the effects of NO inhibitors and the effect of NO donors.

Previous studies have suggested nitric oxide may play a central role in human sepsis and that inhibition of NO synthesis might be beneficial in patients with septic shock. However, most clinical studies only describe initial effects on hemodynamics and not much is known about effects of NO synthase inhibitors on organ function, myocardial performance, pulmonary gas exchange and metabolism in human sepsis. In the next chapters we present the results of a clinical study that evaluated the effects of prolonged inhibition of NO synthesis with the NO inhibitor L-NAME in patients with septic shock. The 'L-NAME trial' was conducted in the intensive care units of the department of Surgery and the department of Internal Medicine of the University Hospital Rotterdam Dijkzigt. Patients were included in the period between January 1995 and February 1996. In **chapter 4** the effects of continuous L-NAME infusion on hemodynamic, biochemical and metabolic parameters are presented.

Several studies in animals and humans have mentioned reductions in cardiac output during inhibition of NO synthesis. So far the precise mechanism underlying this reduction in cardiac output remains unclear. Furthermore, NO plays a role in the pulmonary vasculature and NO inhalation can improve oxygenation in patients with ARDS.[151] This could suggest that

inhibition of NO synthase in septic shock may have negative side effects on pulmonary function. In **chapter 5** we present the effects of continuous L-NAME infusion on cardiac performance and pulmonary gas exchange.

In **chapter 6** the dose related side effects of NO synthase inhibition are described in a postoperative patient with severe septic shock who was given a high dose continuous infusion of L-NAME.

Besides the impact on vascular tone, NO has important immunological functions.[32-34] In septic rats NO may have a negative feedback on cytokine release and inhibition of NO synthesis may result in detrimental increases in pro-inflammatory cytokine levels of TNF-α and IL-6.[176,177] The immunologic effects of NOS inhibition in human sepsis remain to be determined. In **chapter 7** we describe the effect of L-NAME on plasma levels of IL-6, IL-8, TNF-α and nitrite/nitrate in human septic shock.

The relation of NO with other vasoactive mediators in humans have not been fully identified. For instance, NO release may directly inhibit the release of endothelin-1 (ET-1), a powerful vasoconstrictor produced by endothelial cells in animals and humans.[178,179] In rats part of the hypertensive response after inhibition of NO synthesis results from increased production of ET-1.[171,172] Since ET-1 levels have been shown to be increased during human sepsis,[180] we speculated that part of the vasoconstriction seen with L-NAME may result from increased levels of ET-1. In **chapter 8** we describe the relation between ET-1 levels and the increase in blood pressure with L-NAME in human sepsis.

Although several others have used L-NAME as an inhibitor of nitric oxide synthesis, no pharmacokinetic information exists on clearance, biodistribution or excretion in humans and the rationale for the dosage regimen in septic patients have been largely empirical. In **chapter 9** we describe distribution and metabolism of L-NAME in the patients with severe sepsis. Furthermore we studied in vitro metabolism of L-NAME in buffer, plasma and whole blood. Measurements of L-NAME and its metabolite L-NA were done using high performance liquid chromatography (HPLC).

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Chapter 2

Inhibition of Nitric Oxide Synthesis Causes Myocardial Ischemia in Endotoxemic Rats

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Abstract

Increased production of nitric oxide (NO) may play a central role in the cardiovascular derangements of septic and endotoxin shock. We have investigated the pathophysiological role of NO and the effect of inhibition of NO synthesis following endotoxemia in the isolated perfused rat heart.

In hearts from endotoxin-treated animals coronary flow was raised 64% and oxygen consumption 20% compared to control hearts. NADH fluorescence imaging was used as indicator of regional hypoperfusion. A homogeneous low surface NADH fluorescence, indicative of adequate tissue perfusion, was observed in both control and endotoxin-treated hearts. The increase in coronary flow and oxygen consumption could only partially be prevented by pre-treatment of the animals with dexamethasone. Addition of N^G-Nitro-L-Arginine (NNLA), an inhibitor of NO synthesis, to the perfusion medium eliminated differences in coronary flow and oxygen consumption between normal and endotoxin-treated hearts. However, NADH surface fluorescence images of endotoxin-treated hearts following NNLA revealed areas of high fluorescence indicating local ischemia, whereas the control hearts remained without signs of ischemia. The ischemic areas were present at various perfusion pressures and disappeared after infusion of L-arginine, the natural precursor of NO, or the exogenous NO donor sodium-nitroprusside. Methylene blue (MB), an inhibitor of soluble guanylate cyclase (GC), also eliminated differences in coronary flow and produced similar areas of local myocardial ischemia in endotoxin-treated hearts but not in control hearts. Reducing coronary flow by direct vasoconstriction with vasopressin resulted in similar patterns of myocardial ischemia as with NNLA and MB.

Our results suggest that the coronary vasodilation in the isolated rat heart following endotoxemia is caused by an increased release of NO. Coronary flow reduction with NNLA, MB or vasopressin causes local areas of myocardial ischemia in endotoxin-treated hearts but not in untreated hearts. These data suggest that endotoxemia promotes myocardial ischemia in vulnerable areas of the heart following inhibition of the NO pathway or direct vasoconstriction.

Introduction

Sepsis remains an important cause of mortality, especially when accompanied by shock or Multiple Organ Failure (MOF), despite advances in antibiotic and vasopressant therapy.[1] The initial cardiovascular changes observed during the hyperdynamic septic state are often characterized by profound vasodilation with normal to high cardiac output, low systemic vascular resistance, and severe hypotension.[2] Persistent hypotension with low vascular resistance refractory to vasopressor therapy is present in approximately 50% of patients who die of septic shock.[2-3] By promoting the release of cytokines and other mediators, bacterial lipopolysaccharides (LPS = endotoxin) initiate many of the cardiovascular changes seen during sepsis both in animals and in the human.[4,5]

Recent evidence suggests that a massive release of nitric oxide (NO), a very potent endogenous vasodilator, causes much of the vascular relaxation and hypotension of sepsis and endotoxemia.[6-8] NO is formed from L-arginine by the constitutive NO synthase present in the vascular endothelium and stimulates the enzyme soluble guanyl-cyclase (GC) in the smooth muscle cell which converts GTP to c-GMP resulting in vascular relaxation. Under physiologic conditions NO plays an important role in the regulation of blood pressure and tissue perfusion. In sepsis and endotoxemia an inducible form of NO synthase, located in vascular smooth muscle, is formed leading to a massive release of NO resulting in profound vasodilation.[6] The induction of this inducible type of NO synthase can be prevented by pre-treatment with glucocorticoids such as dexamethasone.[7,8]

In the coronary vessels NO plays a role in the regulation of myocardial blood flow.[9-13] In human septic shock inappropriately high coronary flow with decreased oxygen extraction has been reported closely mimicking the peripheral circulatory anomalies of vascular relaxation.[14,15] This pattern of disordered coronary flow regulation may be due to the release of a putative vasodilator substance such as NO.[8,16]

Methylated or nitro-substituted analogues of L-arginine competitively inhibit the formation of NO from L-arginine by NO synthase.[17,18] Furthermore, at the end of the NO pathway the effect of NO can be inhibited by methylene blue (MB) which inhibits the enzyme soluble guanylate cyclase (GC) in the vascular smooth muscle cell.[19] It has been suggested that such

inhibitors have therapeutic value in the treatment of septic shock since hypotension is corrected following administration of these L-arginine analogues and MB.[20-23] However, complete inhibition of NO formation can increase mortality in endotoxemic animals.[24-26] Also, many investigators have reported reductions in cardiac output following inhibition of NO production.[21-25,27] Combined with the increased vascular resistance seen after inhibition of NO formation such reductions in cardiac output may compromise myocardial and tissue perfusion even further. Decreased coronary flow causing myocardial ischemia has been hypothesised to contribute to the fall in cardiac output seen after administration of inhibitors of NO synthesis during sepsis and endotoxemia; however, this has never been shown directly.[25,28]

In this study we investigated the effect of N^G-Nitro-L-Arginine (NNLA)[18] and MB[19] on myocardial metabolism and coronary flow in hearts of normal and endotoxin-treated rats using a Langendorff heart preparation. To identify any inadequacies in oxygen supply causing myocardial ischemia in the perfused hearts, we used the fluorescence properties of reduced pyridine nucleotide (NADH), an indicator of mitochondrial respiration.[29,30] We used this technique recently to visualise the existence of microcirculatory units vulnerable to ischemia in the isolated rat heart.[29] In the present study we hypothesize that such units might be susceptible for ischemia during endotoxemia when the NO pathway is inhibited. Preliminary results have been presented elsewhere.[31]

Materials and Methods

Experimental set-up. The protocol was approved by the animal ethics board. Male Wistar rats (300-360 g) received either 0.7 mg/kg of body weight (BW) E. Coli endotoxin (LPS, 0127:B8, Sigma St. Louis, MO) or saline by intraperitoneal (i.p.) injection 12 hours before the initiation of the experiments. Control and endotoxin-treated animals were used for *in vivo* measurement of blood pressure and heart rate. Following anesthesia with pentobarbitone (50 mg/kg BW, i.p.) the right carotid artery was cannulated and blood pressure was measured on a pressure transducer (Hewlett Packard 8805B Corner Amplifier, MA) in control and endotoxemic rats.

Animals to be used for isolated perfusion of the heart were anesthetized with ether and

rectal temperature was measured. These animals were heparinized (0.5 ml, 100 IU) and 1 ml of blood was withdrawn from the descending aorta. To assess the adequacy of endotoxin administration, collected blood samples were analyzed for lactate using an enzymatic and colorimetric procedure.[32] For indirect determination of global NO release, measurement of plasma nitrite and nitrate, the stable end products of NO oxidation, were done using an automated procedure based on the Griess reaction.[33] The hearts were removed, immediately cooled in ice-cold perfusion medium and rapidly arranged for constant pressure perfusion at 37°C according to the Langendorff technique. The perfusion medium consisted of (mM) 128 NaCl, 4.7 KCl, 1 $MgCl_2$, 0.4 NaH_2PO_4, 20.2 $NaHCO_3$, 1.3 $CaCl_2$, 5.0 glucose, 2.0 pyruvic acid, and was oxygenated with 95% O_2 and 5% CO_2. All hearts were continuously paced at 5 Hz. To minimize metabolic demand, the left ventricle was drained by a cannula in the apex so that no external work was performed. Flow rates (expressed as ml/min/g of ventricle wet weight) were measured by an electromagnetic flow probe (Skalar-Medical, Delft, The Netherlands) in the aortic perfusion line. For the determination of effluent oxygen pressure the right pulmonary artery was cannulated and a constant fraction of the coronary effluent was led along a Clark-type electrode (YSI Biological Oxygen Monitor, model 5300, Yellow Springs Instruments, OH). The coronary influent was similarly monitored for oxygen pressure via a side arm of the aortic cannula. Oxygen consumption (μmol/min/g of ventricle wet weight) was calculated from the product of the influent-effluent concentration difference and coronary flow using the Bunsen solubility coefficient of oxygen in Krebs-Henseleit buffer (22.7 μl O_2/ml per atmosphere at 37°C).

NADH videofluorimetry. NADH is a key intermediate in the transfer of reducing equivalents from metabolic substrates in the cytosol to oxygen in the mitochondria in the myocardial cells. During hypoxia less NADH is oxidized to NAD^+ leading to accumulation of NADH. When excited with light at 360 nm the NADH, unlike NAD^+, emits fluorescence light at wavelengths centered around 460 nm. Hypoxic regions therefore will appear as areas of high fluorescence on the surface of the heart. NADH surface fluorescence provides a sensitive method to visualise any regional inadequacies in oxygen supply and beginning myocardial ischemia. NADH video-fluoroscopy allows on-line evaluation of spatial heterogeneity in hypoxia, not detected by global parameters of flow, pressure and metabolites. NADH

surface fluorescence imaging was done by excitation of about 1 cm^2 of the left ventricle of the Langendorff rat heart with light of 360 nm and measurement of the emitted fluorescence (460-490 nm) using an image intensified CCD videocamera as described elsewhere.[30] Fluorescence images were recorded on a video-recorder and computer-analyzed off-line. A substraction routine was used in all pictures to enhance contrast.

Table 1. Effects of endotoxin administration.

	Control (n)	Endotoxin (n)
Temperature (°C)	36.9±0.3 (8)	38.1±0.6*(8)
MABP (mmHg)	112.2±4.2 (5)	95.2±4.8*(5)
Heart rate (BPM)	384±18 (5)	408±19*(5)
Plasma lactate (mM)	1.5±0.3 (8)	4.3±0.6*(8)
Serum $NO_2^-+NO_3^-$ (μM)	23±8 (8)	214±36*(8)

All values represent means ± SD. Numbers of rats are presented in parenthesis. MABP=mean arterial blood pressure, BPM=beats per minute. For all parameters the endotoxin-treated group differed significantly from the control group. *$p<0.05$.

Experimental protocol Perfusion pressure is an important determinant of both oxygen delivery and oxygen consumption in the isolated Langendorff rat heart.[34] Therefore, we investigated myocardial metabolism over a range of perfusion pressures in eight control and eight endotoxin-treated hearts. Hearts were perfused for a total of 130 min. Following a period of 30 min allowing coronary flow to stabilize at 60 mmHg, perfusion pressure was first lowered to 20 mmHg and subsequently increased by stepwise changes in perfusion pressure of 10 mmHg at 3 min intervals to a maximum of 100 mmHg. At the end of this series of pressure changes, perfusion pressure was set at the initial value of 60 mmHg. When the flow was stable, perfusion medium was switched to one containing NNLA in a concentration of 100 μM. After a 30 min interval, when a new steady state was achieved, the stepwise changes in perfusion pressure were repeated once again. In three hearts the effects of either L-arginine (100 μM), the precursor of NO production,[6] or nitroprusside (10 μM), a nitrovasodilator that acts as an exogenous NO donor,[35] were studied following 30 min of NNLA. To study the effect of inhibition of soluble guanylate-cyclase (GC), the effector enzyme of NO, we used

methylene blue (MB) in four control and four endotoxin-treated hearts. MB was used in a concentration of 5 μM since higher concentrations (>20 μM) caused a paradoxal increase in coronary flow in control hearts, probably by nonspecific toxic effects.[36] To see whether the effect of direct vasoconstriction was different from the effect of inhibition of NO metabolism, in three separate hearts we switched to vasopressin, a direct smooth muscle vasoconstrictor, in a concentration of ~1 nM in order to reach approximately the same reduction in flow as did NNLA.

The LPS-stimulated induction of NO synthase can be prevented by pre-treatment with dexamethasone without affecting NO release of the constitutive enzyme.[7,8] To investigate the effect of dexamethasone pre-treatment, five rats were injected with dexamethasone 4 mg/kg intravenously 90 min prior to LPS infusion as compared to three rats of a control group that received only dexamethasone in combination with saline.

Histologic examination by light microscopy was done following fixation with 10% formaldehyde and hematoxylin-eosine staining to assess if histo-pathologic differences were present between control and endotoxin-treated hearts.

Chemicals NNLA, MB, arginine-vasopressin and dexamethasone were obtained from Sigma (St. Louis, MO). All the other chemicals were obtained from Merck (Darmstadt, Germany).

Statistics All values are expressed as mean ± S.D. Student's t-test for group comparisons or paired t-test where appropriate were used to statistically compare values. To test whether drug effects were perfusion pressure dependent, we used analysis of covariance. $p < 0.05$ was taken as statistically significant. NS is used to denote not significant.

Results

Effects of endotoxin administration. Although most animals of the endotoxin-treated group showed signs of lethargy, all animals survived. As shown in Table 1 endotoxin-treated animals showed raised rectal temperature, decreased mean arterial blood pressure, increased heart rate and increased plasma levels of lactate. Serum nitrite+nitrate ($NO_2^- + NO_3^-$) were elevated in endotoxin-treated animals as a sign of increased systemic release of NO (Table 1).

Histologic examination of the hearts following perfusion revealed no signs of capillary thrombosis, leukocyte infiltration, intracellular edema or signs of tissue damage in control (n=3) or endotoxin-treated (n=3) hearts.

Effect of endotoxin and dexamethasone pre-treatment on coronary flow, effluent pO_2 and oxygen consumption. In steady state conditions at a perfusion pressure of 60 mmHg, the coronary flow was raised 64% in endotoxin-treated hearts (n=8) (from 9.2±0.6 to 15.0±1.6 ml/min/g wet weight; Figure 1a) and oxygen consumption was raised 20% (from 4.0±0.2 to 4.8±0.3 μmol/min/g wet weight; Figure 1c) as compared to control hearts (n=8). Since the relative rise in coronary flow was greater than in oxygen consumption, this was accompanied by a rise in coronary effluent pO_2 (from 374±20 to 463±37 mmHg; Figure 1b). To see whether increased coronary flow could be prevented with glucocorticoids, we pre-treated rats (n=5) with dexamethasone 90 min before the injection of endotoxin. Dexamethasone pre-treatment could only partially prevent the increase in coronary flow and oxygen consumption in the endotoxin-treated hearts (11.3±1.9 ml/min/g and 4.3±0.3 μmol/min/g, respectively; Figure 1). Dexamethasone pre-treatment was without effect on coronary flow and oxygen consumption in control hearts (n=3) (8.9±0.7 ml/min/g and 4.0±0.3 μmol/min/g, respectively, NS).

Effect of perfusion pressure on coronary flow, effluent pO_2, oxygen consumption and mean NADH fluorescence. During perfusion at different perfusion pressures, ranging from 20 to 100 mmHg, coronary flow (Figure 2a) and oxygen consumption (Figure 2c) were significantly raised in hearts from endotoxic animals at perfusion pressures above 20 mmHg. Decreased oxygen extraction resulted in a rise of coronary effluent pO_2 (Figure 2b). Mean NADH surface fluorescence was not significantly different between the control and endotoxin-treated hearts for all perfusion pressures. Signs of local ischemia, detected as small areas of high fluorescence and a rise of mean NADH surface fluorescence, were only present at 20 mmHg perfusion pressure. At higher perfusion pressures a homogeneous image with low NADH fluorescence intensity was seen in both groups (Figures 5a and 5c).

Effect of inhibiting NO synthesis with NNLA on coronary flow, effluent pO_2 and oxygen consumption. Figure 3 shows two representative tracings of the response in time of coronary flow following inhibition of NO synthesis with 100 μM NNLA in an endotoxin-treated and

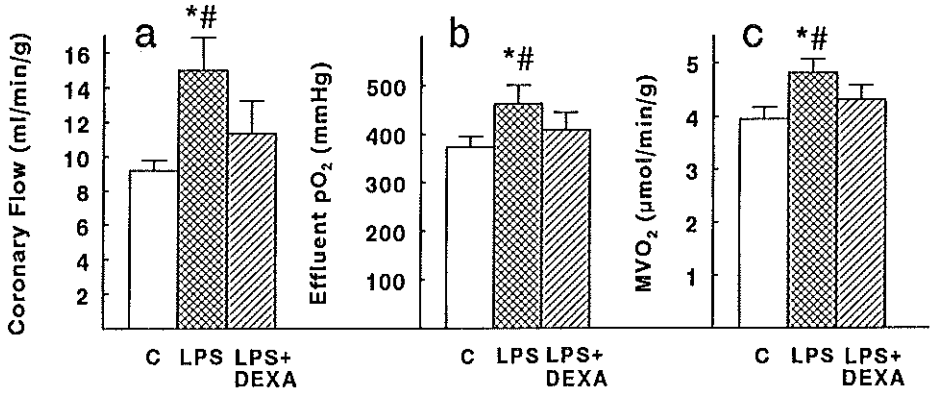

Figure 1. Effect of endotoxin (LPS) and dexamethasone pre-treatment (LPS+DEXA) on coronary flow, effluent pO_2, and oxygen consumption (MVO_2) as compared to untreated control hearts (C), at steady state perfusion pressure of 60 mmHg. Each column represents the mean, vertical bars indicate S.D. of control (n=8), endotoxin (n=8) or LPS+DEXA (n=5) hearts. * Statistically significant difference of LPS against C ($p<0.05$). # Statistically significant difference of LPS+DEXA against LPS ($p<0.05$).

control heart. In the endotoxin-treated heart there is an initial steep reduction of 4.0 ml/min/g in coronary flow the first 3 min with a more sustained decrease in flow the following 20 min. In the control heart a gradual decline in coronary flow of only 0.5 ml/min/g is seen during the first 3 min following NNLA. As early as 5 min after starting NNLA administration the endotoxin-treated heart had reached approximately the same coronary flow as the control heart. Figure 4 shows the steady state situation 30 min after inhibition of NO synthesis. NNLA decreased coronary flow by 8.5 ml/min/g (57%) in the endotoxin group ($p<0.05$) and by 3 ml/min/g (34%) in the control group ($p<0.05$). Thirty min following NNLA, coronary flow in endotoxin-treated hearts (6.5±0.6 ml/min/g) was not significantly different from control hearts (6.1±0.6 ml/min/g). Effluent pO_2 was reduced 35% to 299±50 mmHg in endotoxin-treated hearts and 26% to 276±27 mmHg in control hearts. Oxygen consumption was significantly reduced in both endotoxin-treated and control hearts (endotoxin 32% reduction to 3.3±0.3 µmol/min/g and control 18% reduction to 3.3±0.3 µmol/min/g wet weight). In hearts of dexamethasone pre-treated endotoxemic animals NNLA reduced coronary flow to 6.6±0.5 ml/min/g and oxygen consumption to 3.4±0.4 µmol/min/g wet weight ($p<0.05$). No significant differences in coronary flow, effluent pO_2, and oxygen consumption were

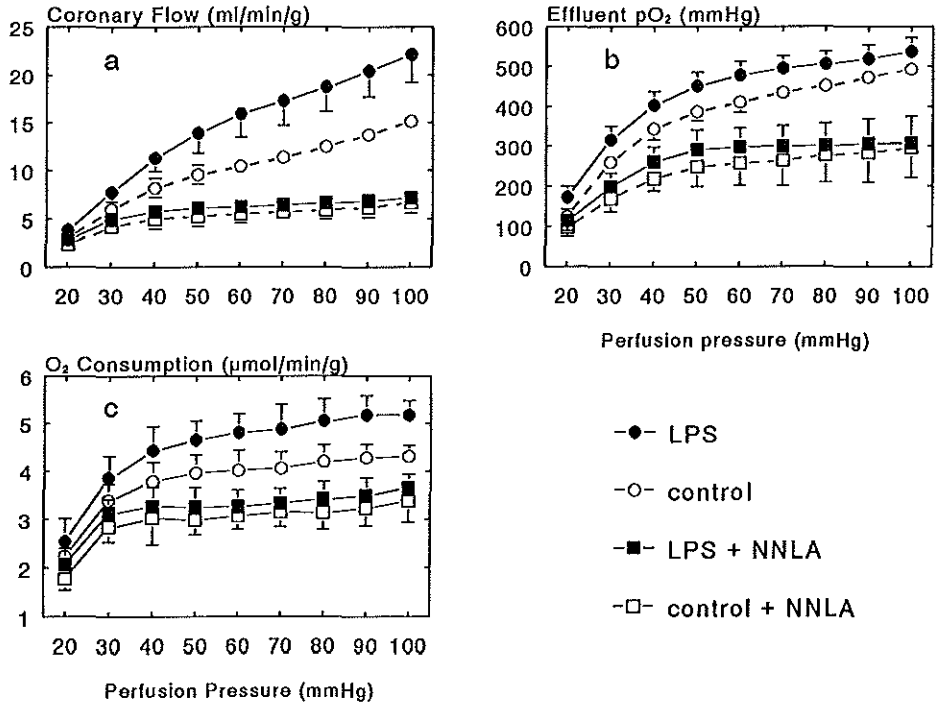

Figure 2. Effect of perfusion pressures on coronary flow (a), effluent pO_2 (b), oxygen consumption (c) in control (-O-) and endotoxin-treated (-●-) rat hearts. Control (-□-) and endotoxin-treated (-■-) hearts after inhibition of NO synthesis with 100 μM N^G-Nitro-L-arginine (NNLA). Each point is the mean ± S.D. of 8 hearts at a given perfusion pressure. * Statistically significant difference of LPS against C ($p<0.05$).

present between the three groups receiving NNLA (Figure 4).

Effect of perfusion pressure following NNLA. Figure 2 shows the effect of different perfusion pressures on coronary flow (Figure 2a), effluent pO_2 (Figure 2b) and oxygen consumption (Figure 2c) following inhibition of NO synthesis with NNLA (100 μM) in endotoxin-treated and control hearts. Relative changes in coronary flow induced by NNLA depended on perfusion pressure: they were larger at higher perfusion pressures in both control and endotoxin-treated hearts (analysis of covariance, $p<0.05$). After NNLA administration no significant differences were present between endotoxic and control hearts at all perfusion pressures.

Effect of NNLA on surface NADH fluorescence of rat hearts. Switching to perfusate

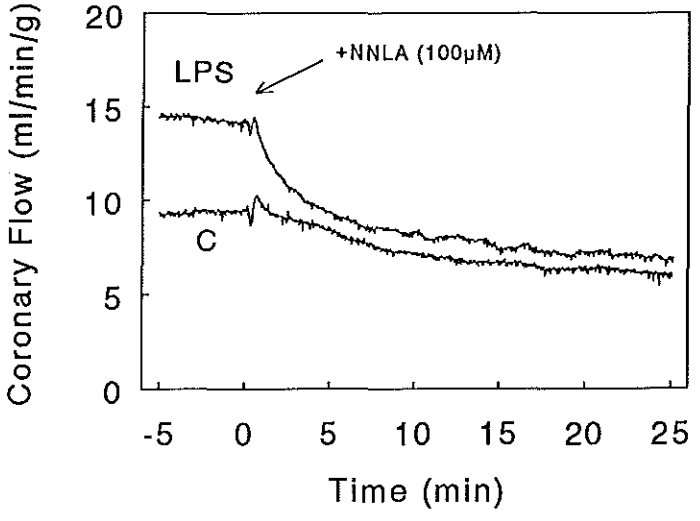

Figure 3. Two representative tracings of coronary flow in a control (C) and an endotoxin-treated (LPS) rat heart after inhibition of NO synthesis with N^G-Nitro-L-arginine (100 μM) at t=0, and a constant perfusion pressure of 60 mmHg.

containing NNLA at 60 mmHg perfusion pressure resulted in the development of small areas of high fluorescence in the endotoxin-treated hearts (Figure 5d) which is indicative of local ischemia. The control hearts (Figure 5b) remained without any sign of ischemia. The ischemic areas in the endotoxic hearts were present at all perfusion pressures. They disappeared after addition of L-arginine, the natural precursor of NO, or sodium-nitroprusside, an exogenous NO donor, indicating that the areas of ischemia are indeed caused by the action of NO. In normal hearts, areas of ischemia appeared only when perfusion pressure was lowered to 20 and 30 mmHg. The mean NADH fluorescence was not significantly different between groups and was raised only at 20 mmHg perfusion pressure in both endotoxin and control group. NNLA did not significantly affect mean NADH fluorescence in either group.

Effect of methylene blue (MB) on coronary flow and surface NADH fluorescence. To study the effect of inhibiting soluble guanylate-cyclase (GC), the effector enzyme of NO, we used MB (5 μM) in control hearts (n=4) and endotoxin-treated hearts (n=4). MB reduced coronary flow from 8.7±0.6 to 7.6±0.9 ml/min/g wet weight (NS) in control hearts and from 16.0±1.3 to 8.9±1.4 ml/min/g ($p<0.05$) in endotoxin-treated hearts at 60 mmHg perfusion pressure. There was no significant difference in coronary flow between control and endotoxin-

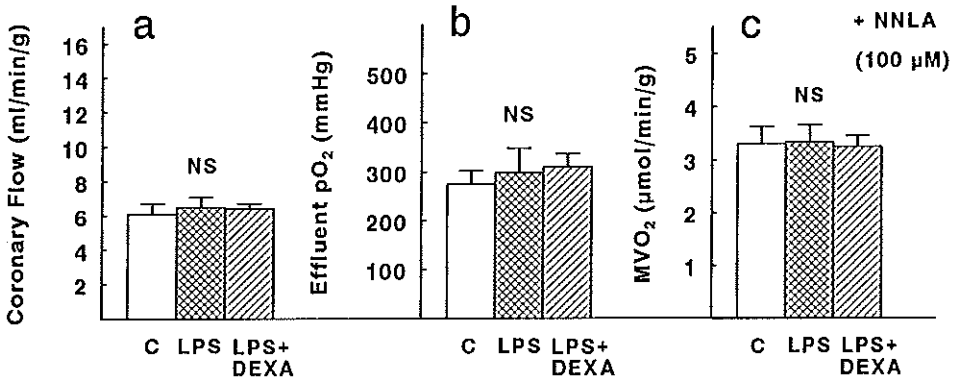

Figure 4. Effect of N^G-Nitro-L-arginine on coronary flow, effluent pO_2 and oxygen consumption (MVO_2) in control (C), endotoxic (LPS) and dexamethasone pre-treated (LPS+DEXA) rat hearts at 60 mmHg perfusion pressure. Each column represents the mean of control (n=8), endotoxin (n=8) or LPS+DEXA (n=5) hearts, vertical bars indicate S.D. Differences between groups were not significant (NS).

treated hearts following MB. However, MB produced areas of local ischemia in endotoxin hearts (Figure 6d) but not in the control group (Figure 6b). The areas of ischemia remained present in endotoxin-treated hearts at all perfusion pressures, whereas control hearts showed signs of ischemia only at 20 and 30 mmHg perfusion pressure. MB in a dose of 10 μM did not further reduce flow in control or endotoxin-treated hearts. Higher doses of MB (20 μM) caused a paradoxal vasodilatation with sustained increase in coronary flow, probably by non-specific toxic effects on cardiomyocytes.[36]

Effect of direct smooth muscle vasoconstriction with vasopressin on surface NADH fluorescence. It has been suggested that prevention of excess vasodilatation might less likely cause ischemia and poor tissue perfusion than the use of powerful vasoconstrictors.[21] We investigated whether the effect of reduction of coronary flow by a vasoconstrictor on local tissue oxygenation was different from the reduction of flow by inhibition of the NO pathway with NNLA or MB in control and endotoxin-treated hearts. We used vasopressin, a direct smooth muscle vasoconstrictor, in a concentration (~1 nM) that achieved a similar reduction in coronary flow to that of 100 μM NNLA at 60 mmHg perfusion pressure. At 60 mmHg perfusion pressure, vasopressin produced areas of local ischemia in endotoxin hearts (Figure 7d) but not in the control group (Figure 7b). With vasopressin the areas of ischemia remained

present in endotoxin-treated hearts at all perfusion pressures, whereas control hearts showed signs of ischemia only at 20 and 30 mmHg perfusion pressure.

Discussion

The myocardial circulation during septic shock is often characterised by an inappropriately high coronary flow.[14,15] Nitric oxide (NO) plays an important role in the regulation of myocardial flow.[9-13] Therefore, we investigated the role of NO in the hyperdynamic changes of coronary flow in hearts of endotoxin-treated rats. The data presented in this study indicate that these vascular changes are predominantly due to the vasodilatory action of NO. However, inhibition of the NO pathway can result in focal areas of ischemia in endotoxin-treated hearts, suggesting an imbalance of local oxygen supply to demand.

A model of endotoxemia, using a sublethal low dose of endotoxin in the rat, was selected that produced a hyperdynamic coronary circulation,[37] which is also seen in human sepsis.[14,15] Comparable to human sepsis the model resulted in raised temperature;[37] reduced blood pressure and increased levels of plasma lactate,[38] and nitrate+nitrite. The 12 hours needed to establish this model provides sufficient time for the inducible type of NO synthase to form, estimated to take a lag-phase of approximately 4 to 6 hours.[39] Pre-treatment with

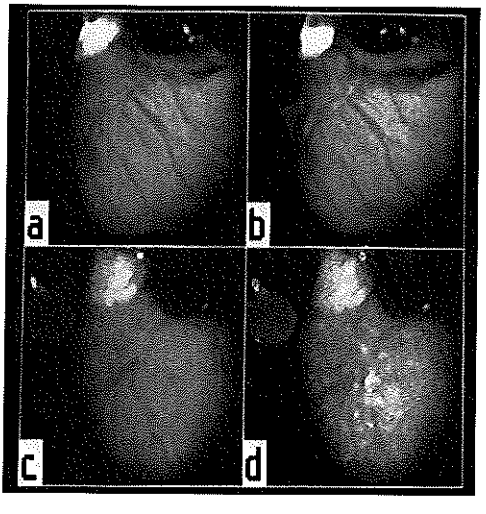

Figure 5.

Effect of inhibiting NO synthesis with N^G-Nitro-L-arginine (NNLA) on NADH fluorescence images in a control and endotoxin-treated rat heart. Before administration of NNLA both the control (A) and the endotoxic (C) heart show a homogeneous low fluorescence. Following NNLA (100 μM) the control heart (B) shows no changes in NADH fluorescence, whereas in the endotoxic heart (D) local areas of high fluorescence are visible, indicative of myocardial ischemia.

dexamethasone, an inhibitor of only the inducible type of NO synthase without affecting the constitutive enzyme,[7,8] inhibited the increase in coronary flow in this model of endotoxemia. This suggests that the inducible type of NO-synthase is to a large extent responsible for the increase in coronary flow. Dexamethasone, however, could not totally prevent the increase in coronary flow suggesting the effect of dexamethasone to be either temporary, or that part of increased NO release is produced by the increased activity of the constitutive NO synthase.[40,41] Since dexamethasone inhibits the LPS-induced synthesis and release of interleukin-1 and tumor necrosis factor alpha[42] -both cytokines stimulate the production of inducible NO synthase[39]- the inhibitory effect could also be the result of diminished release of these mediators in addition to direct inhibition of expression of the enzyme.[43]

Not only the coronary vessels but also the myocardium itself has the capacity to express the constitutive and inducible NO synthase.[39,44,45] The constitutive form of NO synthase has been shown to be present in normal rat myocardium and the inducible NO synthase is expressed following endotoxemia.[39] Isolated myocytes of the rat express the inducible NO synthase only after stimulation with cytokines which could be inhibited with dexamethasone.[39] Increased release of NO by the inducible NO synthase within the cardiac myocytes themselves has been suggested to contribute to the reduced contractility of isolated guinea pig cardiac ventricular myocytes following endotoxemia.[44] Human ventricular tissue of patients with dilated cardio-

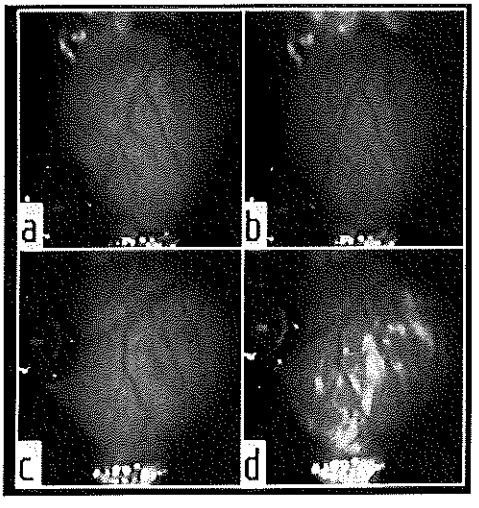

Figure 6.

Effect of methylene blue (MB), an inhibitor of soluble guanylate-cyclase, on NADH fluorescence images in a control and endotoxin-treated rat heart. Before administration of MB both the control (A) and the endotoxic (C) heart show a homogeneous low fluorescence. Following MB (5 μM) the control heart (B) shows no change in NADH fluorescence, whereas in the endotoxic heart (D) local areas of high fluorescence are visible, indicative of myocardial ischemia.

myopathy shows a significant activity of the inducible enzyme accompanied by a low activity of the constitutive NO synthase.[45] This suggests that the inducible enzyme plays a pathological role in the myocardium, whereas the constitutive enzyme plays a more physiological role. To what extent the direct expression of NO synthase within cardiac myocytes itself contributed to the vascular relaxation in our model of endotoxemia remains to be established.

Our data indicate that reduction of excess coronary flow in endotoxemia, due to inhibition of the NO pathway with NNLA or MB, can cause local myocardial ischemia. In a recent study by Duncker et al.[46] using radioactive microspheres, NNLA was shown to exacerbate myocardial hypoperfusion during exercise in the presence of a coronary stenosis in awake dogs. Administration of L-N^G-Monomethyl-Arginine (L-NMMA), another inhibitor of NO synthesis, has been reported to exacerbate global myocardial ischemia *in vivo* in a rabbit model of endotoxemia as indicated by changes on the electrocardiogram.[24] In our study, foci of ischemia are indicated by areas of enhanced NADH fluorescence. This inhomogeneity of NADH fluorescence may be explained by a heterogeneously distributed blood flow throughout the heart,[47,48] leading to an uneven distribution of ischemic areas in the myocardium during low coronary perfusion pressure or coronary flow restriction.[49] This study shows that heterogeneously distributed ischemia also exists following flow restriction by inhibition of the NO pathway in endotoxin-treated rat hearts but not in normal hearts. Since histologic examination

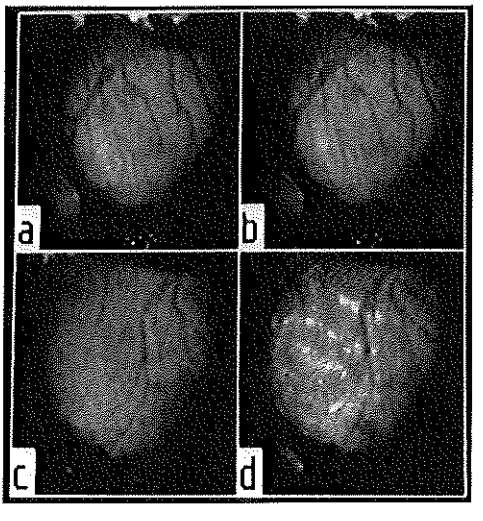

Figure 7.

Effect of direct smooth muscle vasoconstriction with vasopressin on NADH fluorescence images in a control and endotoxin-treated rat heart. Before vasopressin both the control (A) and the endotoxic (C) heart show a homogeneous low fluorescence. Following smooth muscle vasoconstriction with vasopressin (~1 nM), the control heart (B) shows no changes in NADH fluorescence, whereas in the endotoxin heart (D) local areas of high fluorescence exist, indicative of myocardial ischemia.

revealed no differences between groups and since overall parameters of coronary flow, effluent pO_2 and oxygen consumption in endotoxin-treated hearts were not significantly different from control hearts following NNLA, the mismatch in local oxygen balance may reflect changes in local myocardial blood flow. The size of the ischemic areas, as revealed by NADH videofluorimetry, are approximately the same size as those seen during near hypoxia in normal hearts. We have recently shown, using NADH videofluorimetry, that such areas represent microcirculatory units at capillary level that are consistently vulnerable to ischemia in the isolated rat heart.[30] Our results suggest that endotoxemia promotes ischemia in these vulnerable areas.

Maldistribution of heterogeneous coronary blood flow has been demonstrated during endotoxin shock *in vivo* and may play a role in the development of depressed cardiac function.[50,51] This idea is supported by the results of the present study. There could be several explanations for the appearance of ischemic foci during inhibition of the NO pathway. Regional maldistribution of coronary flow leading to under-perfusion of myocardial tissue could have caused a local decrease in oxygen supply in endotoxin-treated hearts. No signs of ischemia, either local or global, were present in the isolated endotoxin-treated rat hearts when the NO pathway was not inhibited. This might indicate that maldistribution of coronary blood flow did not exist prior to flow limitation, or that maldistribution of blood flow was compensated by increased overall coronary flow. Reducing coronary flow by inhibiting NO metabolism or direct smooth muscle vasoconstriction could have unmasked relatively under-perfused (low-flow) areas in the endotoxin-treated hearts by causing myocardial ischemia. These ischemic areas probably represent the vulnerable units in the capillary network of the rat myocardium which are the first to be compromised when oxygen supply becomes limited.[30] An alternative explanation may be that oxygen demand is higher whilst distribution of flow remains the same. However, our results showed equal oxygen consumption for both groups following inhibition of NO synthesis. Increasing coronary flow by restoring NO synthesis with L-arginine or providing exogenous NO with the NO-donor nitroprusside restored perfusion in the ischemic areas in both cases. These findings support the idea that NO plays a role in maintaining organ perfusion.[24,26]

In general it is thought that the constitutive NO synthase plays a role in maintaining

organ perfusion, whereas the expression of the inducible form (as in sepsis and endotoxemia) leads to the production of excessive amounts of NO resulting in vascular relaxation and tissue damage.[24] The therapeutic use of NO inhibitors during sepsis should, therefore, be directed to inhibition of the inducible NO synthase. In this study we used inhibitors that affect both the constitutive and the inducible NO synthase. The inhibition of only the inducible NO synthase might have prevented myocardial ischemia in endotoxin-treated hearts. However, to our knowledge, no selective inhibitors of only the inducible NO synthase are currently available. To prevent tissue hypoperfusion, total inhibition of NO synthesis combined with an exogenous NO donor could be helpful.[24] In our study the exogenous NO donor sodium nitroprusside restored myocardial hypoperfusion when endogenous NO synthesis was totally inhibited with NNLA. Development of selective inhibitors of inducible NO synthase must be awaited; such selective inhibitors may be of special value in the future management of septic patients. For now, we suggest that the therapeutic use of non-selective NO inhibitors during sepsis must be done with caution and under careful cardiac monitoring.

The present study supports the notion that the massive coronary vasodilation seen in the isolated endotoxin-treated rat heart is caused by an increased release of NO. Inhibition of NO metabolism with NNLA or MB following endotoxemia reduced the coronary flow and caused areas of myocardial ischemia. Reducing coronary flow by direct vasoconstriction resulted in similar areas of myocardial ischemia, as did inhibition of the NO pathway. This finding suggests that the local balance between oxygen supply and oxygen demand in endotoxin-treated hearts is disturbed following flow restriction. Inhibition of NO synthesis during sepsis and endotoxemia has been reported to be accompanied by reductions in cardiac output[21-25,27] and increased mortality.[24-26] It could be that localized myocardial ischemia reported in this study may have contributed to these deleterious effects. Conversely, it could be that the induction of NO synthase in the myocardium during endotoxemia may be a protective mechanism that prevents the development of local ischemia of malperfused cardiac tissue and that inhibition of NO synthesis could act counter-productive.

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Chapter 3

Nitric Oxide Causes Dysfunction of Coronary Autoregulation in Endotoxemic Rats

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Abstract

This study tested the hypothesis that overproduction of endogenous nitric oxide (NO) during endotoxemia may modulate coronary autoregulation and myocardial reactive hyperemia. Hearts of endotoxin-pretreated rats and controls were isolated and arranged for perfusion in a Langendorff preparation. Autoregulation was studied by examining flow-pressure relations during stepwise changes in perfusion pressure. The contribution of nitric oxide was examined by pefusion with N^G-nitro-L-arginine (NNLA), an inhibitor of nitric oxide synthesis and methylene blue (MB), an inhibitor of soluble guanylate-cyclase.

Endotoxin-treated hearts showed massive coronary vasodilatation and autoregulatory function was impaired at perfusion pressures from 20 to 60 mmHg. Both NNLA and MB reduced coronary flow, improved autoregulation and eliminated differences in coronary flow and autoregulation between the control and endotoxin-treated group. Vasoconstriction with vasopressin, a direct smooth muscle constrictor, could not eliminate differences in autoregulation between groups. Reactive hyperemia following coronary occlusion in endotoxin-treated hearts showed decreased duration, flow repayment and repayment ratio. In the presence of NNLA or MB, however, no significant differences in reactive hyperemic flow patterns were present.

These observations suggest that massive coronary vasodilatation due to increased myocardial NO synthesis can result in autoregulatory dysfunction and altered myocardial reactive hyperemia during endotoxemia.

Introduction

Coronary autoregulation is the ability of the coronary vasculature to maintain blood flow relatively constant during changes in perfusion pressure.[1,2] Among the physiological mechanisms that are known to influence autoregulation are metabolic factors (i.e., oxygen tension and/or tissue metabolites) and the so-called myogenic response, which mediates constriction in response to increased transmural pressure.[3] By increasing vascular tone in response to increasing perfusion pressure, the myogenic autoregulatory response may be an important mechanism in keeping capillary hydrostatic pressure relatively constant and preventing excess flow to the microcirculation.[4,5]

Nitric oxide is a major endothelium derived relaxing factor (EDRF), formed from the precursor amino acid L-arginine and stimulates the enzyme soluble guanylate-cyclase (GC) in the smooth muscle cell which converts GTP to cGMP resulting in vascular relaxation.[6] In the coronary vessels NO plays an important role in the regulation of vascular tone and myocardial blood flow both *in vivo* and in the isolated heart preparation.[7,8] The increased release of NO is suggested to be responsible for the vascular relaxation and hypotension seen in states of sepsis and endotoxemia.[6] Recently it was found that endothelium derived NO can counteract coronary autoregulation by opposing myogenic tone and that inhibition of NO synthesis results in improved coronary autoregulation in the isolated guinea pig heart and rabbit heart.[9,10] Furthermore NO plays a role in myocardial reactive hyperemia,[11,12] i.e. the increase in flow following a period of coronary arterial occlusion.

During sepsis and endotoxemia the coronary circulation is often characterized by inappropriately high coronary flow rates secondary to coronary vasodilatation with uncoupling of flow from metabolic demand.[13,14] This massive coronary dilatation may depend on accelerated cardiac production of NO.[15,16] We recently showed that inhibition of this increased NO synthesis can result in focal myocardial ischemia.[17] Since the basal release of NO can attenuate coronary autoregulation,[9,10] it is feasible to hypothesize that increased synthesis of NO, as in sepsis and endotoxemia, can interfere with the physiological process of flow regulation leading to dysfunction of coronary autoregulation and altered reactive hyperemic response.

In this study, we investigated coronary myogenic autoregulatory function in response to step-wise changes in perfusion pressure and myocardial reactive hyperemia after a stop of coronary flow in isolated hearts of normal and endotoxin-treated rats. To investigate the role of NO, we used the L-arginine analogue N^G-nitro-L-arginine (NNLA),[18] to inhibit NO synthesis and methylene blue (MB),[19] to inhibit soluble guanylate-cyclase. To see if the effect of direct vasoconstriction was different from the effect of inhibition of NO synthesis, we used vasopressin, a direct smooth muscle constrictor that acts independent of the L-arginine/NO/cGMP pathway.[20]

Materials and methods

Experimental set-up This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The protocol was approved by the animal ethics board of our university. Male Wistar rats (300-360 g) received either a sublethal dose of 0.7 mg/kg of body wt *Escheria Coli* endotoxin (LPS, 0127:B8, Sigma Chemical Co) or saline by intraperitoneal injection 12 hours before the initiation of the experiments. *In vivo* measurements of hemodynamic parameters and plasma metabolites have been previously presented.[17] Endotoxin-treated animals showed elevated rectal temperature, decreased mean arterial blood pressure, increased heart rate, and increased plasma levels of lactate. Serum nitrite+nitrate ($NO_2^-+NO_3^-$), the stable end products of NO production, were elevated in endotoxin-treated animals as a sign of increased systemic release of NO. Rats were anesthetized with ether, heparinized (0.5 mL, 100 IU), the hearts were removed, immediately cooled in ice-cold perfusion medium and rapidly arranged for constant pressure perfusion at 37°C according to Langendorff. The perfusion medium consisted of (mmol/L) NaCl 128, KCl 4.7, $MgCl_2$ 1, NaH_2PO_4 0.4, $NaHCO_3$ 20.3, $CaCl_2$ 1.3, glucose 5.0, pyruvate 2.0, and was oxygenated with 95%O_2/5%CO_2. All hearts were continuously paced at 5 Hz. To minimize the metabolic component of coronary autoregulation, the left ventricle was drained by a cannula in the apex so that no external work was performed. Flow rates (expressed as milliliters per minute per gram of ventricular wet weight) were measured by an electromagnetic flow probe (Skalar-Medical) in the aortic perfusion line. For the determination

of effluent oxygen pressure the right pulmonary artery was cannulated and a constant fraction of the coronary effluent was led along a Clark-type electrode (YSI Biological Oxygen Monitor, model 5300, Yellow Springs Instruments). The coronary influent was similarly monitored for oxygen pressure via a side arm of the aortic cannula. Oxygen consumption (in micromoles per minute per gram of ventricular wet weight) was calculated from the product of influent-effluent oxygen concentration difference and coronary flow.

Autoregulatory function protocol Autoregulatory function was investigated by studying adjustments in coronary flow in response to step-wise changes in perfusion pressure.[21] Following stabilization at 60 mmHg perfusion pressure, perfusion pressure was first lowered to 20 mmHg and subsequently increased by step-wise changes in perfusion pressure of 10 mmHg at 3-minute intervals to a maximum of 100 mmHg. At the end of this series of pressure changes, perfusion pressure was set at the initial value of 60 mmHg. When the flow was stable, the perfusion medium was switched to one containing 100 μmol/L NNLA or 5 μmol/L MB. After a 30-minute interval to reach a new steady state, the step-wise changes in perfusion pressure were repeated. Hearts were perfused for a total of 130 minutes. MB was used in a concentration of 5 μmol/L since higher concentrations ($>$20 μmol/L) caused a paradoxical increase in coronary flow in control hearts, probably by non-specific toxic effects.[22]

In five hearts of the control and endotoxin-treated group, a supramaximum dose of nitroprusside (10 μmol/L), a nitrovasodilator that acts as an exogenous NO donor, was tested to obtain maximum vasodilatation. To see if the effect of direct vasoconstriction was different from the effect of inhibiting the release of the endogenous vasodilator NO, we repeated the protocol of pressure steps switching to vasopressin, a direct smooth muscle constrictor that acts independent of the L-arginine/NO/cGMP pathway,[20] in a concentration of 1 nmol/L that caused approximately the same reduction in flow as NNLA did in four hearts of the control and endotoxin-treated group. In four separate hearts of the control group the effect of increasing flow similar to endotoxin-treated hearts with nitroprusside (0.1 μmol/L) was tested. NNLA, MB, vasopressin and nitroprusside were diluted in the perfusate in their final concentration.

Hyperemic flow response Myocardial reactive hyperemia is the increase in coronary flow following a period of coronary artery occlusion.[23,24] Reactive hyperemic flow response

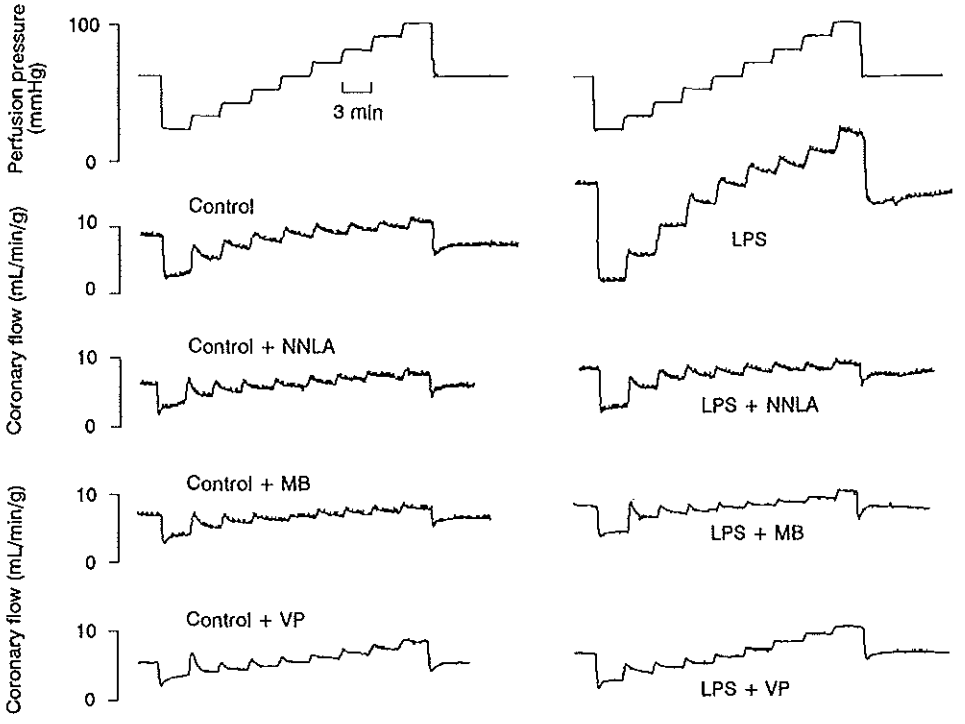

Figure 1. Representative tracings of coronary flow (four lower tracings) in response to step-wise changes in perfusion pressure (upper tracings) of an isolated control heart and endotoxin-treated (LPS) heart before and after inhibition of the L-arginine/NO/cGMP pathway with 100 μmol/L N^G-nitro-L-arginine (NNLA) or 5 μmol/L methylene blue (MB) and direct vasoconstriction with 1 nmol/L vasopressin (VP). Note that endotoxin-treated hearts showed increased pressure induced flow changes as compared to control which could be prevented by treatment with NNLA or MB, whereas vasopressin was less effective.

was investigated after a stop of coronary flow for 90 seconds in hearts of control and endotoxin-treated animals (n=8 in both groups). To study the contribution of NO in reactive hyperemic response, the flow stop was repeated after the perfusion medium was switched to one containing 100 μmol/L NNLA or 5 μmol/L MB in four hearts each. In four separate hearts of the control group the effect of increasing flow similar to endotoxin-treated hearts on reactive hyperemic flow response was tested with nitroprusside (0.1 μmol/L). In four separate hearts of the endotoxin-treated group the effect of reducing flow similar to NNLA with vasopressin was tested.

Definitions and calculations Autoregulatory function was quantified by an index (ArI) first described by Norris et al.[21] which compares the observed change in vascular conductance for a given change in pressure in relation to the calculated change in conductance assuming that flow remained constant: ArI = 1 - $(\Delta F/F_i) / (\Delta P/P_i)$, where F is flow at pressure P, F_i and Pi are initial flow and pressure, respectively, $\Delta F = F_i - F$ and $\Delta P = P_i - P$. If conductance is unchanged or increases when pressure is increased (passive vascular bed), then ArI ≤ 0 and there is no autoregulation. If conductance decreases when pressure increases, then ArI > 0 with a maximum value for ArI of 1.0, where coronary flow remains constant irrespective of pressure changes indicating perfect autoregulation.[1] Quantitative analyses of features characterizing the myocardial reactive hyperemic flow response were performed according to the criteria described by Coffman and Gregg[23] where flow debt (mL) = control flow rate (mL/s) x occlusion time (s); Flow repayment (mL) = total flow volume during reactive hyperemia (mL) - control flow rate (mL/s) x duration of reactive hyperemia (s); Flow repayment ratio = flow repayment / flow debt.

Statistics All values are expressed as mean ± SEM. Student's t-test for group comparisons or paired t-test where appropriate were used to statistically compare values. Linear regression analysis was performed to determine the relation between myocardial oxygen consumption and coronary flow. $p < 0.05$ was taken as statistically significant. NS is used to denote not significant.

Chemicals NNLA, MB, vasopressin and nitroprusside were obtained from Sigma. All the other chemicals were obtained from Merck.

Results

Effect of endotoxemia on coronary flow, oxygen consumption and autoregulatory function at varying perfusion pressure. Figure 1 shows representative tracings of coronary flow responses to step-wise changes in perfusion pressure. During the different perfusion pressures ranging from 20 to 100 mmHg, coronary flow (Figure 2a) and oxygen consumption were significantly raised in hearts from endotoxin-treated animals at all perfusion pressures

studied. At 60 mmHg perfusion pressure coronary flow was raised 58±3% (from 10.5±0.2 to 16.6±0.5 mL/min per gram wet weight) and oxygen consumption 23±3% (from 4.0±0.1 to 4.9±0.1 μmol/min per gram wet weight) in hearts from endotoxin-treated animals (n=16) as compared to control (n=15). As shown in Figure 3 there was a linear relation between oxygen consumption and coronary flow in control ($Y=0.130X+2.58$, $R^2=0.76$, $p<0.001$) and endotoxin-treated hearts ($Y=0.125X+2.74$, $R^2=0.78$, $p<0.001$). The endotoxin-treated hearts showed significantly reduced autoregulatory function at perfusion pressures ranging

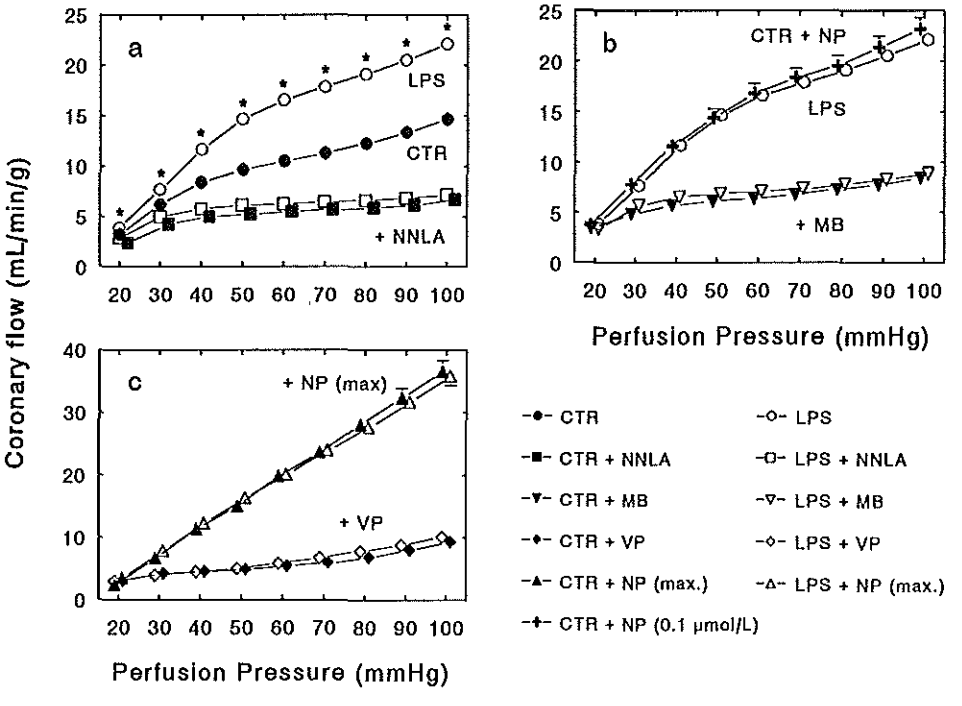

Figure 2. Line graphs showing effect of perfusion pressure on coronary flow in control and endotoxin-treated hearts with and without inhibition of the L-arginine/NO/cGMP pathway. (a) Coronary flow-pressure relations in (n=15) control (CTR) (-●-) and (n=16) endotoxin-treated (LPS) (-○-) hearts. Coronary flow following inhibition of NO-synthesis with 100 μmol/L N^G-nitro-L-arginine (NNLA) in (n=8) control (-■-) and (n=8) endotoxin-treated (-□-) hearts. (b) Coronary flow following 5 μmol/L methylene blue (MB), an inhibitor of soluble guanylate-cyclase, in (n=4) control (-▼-) and (n=4) endotoxin-treated (-▽-) hearts and coronary flow in (n=4) untreated hearts with 0.1 μmol/L nitroprusside (NP) (-+-). (c) Coronary flow in a "passive" vascular bed following maximum vasodilatation with 10 μmol/L nitroprusside (NP) in (n=5) control (-▲-) and (n=5) endotoxin-treated (-△-) hearts and with the direct smooth muscle constrictor vasopressin (VP) 1 nmol/L in (n=4) control (-◆-) and (n=4) endotoxin-treated (-◇-) hearts. Values are mean±SEM. *$p<0.05$ for LPS *v.* control.

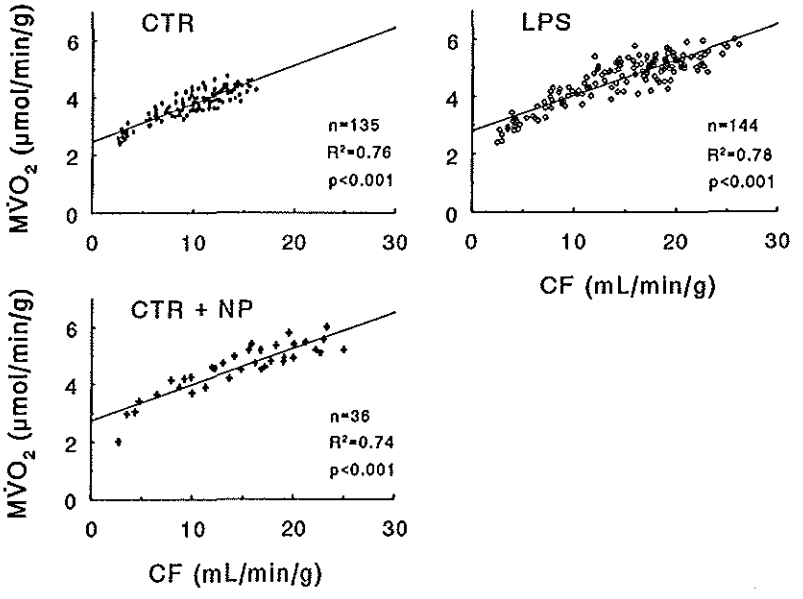

Figure 3. Scatterplot showing the relation of coronary flow and oxygen consumption in control hearts (CTR, -●-), endotoxin-treated hearts (LPS, -O-), and normal hearts with 0.1 µmol/L nitroprusside (CTR+NP, -+-). Each point represents a single measurement at a given perfusion pressure ranging from 20 to 100 mmHg (nine measuments per separate heart). In all three groups there was a linear relation between coronary flow and oxygen consumption (all $p<0.001$). Regression lines were not significantly different between groups with regard to slope or intercept.

from 20 to 60 mmHg (Figure 4a). At perfusion pressures of 70 mmHg and higher autoregulatory function was not significantly different from control hearts. Attaining maximum coronary flow with a supra-maximum dose of nitroprusside (10 µmol/L) caused a rise of coronary flow of 9.4±0.5 mL/min per gram (89%) in control hearts (n=5) and 3.5±0.7 mL/min per gram (21%) in endotoxin-treated hearts (n=5), which resulted in similar flow rates (Figure 2c) and absence of autoregulation (ARI<0 at all perfusion pressures studied) in control and endotoxin-treated hearts.

Effect of nitroprusside (0.1 µmol/L) on coronary flow, oxygen consumption and autoregulatory function of control hearts. The effect of increasing flow in normal hearts similar to the flow level of endotoxin-treated hearts at 60 mmHg was tested using nitroprusside (0.1 µmol/L) (n=4). Normal hearts with 0.1 µmol/L nitroprusside had increased coronary flows (Figure 2b). Oxygen consumption increased parallel to the increase in flow (4.8±0.1

μmol/min per gram wet weight at 60 mmHg) with a linear relation between oxygen consumption and coronary flow ($Y=0.127X+2.71$, $R^2=0.74$, $p<0.001$) (Figure 3). Regression lines were not significantly different between goups with regard to slope and intercept (Figure 3). Normal hearts with nitroprusside (0.1 μmol/L) showed decreased ArI at perfusion pressures from 20 to 60 mmHg ($p<0.05$ compared to control) similar to endotoxin-treated hearts (Figure 4b).

Effect of NNLA, MB and vasopressin on coronary flow and autoregulation. Switching to perfusion medium containing 100 μmol/L NNLA reduced coronary flow at all perfusion pressures to similar levels in control and endotoxin-treated hearts (Figure 1 and 2a). Parallel to the reduction in flow there was a reduction in oxygen consumption (per gram wet weight) to 3.1 ± 0.1 μmol/min in control hearts and 3.2 ± 0.1 μmol/min in endotoxin-treated hearts at 60 mmHg perfusion pressure, which is not significantly different between groups. With NNLA autoregulation index was increased at all perfusion pressures in both control and endotoxin-treated hearts ($p<0.05$) to similar levels in both groups (Figure 4c) resulting in a plateau of considerable autoregulation at perfusion pressures above 40 mmHg

Inhibition of soluble guanylate-cyclase with 5 μmol/L MB reduced coronary flow at all perfusion pressures to a similar level in both groups (Figure 1 and 2b). Autoregulation index with MB (Figure 4d) was increased at perfusion pressures from 20 to 40 mmHg and above 70 mmHg in control hearts ($p<0.05$) and at all perfusion pressures in endotoxin-treated hearts ($p<0.05$) to similar levels at all perfusion pressures. ArI following MB compared to before MB was not increased at 50 and 60 mmHg perfusion pressure ($p=$NS).

Direct smooth muscle constriction with vasopressin in a concentration of 1 nmol/L that reduced flow to a similar level as NNLA at 60 mmHg perfusion pressure (figure 1 and 2c) increased ArI at 20 mmHg perfusion pressure and decreased ArI at perfusion pressures above 40 mmHg in control and endotoxin-treated hearts. Vasopressin could not eliminate differences in ArI between control and endotoxin-treated hearts in the pressure range between 30 and 70 mmHg ($p<0.05$ of LPS versus control) (Figure 4e). In all hearts coronary flow returned to the baseline value, when at the end of the series of pressure step changes, perfusion pressure was set at the initial value of 60 mmHg.

Effect of endotoxemia on reactive hyperemic flow response. Endotoxemia resulted in

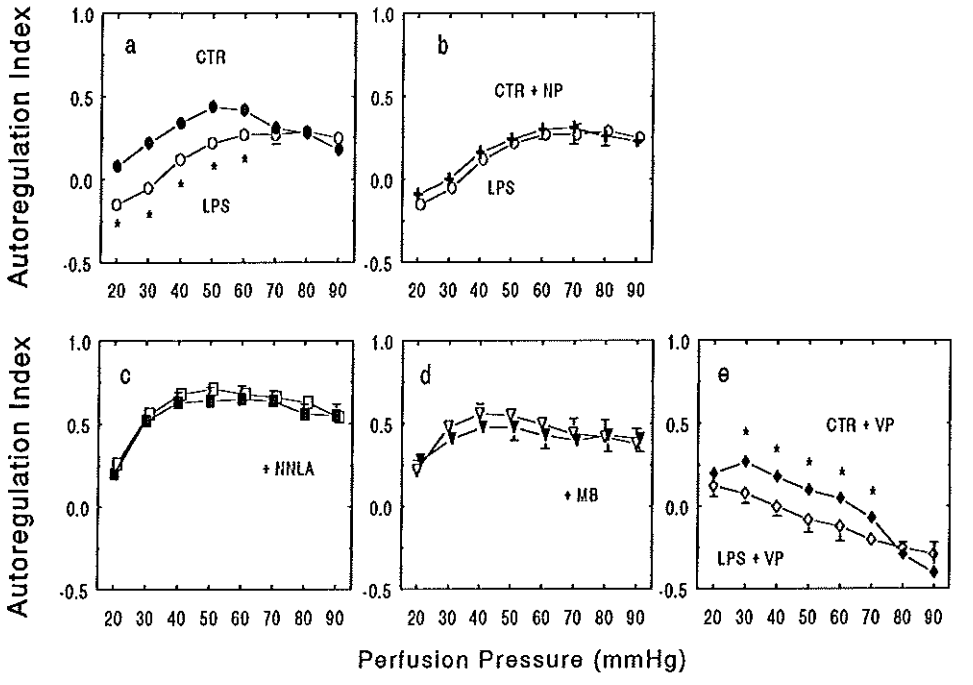

Figure 4. Line graphs showing the effect of perfusion pressure on coronary autoregulation. "Autoregulation index" was calculated from the data in the pressure-flow relations of control and endotoxin-treated hearts before and after inhibition of the L-arginine/NO/cGMP pathway. (a) Autoregulation index in control (CTR) (-●-) and endotoxin-treated (LPS) (-○-) hearts. (b) Autoregulation index in untreated hearts with 0.1 μmol/L nitroprusside (NP) (-+-). (c) Autoregulation index with 100 μmol/L N^G-nitro-L-arginine (NNLA) in control (-■-) and endotoxin-treated (-□-) hearts. (d) Autoregulation index with 5 μmol/L methylene blue (MB) in control (-▾-) and endotoxin-treated (-▿-) hearts. (e) Autoregulation index with 1 nmol/L vasopressin (VP) in control (-♦-) and endotoxin-treated (-◇-) hearts. Values are mean±SEM. *$p<0.05$ for LPS *v.* control.

a changed pattern of reactive hyperemia (Figure 5a). As displayed in Table 1, endotoxemia resulted in 59±7% increased basal flow and flow debt, 66±7% decreased duration of reactive hyperemia, 65±7% decreased flow repayment and 80±6% decreased repayment ratio (all $p<0.01$ compared to control). Maximum hyperemic flow was not significantly different from control hearts (Table 1). The changed pattern of reactive hyperemia could be mimicked in untreated hearts by increasing flow equally to endotoxin-treated hearts with 0.1 μmol/L nitroprusside (Figure 5d). Quantitative analyses of reactive hyperemic response showed no significant differences of untreated hearts with 0.1 μmol/L nitroprusside versus endotoxin treated hearts (p=NS, Table 1).

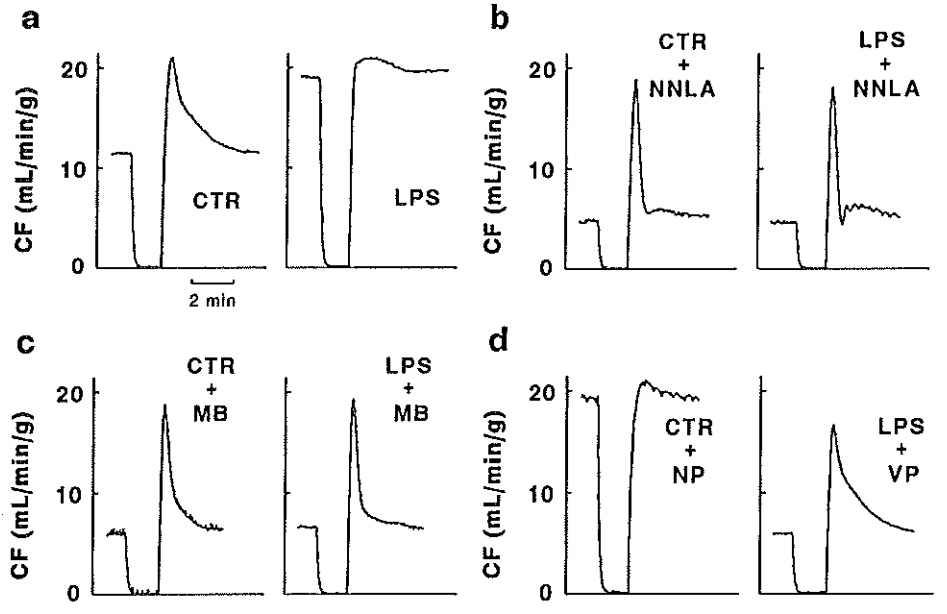

Figure 5. Representative tracings of myocardial reactive hyperemic response in control and endotoxin-treated hearts following a 90 seconds occlusion period and effect of inhibition of the L-arginine/NO/cGMP pathway. (a) Reactive hyperemic response in control (CTR) and endotoxin-treated (LPS) heart. (b) Reactive hyperemic response in control and endotoxin-treated heart with 100 μmol/L N^G-nitro-L-arginine (NNLA). (c) Reactive hyperemic response in control and endotoxin-treated heart with 5 μmol/L methylene blue (MB). (d) Reactive hyperemic response in normal heart with 0.1 μmol/L nitroprusside (CTR+NP) and endotoxin-treated heart with 1 nmol/L vasopressin (LPS+VP). For quantitative analyses of hyperemic responses see Table 1.

Effect of NNLA, MB and vasopressin on reactive hyperemic response. Perfusion of the hearts with 100 μmol/L NNLA or 5 μmol/L MB resulted in similar reactive hyperemic flow patterns of control and endotoxin-treated hearts (Figure 5b and 5c). In the presence of NNLA or MB reductions in basal flows, flow debt and maximum hyperemic flow were seen in control and endotoxin treated animals ($p<0.05$, Table 1). NNLA reduced the duration of reactive hyperemia and flow repayment in control hearts and increased flow repayment in control and endotoxin-treated hearts ($p<0.05$, Table 1). NNLA did not change the duration of reactive hyperemia in endotoxin-treated hearts (p=NS, Table 1). MB reduced the duration of reactive hyperemia in control hearts but increased the duration of reactive hyperemia in endotoxin-treated hearts ($p<0.05$, Table 1). MB increased flow repayment in endotoxin-treated hearts ($p<0.05$) but did not change flow repayment in control hearts (p=NS, Table 1). MB resulted

in increased flow repayment ratio in both control and endotoxin hearts ($p < 0.05$, Table 1).

In the presence of NNLA or MB no significant differences in basal flow, flow debt, maximal reactive flow, duration of hyperemia, flow repayment and repayment ratio were present between control and endotoxin-treated hearts (p=NS, Table 1). In endotoxin-treated hearts, the presence of 1 nmol/L vasopressin caused a similar reduction in basal flow, flow debt and maximal reactive flow as was seen with NNLA (p=NS of NNLA versus vasopressin, Table 1) and increased duration of reactive hyperemic flow, flow repayment and repayment ratio ($p < 0.05$ of NNLA versus vasopressin, Table 1).

Discussion

The main finding of this study is that the excessive myocardial production of NO in isolated hearts from endotoxemic rats leads to massive coronary vasodilatation that results in dysfunction of coronary autoregulation and attenuation of myocardial reactive hyperemia. Reduction of coronary flow by inhibition of the L-arginine/NO/cGMP pathway with NNLA or MB could restore this autoregulatory dysfunction, whereas direct smooth muscle constriction with vasopressin could not. These findings may bring some new insights into the pathophysiological processes leading to cardiovascular dysfunction in sepsis and septic shock.

Coronary flow autoregulation has been extensively studied both *in vivo* and *in vitro* in the isolated vessel preparation and isolated perfused heart preparation.[1,3] The present study used an isolated perfused heart preparation. In this preparation, which does no external work, oxygen demand is low so that perfusion at low pressures is tolerated without causing severe ischemia. Furthermore oxygen consumption is kept as constant as possible minimizing variations in flow through metabolic coronary vasodilatation. Compared to the isolated vessel preparation this whole organ preparation has the advantage that it contains all consecutive vascular segments including larger coronary arteries and small arterioles, since autoregulation of blood flow can be spatially organized.[25,26] However, some reservations must be made to extrapolate the findings of the present study to the *in vivo* situation. The isolated non-working heart preparation that was used misses the neurohumoral and metabolic input which are known to influence autoregulation *in vivo*.[1] Furthermore, changes in NO production may directly in-

Table 1. Basal flow and parameters for reactive hyperemia following 90 seconds of coronary occlusion in control and endotoxin-treated (LPS) hearts before and after N^G-nitro-L-arginine (NNLA), methylene blue (MB), nitroprusside (NP) and vasopressin (VP).

	before		+ NNLA		+ MB		+ NP	+ VP
	control (n=8)	LPS (n=8)	control (n=4)	LPS (n=4)	control (n=4)	LPS (n=4)	control (n=4)	LPS (n=4)
Basal flow (mL/min/g)	11.5±0.2	18.3±0.4*	4.8±0.2	5.0±0.4	6.0±0.3	6.8±0.5	18.5±0.1	5.3±0.3
Flow debt (mL/g)	17.2±0.3	27.5±0.5*	7.2±0.2	7.5±0.5	8.9±0.5	10.2±0.5	27.8±0.2	7.9±0.6
Maximal flow (mL/min/g)	20.7±0.4	20.1±0.4	17.5±0.6	17.3±0.4	18.8±0.2	19.1±0.3	20.5±0.7	17.6±0.4
Duration (s)	119±10	40±4*	46±3	50±3	77±4	75±10	29±4	117±12
Flow repayment (mL/g)	11.0±0.8	3.8±0.7*	7.4±0.4	8.4±0.6	10.3±0.9	11.1±2.0	3.3±1.0	10.3±0.6
Repayment ratio†	0.64±0.05	0.14±0.03*	1.04±0.04	1.13±0.08	1.14±0.07	1.07±0.18	0.12±0.04	1.32±0.06

Values are means ± SEM. *$p<0.05$ of LPS *v.* control. †Repayment ratio = flow repayment/flow debt.

fluence myocardial contractility,[27] which was not measured in the preparation used. Therefore we cannot rule out that any changes in autoregulation that have been found during isolated perfusion may have been compensated for in the *in vivo* situation.

A hyperdynamic model of endotoxemia was used that showed increased coronary flow together with increased myocardial oxygen consumption. Although the origin of this high oxygen consumption in hearts of endotoxin-treated animals is still speculative, the increases in oxygen consumption could have directly resulted from increases in coronary flow, the so-called Gregg phenomena.[28,29] This is supported by the finding that isolated hearts showed a linear increase in oxygen consumption with increases in coronary flow and increased oxygen consumption following addition of the vasodilator nitroprusside (Figure 3). Parallel with these observations, reduction of coronary flow by NNLA resulted in decreased oxygen consumption. Similar findings were done by Pohl et al.[9] in the isolated rabbit heart where NNLA reduced oxygen consumption during constant pressure perfusion but was without effect during constant flow perfusion excluding a direct inhibitory effect of NNLA on myocardial metabolism.

Coronary autoregulation depends on the balance between competing constricting and dilating influences.[1] Autoregulatory dysfunction in endotoxin-treated hearts could therefore have resulted from defective vasoconstrictive mechanisms or an overshoot of vasodilator mechanisms. The myogenic response mediates vasoconstriction in response to increased transmural pressure and is considered a major constricting force in the coronary vessels.[3] Myogenic dilation following a period of occlusion and myogenic constriction after restoration of flow is one of the mechanisms contributing to reactive hyperemia in the coronary vessels.[24] The mechanism responsible for the myogenic response is still incompletely understood but is probably located in the vascular smooth muscle cells itself, and mediated by an increase in intracellular calcium through influx via either stretch-activated or voltage gated ion-channels.[30,31] Endotoxin could have induced defects in the myogenic response that may have resulted in a loss of vascular tone, autoregulatory dysfunction and altered reactive hyperemia. However, inhibition of the L-arginine/NO/cGMP pathway with NNLA or MB reduced basal coronary flow and prevented pressure induced increases in coronary flow of endotoxin-treated hearts resulting in similar levels of autoregulation as in control hearts with NNLA or MB whereas with vasopressin, a direct smooth muscle constrictor, differences in autoregulation

between control and endotoxin-treated hearts remained. Furthermore in endotoxin-treated hearts in the presence of NNLA or MB there was only a brief reactive hyperemic response following occlusion after which coronary flow returned to baseline levels. These results suggest that myogenic tone was well preserved following inhibition of the L-arginine/NO/cGMP pathway. An overshoot in NO mediated vasodilating influences thus counteracting myogenic vascular tone is more likely to have caused the autoregulatory dysfunction and altered reactive hyperemic response in endotoxin-treated hearts. This view is further supported by the present finding that vasodilation with the exogenous NO donor nitroprusside in control hearts resulted in similar autoregulatory dysfunction and altered reactive hyperemic response as in endotoxin-treated hearts.

Earlier studies have shown that under normal (i.e., non-septic or non-endotoxemic) conditions the endogenous production of NO can attenuate autoregulation by opposing myogenic tone in the isolated perfused rabbit ear,[32] guinea pig heart[10] and rabbit heart,[9] and in the canine heart *in vivo*.[33] The present study is the first that shows that autoregulatory dysfunction may exist in endotoxemia resulting from excessive production of NO. However, NO is not the only regulator of vascular tone. Other vasoactive factors, such as adenosine, prostaglandins, pO_2 and pCO_2, are known to influence vascular tone.[1,34] To what extent one or more of these metabolites may have contributed to vasodilatation and derangements in autoregulation and reactive hyperemia in the endotoxin-treated hearts is unknown.

Under physiological conditions NO plays an important role in myocardial reactive hyperemia.[11,12] In the present study inhibition of the L-arginine/NO/cGMP pathway in normal hearts resulted in decreased peak reactive flow, decreased duration of the hyperemic response and decreased total reactive hyperemic blood flow which demonstrates the contribution of NO to myocardial reactive hyperemia. Although endotoxin-treated hearts showed a pattern of reactive hyperemia (Figure 5), flow repayment was only one third of that of control hearts. Vasoconstriction through inhibition of the L-arginine/NO/cGMP pathway with NNLA or MB could improve flow repayment in endotoxin-treated hearts and resulted in similar reactive hyperemic flow patterns as in control hearts with NNLA or MB. However, also vasopressin, a direct smooth muscle vasoconstrictor, could improve flow repayment and resulted in a 'normal' reactive hyperemia pattern. These results suggest that an excessive coronary flow

secondary to endogenous NO production minimized flow reserve and blunted the reactive hyperemic response in endotoxin-treated hearts.

In conclusion, the massive coronary vasodilatation due to increased myocardial production of NO during sepsis and endotoxemia may result in dysfunction of coronary autoregulation. Increasing vascular tone by inhibition of the L-arginine/NO/cGMP pathway with NNLA or MB can restore these changes, unlike direct smooth muscle constriction with vasopressin. These findings may help us understand some of the pathophysiological processes that lead to cardiovascular dysfunction during sepsis and endotoxemia. Improvement of vascular autoregulation may be one of the mechanisms by which inhibitors of the L-arginine/NO/cGMP pathway contribute to correction of the cardiovascular derangements during human septic shock.[35]

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Chapter 4

Prolonged inhibition of nitric oxide synthesis in severe septic shock: A clinical study

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Abstract

Inhibitors of nitric oxide synthesis have suggested to be of value in the treatment of hypotension during sepsis. However, earlier clinical reports only describe initial effects of these nitric oxide inhibitors. This non-randomised clinical study was designed to examine the effects of prolonged inhibition of nitric oxide synthesis with N^G-nitro-L-arginine methyl ester (L-NAME) in patients with severe septic shock. Eleven consecutive patients with ongoing hyperdynamic septic shock, unresponsive to fluid resuscitation and vasopressor therapy were included. Measurements of hemodynamic, hematological, and biochemical variables were made before, during and after a continuous intravenous infusion of 1 mg/kg/h of N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthesis, for 12 hours.

Continuous infusion of L-NAME resulted in a direct increase in mean arterial blood pressure from 65 ± 3 (SEM) to 93 ± 4 mmHg and systemic vascular resistance from 426 ± 54 to 700 ± 75 dyne·s/cm^5 reaching a maximum in 0.5 h. Pulmonary artery pressure was increased from 31 ± 2 to a maximum of 36 ± 2 mmHg at 1 h and pulmonary vascular resistance increased from 146 ± 13 to a maximum of 210 ± 23 dyne·s/cm^5 at 3 h. Parallel to these changes, cardiac output decreased from 10.8 ± 0.8 to 8.7 ± 0.7 l/min and oxygen delivery decreased from 1600 ± 160 to 1370 ± 130 mL/min (for all changes $p < 0.05$ as compared to the baseline value). Heart rate, cardiac filling pressures, oxygen consumption, urine production, arterial lactate and other biochemical parameters were not significantly changed by L-NAME (all $p > 0.05$). During L-NAME infusion the dosage of catecholamines could be reduced ($p < 0.05$). Although sustained hemodynamic effects were seen, L-NAME was most effective during the early stages of administration and the effect of L-NAME on blood pressure and vascular resistance tended to diminish during continued infusion. Seven of eleven patients ultimately died with survival time ranging from 2 to 34 days.

We conclude that nitric oxide appears to play a role in the cardiovascular derangements during human sepsis. The increase blood pressure and vascular resistance are sustained during prolonged inhibition of nitric oxide synthesis with L-NAME in patients with severe septic shock, although the hemodynamic changes are most significant in the early stages of L-NAME infusion. The high mortality rate suggests that L-NAME has only limited effects on outcome.

Introduction

Human septic shock is characterised by massive systemic vasodilatation with low vascular resistance, increased cardiac output, hypotension and high mortality rate.[1] Increased synthesis of the potent vasodilator nitric oxide (NO) has been incriminated in the hypotension and vasodilatation during sepsis and endotoxemia.[2] Under normal conditions small amounts of NO are formed from L-arginine by the constitutive NO synthase (eNOS) present in the vascular endothelium and stimulate the enzyme soluble guanylate cyclase (GC) in the smooth muscle cell which converts guanosine triphosphate (GTP) to cyclic guanosine monophosphate (c-GMP) resulting in vascular relaxation.[3] This endothelium derived NO (EDNO) plays a role in the regulation of blood pressure and tissue perfusion by maintaining vasodilatory tone. Upon stimulation by endotoxins (LPS) and cytokines such as TNF and IL-1, an inducible form of NO synthase (iNOS) is formed in macrophages and vascular smooth muscle cells.[2] This inducible enzyme differs from the constitutive isoform in that it releases massive amounts of NO resulting in profound vasodilatation with hypotension, dysfunction of vascular autoregulation, resistance to catecholamines and tissue damage.[2,4]

L-arginine analogues, like N^G-monomethyl-arginine (L-NMMA), N^G-nitro-L-arginine (L-NA) and N^G-nitro-L-arginine methyl ester (L-NAME), competitively inhibit the production of nitric oxide from L-arginine by both isoforms of nitric oxide synthase.[5,6] Animal studies have shown that these inhibitors of nitric oxide synthesis can prevent or reverse endotoxin or cytokine induced hypotension.[7,8] Short-term administration of these analogues of L-arginine in patients with sepsis, can increase blood pressure and systemic vascular resistance.[9,10] Furthermore methylene blue, an inhibitor of soluble guanylate cyclase, has been shown to temporarily raise blood pressure in patients with septic shock.[11] Therefore inhibitors of NO synthesis have been suggested to be of value in the treatment of hypotension during human septic shock. However, no data are present about the effect of continued inhibition of nitric oxide synthesis in human sepsis and effects on mortality and organ function remain unclear.[12,13]

The goal of the present study was to asses the effects of prolonged inhibition of nitric oxide synthesis during continuous infusion of L-NAME for 12 hours in patients with severe septic shock.

Materials and Methods

Subjects. The study was approved by the hospital's Medical and Ethics Committee. A total of 11 adult critically ill patients of the intensive care unit of our hospital were enrolled into the study (Table 1). Because informed consent could not be obtained personally from these severely ill patients, direct family members were informed of the nature of the study and gave informed consent. All patients met the criteria of sepsis as described by Bone et al.[14] These criteria include evidence of infection, tachycardia (>90 beats/min in the absence of β-adrenergic receptor blockade), tachypnea (respiratory rate >20 breaths/min or the requirement of mechanical ventilation), fever or hypothermia (temperature >38.3°C or <35.6°C), plus at least one of the following signs of inadequate organ perfusion: $Pao_2/Fio_2 < 280$ (without other pulmonary or cardiovascular disease as the cause), increased blood lactate levels (>2 mmol/L), and oliguria (<0.5 mL/kg/h) for at least 1 hr. All patients were in shock (systolic BP <90 mmHg or a decrease >40 mmHg from baseline unresponsive to a fluid challenge) on admission requiring therapy with pressor agents. At the time of the study all patients were receiving dopamine >15 μg/kg/min, noradrenaline >0.1 μg/kg/min or a combination of both. All patients had respiratory failure and required mechanical ventilation. Patients received standard antibiotic therapy adjusted to bacterial cultures. Patients received total parenteral feeding in six patients and enteral feeding in five patients. Both enteral and parenteral feeding contained standard concentrations of L-arginine and administration was continued during L-NAME infusion. Four patients required continuous hemodialysis because of renal failure. Only patients with cardiac index >3.0 L/min/m^2 were included in the study since earlier reports have shown reductions of cardiac output during inhibition of NO synthesis.[9,10] We hypothesized that a further reduction in cardiac output would be undesirable and even dangerous in a state where cardiac output is reduced. Exclusion criteria for the study were severe coronary artery stenosis (angina pectoris grade III according to NYHA classification), pregnancy and cardiac index <3.0 L/min/m^2.

Study Protocol. All patients underwent continuous electrocardiographic monitoring and had indwelling radial artery and pulmonary artery catheter. Mean systemic arterial blood pressure (MAP), central venous pressure (CVP) and mean pulmonary artery pressure (MPAP)

Table 1. Patient characteristics and bacteriological data.

Pt	Gender	Age (yr)	Underlying disease	APACHE II Score	Sepsis Severity Score[a]	Predominant Organism	Outcome (Survival 28d)
1	M	30	Peritonitis from bowel perforation after severe trauma	27	26	Proteus vulgaris (B), Enterococcus	Died
2	M	59	Acute necrotising pancreatitis	21	20	Pseudomonas aeruginosa (B), Enterococcus	Survived
3	M	45	Pseudomembranous colitis and ARDS after severe trauma	23	21	Clostridium difficile, Streptococcus pneumoniae,	Died
4	M	69	Ischemic colitis after vascular graft for ruptured abdominal aneurysm	19	18	Staphylococcus aureus (B)	Survived
5	M	60	Bronchopneumonia following liver transplantation for cryptogenic cirrhosis	24	24	Enterococcus	Died
6	M	47	Acute necrotising pancreatitis	18	22	Klebsiella pneumoniae (B), Enterococcus	Died
7	M	49	ARDS and thoracal empyema following aspiration pneumonia	20	24	Klebsiella pneumoniae, Staphylococcus epidermidis	Died
8	F	40	Peritonitis from bowel perforation following abdominal hysterectomy	17	16	Enterococcus (B), Bacteroides species	Survived
9	M	57	Pelvic abscess following ileocolectomy for Non-Hodgekin's lymphoma	16	17	Escherichia coli, Staphylococcus epidermidis	Survived
10	M	38	Peritonitis from bowel perforation after severe trauma	23	22	Enterococcus, Staphylococcus epidermidis	Survived
11	M	75	Mediastinitis after esophageal resection for carcinoma	20	19	Enterococcus	Died

Pt = patient, APACHE II = Acute Physiology and Chronic Health Evaluation, Knaus et al. [16], ARDS = adult respiratory distress syndrome, B = blood culture, [a] Sepsis Severity Score as described by Elebute and Stoner [17].

were continuously measured. Triplicate measurements of cardiac output (CO) were made according to the thermodilution method, and the mean value reported. Data were recorded on a computerised data system (Mennen Medical Systems, USA). Cardiac output and pulmonary artery occlusion pressure (PAOP) were measured three an six hours before L-NAME infusion (T= -6 and -3 h), at baseline (T= 0), during L-NAME infusion (T= 0.5, 1, 3, 6, and 12 h) and three and six hours following L-NAME infusion (T= 15 and 18 h). L-NAME was purchased from Sigma Chemicals (St. Louis, MO, USA). The pharmacy of our hospital prepared a sterile and pyrogen-free solution of L-NAME 10 mg/mL, ready for infusion. Following baseline measurements L-NAME 1 mg/kg/h was infused for 12 hours. If mean arterial blood pressure increased above 100 mmHg, the administration of vasopressors was gradually reduced and the dosage noted. Concomitant therapy was at the discretion of the clinician managing the patient. Continuous infusion of L-NAME was chosen since the effect of a bolus injection of 0.15 mg/kg L-NAME lasted only 5-10 minutes. Before and after administration of L-NAME, blood was withdrawn for routine measurement of serum electrolytes, urea and creatinine, liver function tests, lactate, hemoglobin, platelets and leukocytes. In eight patients arterial and central venous blood was withdrawn at T= 0, 1, 3, 6, 12 and 15 h and blood gases and hemoglobin content analyzed for determination of oxygen delivery and oxygen consumption. For pharmacokinetic analysis, in three patients blood samples were immediately centrifuged, plasma was separated and stored at -80 °C. Before analysis plasma was deproteinized with perchloric acid ($HClO_4$, 4.4 M) and levels of L-NAME and L-NA, were measured by means of high performance liquid chromatography using a nucleosil 5SA column (200x4mm, Machery-Nagel, Germany), a NaH_2PO_4 mobile phase with 10% methanol (200mM, pH 2.3, 1.5 ml/min) and UV detection at a wavelength of 268 nm.[15] The method was specific for L-NAME and L-NA as proved by monitoring the UV spectrum of the substance during its determination in plasma samples.

Calculations. Systemic vascular resistance (SVR) and pulmonary vascular resistance (PVR) were calculated according to standard formulae: SVR (dyne·s/cm^5) = 80 x (MAP-CVP)/CO and PVR (dyne·s/cm^5) = 80 x (MPAP-PAOP)/CO. Arterial (CaO_2) and venous (CvO_2) oxygen content were calculated using a standard equation (oxygen content = hemoglobin x 1.34 x oxygen saturation + PO_2 x 0.0031). Oxygen delivery (DO_2) was calculated as

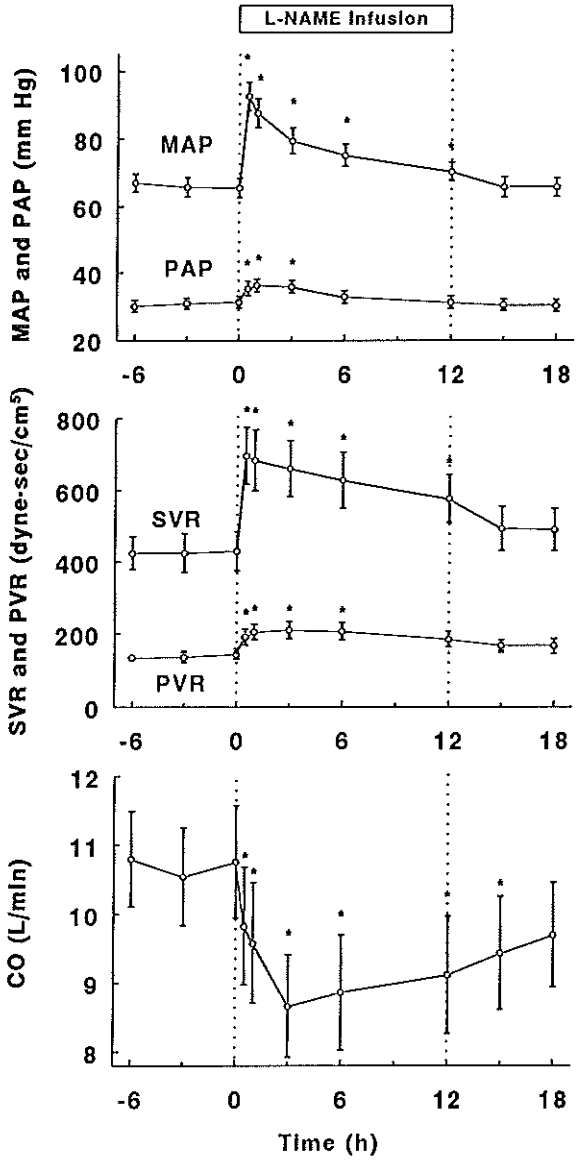

Figure 1. Effects of continuous infusion of 1 mg/kg/h of N^G-nitro-L-arginine methyl ester (L-NAME) for twelve hours on hemodynamics in eleven patients with severe septic shock. Time of L-NAME infusion is from t=0 to t=12 hours. L-NAME caused an increase in mean arterial pressure (MAP) and pulmonary artery pressure (PAP) (*top figure*), systemic vascular resistance (SVR) and pulmonary vascular resistance (PVR) (*middle figure*) and a reduction of cardiac output (CO) (*bottom figure*). Values are displayed as mean±SEM, $*p<0.05$ for comparison with the baseline value.

the product of cardiac output and CaO_2 (DO_2 = 10 x CaO_2 x CO) and oxygen consumption (VO_2) was calculated as the product of cardiac output and arterio-venous oxygen content difference: $VO_2 = (CaO_2 - CvO_2)$ x CO x 10. All of the recorded variables (mean±SEM) were compared with baseline values using Student's paired *t*-test with Bonferroni correction and a *p*-value <0.05 was considered statistically significant.

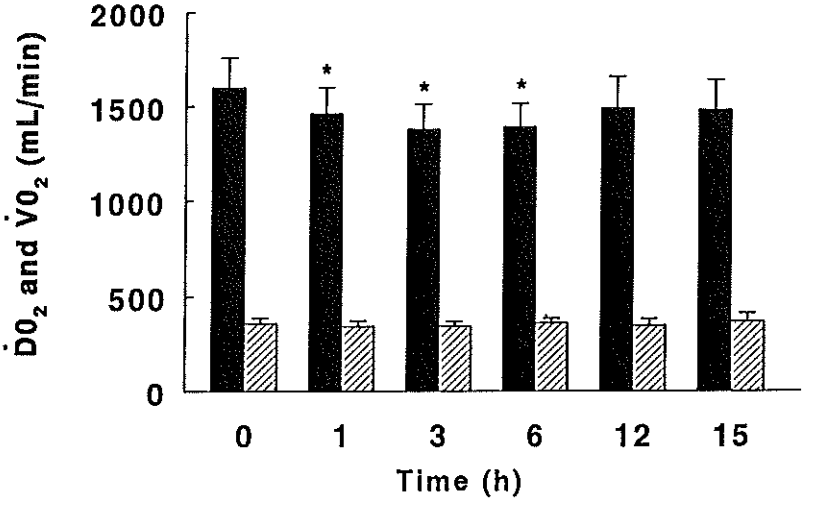

Figure 2. Effect of continuous infusion of 1 mg/kg/h of N^G-nitro-L-arginine methyl ester (L-NAME) for twelve hours on oxygen delivery (DO_2) (*solid columns*) and oxygen consumption (VO_2) (*hatched columns*) in eight patients with severe septic shock. Time of L-NAME infusion is from t=0 to t=12 hours. Values are displayed as mean±SEM, $*p<0.05$ for comparison with the baseline value.

Results

Patient characteristics are shown in Table I. The majority of patients had an intra-abdominal infection as the cause for sepsis. The Acute Physiology and Chronic Health Evaluation (APACHE) II score[16] was 21±1 (mean±SEM) and the Sepsis Severity Score[17] was 21±2. Cultures revealed both gram negative and gram positive bacteria with positive blood cultures in 45% of patients. Mortality at 28 days was 55% and one patient died at day 34 when treatment was discontinued because of inoperable surgical problems. Two patients died 2 days following L-NAME infusion, the others at 3, 5, 8 and 22 days respectively, because of a

combination of persistent septic shock and multiple organ failure. These deaths were not likely related to drug infusion but probably represent the severity of illness.

Continuous infusion of L-NAME resulted in a direct increase (within 5 min) in blood pressure, systemic vascular resistance and pulmonary artery pressure reaching a maximum within one hour (Figure 1 and Table 2). Parallel to these changes cardiac output and oxygen delivery decreased whereas pulmonary vascular resistance increased with a maximum change at t=3 h (Figure 1 and 2, Table 2). Heart rate, central venous pressure, pulmonary artery occlusion pressure and oxygen consumption, were not significantly changed (Figure 2 and Table 2). Vasopressors could be significantly reduced during L-NAME administration (Figure 3). Arterial oxygenation was significantly improved in the first six hours of L-NAME infusion (Figure 4) without significant changes in FiO_2 or PEEP (data not shown). In seven patients

Table 2. Effects of L-NAME on hemodynamic parameters in severe sepsis.

	baseline [t=0]	maximum change with L-NAME	time of max. change	end of infusion [t=12 h]
MAP (mmHg)	65±3	93±4[a]	0.5 h	71±3[a]
PAP (mmHg)	31±2	36±2[a]	1 h	31±2
CO (L/min)	10.8±0.8	8.7±0.7[a]	3 h	9.1±0.9[a]
SVR (dyne·s/cm^5)	426±54	700±75[a]	0.5 h	577±70[a]
PVR (dyne·s/cm^5)	146±13	210±23[a]	3 h	184±21
HR (beats/min)	112±4	108±4	1 h	110±4
CVD (mmHg)	12.9±1.0	14.1±1.3	3 h	11.7±1.3
PAOP (mmHg)	12.7±1.1	14.4±1.2	3 h	12.3±1.1
PaO_2 (torr)	81±5	107±15[a]	3 h	85±6
DO_2 (mL/min) (n=8)	1604±160	1372±131[a]	3 h	1492±173
VO_2 (mL/min) (n=8)	360±26	344±30	1 h	348±36

L-NAME = continuous infusion of 1 mg/kg/h of N^G-nitro-L-arginine methyl ester. MAP = mean arterial pressure, PAP = pulmonary artery pressure, CO = cardiac output, SVR = systemic vascular resistance, PVR = pulmonary vascular resistance, HR = heart rate, CVD = central venous pressure, PAOP = pulmonary artery occlusion pressure, PaO_2 = arterial oxygenation, DO_2 = oxygen delivery, VO_2 = oxygen consumption. Values are displayed as mean±SEM of eleven patients, [a]$p<0.05$ for comparison with the baseline value.

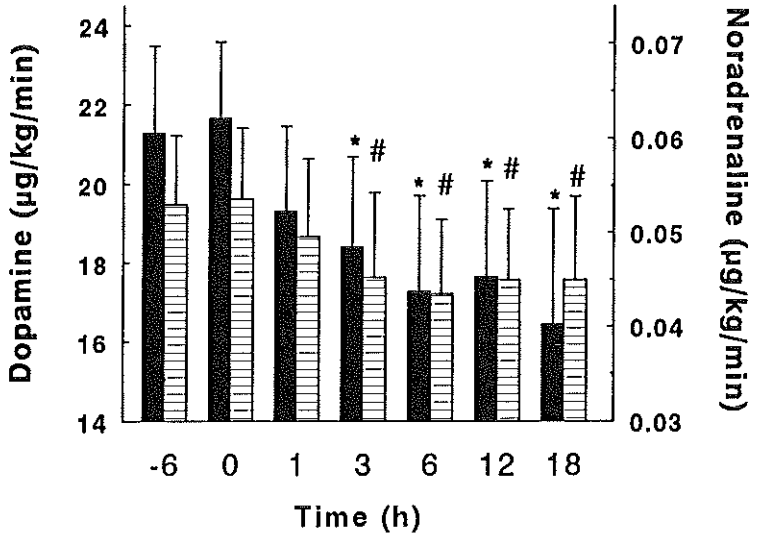

Figure 3. Dopamine (*solid columns*) and noradrenaline (*hatched columns*) administration in eleven patients with severe septic shock during continuous infusion of 1 mg/kg/h of N^G-nitro-L-arginine methyl ester (L-NAME) for twelve hours. Time of L-NAME infusion is from t=0 to t=12 hours. Values are displayed as mean±SEM, * and # $p<0.05$ for comparison with the baseline value.

who did not require continuous hemodialysis, urine output showed a tendency to increase during L-NAME infusion although the difference was not significant ($p=0.09$ at t=6 h, Figure 5). During continuous infusion of L-NAME the hemodynamic effects persisted, although the effects on blood pressure and systemic vascular resistance were most pronounced in the early stages of L-NAME administration and effects diminished after the first hour of L-NAME. Cardiac output remained depressed throughout the whole 12 hour infusion period. During administration of L-NAME no changes were seen in the electrocardiogram. Serum urea and creatinine, liver function tests, lactate, arterial pH, platelets and leukocytes were not significantly changed (Table 3). No side effects were seen during administration of L-NAME. Pharmacokinetic analysis in three patients revealed a mean plasma level of 1.1±0.2 µg/mL L-NAME and 7.3±0.4 µg/mL L-NA at the end of the infusion period. 24 H after L-NAME infusion had been stopped, mean plasma level of L-NA was 3.1±0.4 µg/mL, whereas L-NAME was below the detection limit of 0.05 µg/mL.

Discussion

Sepsis or the septic syndrome is the number one cause of mortality in today's intensive care. Overall mortality of sepsis is estimated to be 40 to 60% and when shock or organ failure is present mortality rate is even higher despite progress in antibiotic and vasopressor therapy.[1,14] New modalities of sepsis treatment offering new therapeutic tools would therefore be desirable for the management of patients with sepsis. Increased production of nitric oxide by the inducible nitric oxide synthase has been implicated as a major factor in the arterial hypotension, low systemic vascular resistance and hyporeactivity to catecholamines associated with endotoxin and cytokine induced shock in animals.[6,7] The present study that used the nitric oxide synthase inhibitor L-NAME, shows that nitric oxide is at least in part responsible for the hemodynamic derangements in patients with severe sepsis and that inhibition of nitric oxide

Table 3. Effect of L-NAME on biochemical and hematological parameters in severe sepsis.

	baseline	after L-NAME	P-value
Urea (mmol/L)	25±4	27±5	NS
Creatinine (μmol/L)	283±56	312±63	NS
Bilirubin (μmol/L)	230±51	228±46	NS
Alk. Phosph. (U/L)	236±106	232±103	NS
γ-GT (U/L)	103±38	106±39	NS
ASAT (U/L)	167±91	141±76	NS
ALAT (U/L)	91±43	71±32	NS
Lactate (mmol/L)	3.7±1.2	3.0±0.7	NS
Arterial pH	7.36±0.02	7.36±0.02	NS
Hemoglobin (mmol/L)	6.8±0.6	6.9±0.6	NS
Platelets (x10^9/L)	139±25	129±23	NS
Leucocytes (x10^9/L)	17.0±2.3	19.1±2.6	NS

L-NAME = continuous infusion of 1 mg/kg/h of NG-nitro-L-arginine methyl ester for 12 hours; Alk. Phosp. = alkaline phosphatase, γ-GT = γ glutamyl transferase, ASAT = aspartate aminotransferase, ALAT = alanine aminotransferase. Values are displayed as mean±SEM of eleven patients, NS = not significant.

synthesis may provide a new therapeutic tool in the management of hypotension and vasodilatation in sepsis.

Sepsis is a complicated multifactorial process resulting from the complex interaction between bacterial cell products like lipopolysaccharides (LPS) and host defense mechanisms

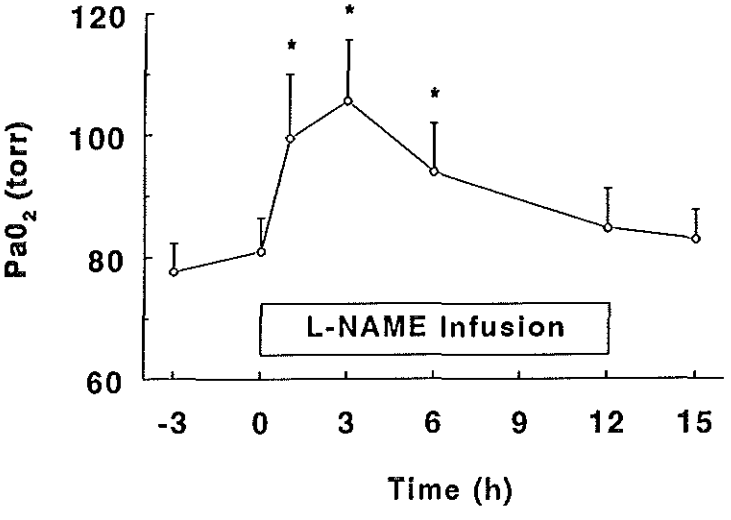

Figure 4. Arterial oxygenation in eleven patients with severe septic shock during continuous infusion of 1 mg/kg/h of N^G-nitro-L-arginine methyl ester (L-NAME) for twelve hours. Time of L-NAME infusion is from t=0 to t=12 hours. Values are displayed as mean±SEM, $*p<0.05$ for comparison with the baseline value.

leading to the release of several cytokines [e.g., tumour necrosis factor (TNF), interleukines (IL-1, IL-2 and IL-6)], myocardial depressant factor (MDF), platelet activating factor (PAF), kinins, arachidonic acid metabolites, endothelin-1 and several other vasoactive mediators.[1,14] Increased synthesis of nitric oxide is only one of many factors contributing to the derangements in the pathophysiological processes of septic shock. Inhibition of nitric oxide synthesis alone will most likely not be sufficient in stopping the cascade of events leading to overactivation of the immune system. This concept may explain the fact that there was no sustained clinical improvement and mortality rate of these severely ill patients remained high, over 60% which is in line of expectation considering APACHE II and Sepsis Severity scores.[16,17] Although our study was not designed to determine the impact on survival, this high mortality rate could suggest that L-NAME had only limited effect on outcome in these

patients. Possibly these patients were already in such a bad clinical condition that either therapy would have failed. Also, the 12 hour infusion period may have been too short to see any clinical improvement and a regimen with repeated infusions might be more effective. However, inhibition of nitric oxide synthesis may be considered as a potent therapeutic tool to gain time for therapy directed at the cause of septic shock e.g. adequate management of the infective site through antibiotics and surgery. New treatment modalities such as the use of selective inhibitors of the inducible type of NO synthase,[18] or the combination of these selective inhibitors with anticytokine drugs, with the aim of blocking the causative inflammatory mediators, may proof effective therapy in the future.[19]

The present study shows that the continuous infusion of N^G-nitro-L-arginine methyl ester (L-NAME), a specific inhibitor of nitric oxide synthesis, raised systemic blood pressure, systemic vascular resistance and pulmonary vascular resistance in patients with severe septic shock. Furthermore vasopressors could be reduced during L-NAME infusion. Our findings that L-NAME can raise blood pressure through preventing excess vasodilatation are consistent with the data from animal studies using L-NAME and other inhibitors of nitric oxide synthesis in endotoxin-induced shock,[8] and from studies using analogues of L-arginine in human sepsis,[9,10] but these reports only describe initial effects. The hemodynamic effects of L-NAME were direct in onset (within 5 min.) and persisted during the 12 hours of infusion, although the effect on blood pressure was most pronounced in the first hour of infusion. The fact that blood pressure gradually declined after the first hour was most probably caused by a concomitant reduction in cardiac output during L-NAME infusion. At the end of the 12 hour infusion period mean arterial blood pressure was only slightly raised as compared to baseline level whereas systemic vascular resistance remained elevated. However, some reservations must be made when interpreting the results of the present study. We did not include a control group (most appropriately receiving D-NAME) for comparison and spontaneous changes in the variables studied may have occurred over time. Furthermore, at this moment it is unclear whether the optimum hemodynamic effect was obtained with the currently used dosage of L-NAME. Possibly a higher or lower dose of L-NAME could have prevented the fall in blood pressure during prolonged infusion, although higher doses resulted in pulmonary hypertension in prior experiments. Also, we do not know to what extent nitric oxide synthesis was inhibited

by L-NAME since no measurements were made of nitrite/nitrate levels, the stable end products of nitric oxide metabolism.[20] New techniques for direct determination of nitric oxide *in vivo* may help to further explore this idea.[21]

Animal studies in dogs and rabbits have shown that L-NAME is converted by serum esterases to L-NA, an active inhibitor of nitric oxide synthase, through esterification of the carboxyl group.[15,22] At the time of our study no data were available about pharmacokinetics of L-NAME in humans. Using high performance liquid chromatography, we could detect high plasma levels of L-NA at the end of L-NAME infusion, whereas plasma levels of L-NAME were much lower, only 15 % of the L-NA plasma concentration. These results suggest conversion of L-NAME to L-NA in humans, similar to what was found in animals.[15,22] Furthermore, 24 hours following the end of L-NAME infusion, plasma levels of L-NA were still about 40 % of maximum whereas L-NAME itself was undetectable suggesting a long half-life of L-NA in humans. At this moment we do not know to what proportion L-NAME was responsible for inhibition of nitric oxide synthesis as compared to the active metabolite L-NA in the patients studied. Also, it is unknown how L-NA is further metabolized and cleared from the human body. To answer these questions further pharmacokinetic studies are warranted.

Cardiac output was depressed during L-NAME infusion as has been reported with other inhibitors of NO synthesis in both animals and humans.[8,10] So far the precise mechanism underlying this reduction in cardiac output remains unclear.[5,7] The reduction in cardiac output could partially have resulted from a reflex change due to the increased systemic vascular resistance and afterload, or a reduction in catecholamine dosage. A direct toxic effect of L-NAME on cardiac myocytes is improbable.[23] Although myocardial depression secondary to coronary vasoconstriction and myocardial ischemia have been suggested as mechanisms for toxic cardiac effects in animal studies using inhibitors of NO synthesis,[24] this pathogenesis is unlikely in the present study since no changes on the electrocardiogram were observed during L-NAME infusion. The reduction in cardiac output and parallel reduction in oxygen delivery may be harmful since tissue perfusion is reduced. Several animal studies have shown harmful effects of nitric oxide synthase inhibitors on organ function and outcome in experimental sepsis possibly as a result of tissue underperfusion related to the unselectivity of these inhibitors for the constitutive nitric oxide synthase (c-NOS).[25,26] L-NAME and L-NA show some preference

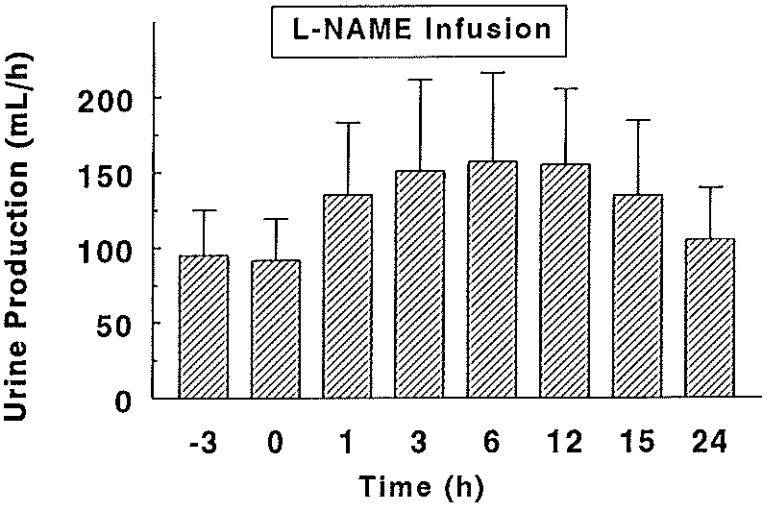

Figure 5. Urine production in seven patients with severe septic shock during continuous infusion of 1 mg/kg/h of N^G-nitro-L-arginine methyl ester (L-NAME) for twelve hours. Time of L-NAME infusion is from t=0 to t=12 hours. Values are displayed as mean±SEM, for all values $p>0.05$ for comparison with the baseline value. Four other patients were not included in the statistical analysis since they had renal failure requiring continuous hemodialysis.

towards c-NOS versus the inducible isoform.[5] However, much of the detrimental effects reported in these studies, depend on the dose of the nitric oxide synthase inhibitor administered, the moment of inhibition and the experimental model used.[12,27] Induction of the inducible nitric oxide synthase (i-NOS) takes a lag-phase of several hours.[28] When administration of nitric oxide synthase inhibitors is done in the early phase of sepsis, i-NOS may not have been expressed and only the constitutive isoform is blocked, resulting in detrimental vasoconstriction.[29] Furthermore, in a situation of hypodynamic shock with reduced cardiac output and reduced organ perfusion, additional vasoconstriction by inhibition of nitric oxide synthesis could be detrimental. This possibility made us include only patients with hyperdynamic sepsis with increased cardiac output, where there is an overperfusion of tissues to begin with. In our study no evidence of compromised tissue oxygenation was found during L-NAME administration since oxygen consumption was unaffected and arterial lactate and pH were unchanged. This finding might suggest that L-NAME preferentially causes vasoconstriction in metabolic inactive tissues. Also no significant adverse effects were seen on renal and liver function. Although not significant, urine output showed a tendency to increase during L-

NAME infusion. The effect of L-NAME on intestinal perfusion remains to be determined and measurement of gastric intramucosal pH, a proposed marker for splanchnic perfusion and an index of patient tissue oxygenation, could be useful in further studies.[30]

The role of nitric oxide is not limited to that of a vasodilator. Nitric oxide has antithrombotic properties that result from inhibition of platelet adhesion and aggregation which can be influenced by inhibitors of nitric oxide synthesis.[31,32] Furthermore nitric oxide may inhibit leukocyte adhesion to the endothelium.[32,33] In the present study platelet numbers and leukocyte count were not significantly changed following 12 hours of L-NAME infusion suggesting that enhanced platelet aggregation and/or leukocyte adhesion by L-NAME played only a minor role. The small changes in platelet numbers and leukocyte count that were seen, probably represent the natural course of the disease and not a direct effect of L-NAME.

A regulatory role of nitric oxide on pulmonary vascular tone has been described and inhaled nitric oxide can be used to reduce pulmonary hypertension and improve oxygenation in patients with Adult Respiratory Distress Syndrome (ARDS).[34,35] That nitric oxide vasodilates the pulmonary vasculature is underscored by the finding that L-NAME increased pulmonary artery pressure and pulmonary vascular resistance. Pulmonary vasoconstriction is an undesirable effect that may lead to pulmonary hypertension with right ventricular failure and reduced left ventricular preload and may also lead to problems in pulmonary gas exchange. In prior dose-finding experiments higher doses of L-NAME resulted in pulmonary hypertension for which L-NAME infusion had to be stopped. However, in our study group no signs of pulmonary hypertension were seen and right atrial pressure and pulmonary artery wedge pressure were unchanged. These results suggest that the detrimental effects of L-NAME on the pulmonary vasculature are dose-related and only found when high doses of L-NAME are used. In fact arterial oxygenation was improved during of L-NAME infusion despite the profound pulmonary vasoconstriction and reduction in cardiac output. This finding may suggest that L-NAME temporarily redistributed intrapulmonary blood to well oxygenated alveoli. Similar effects of L-NAME on arterial oxygenation were described by Sprague et al.[36] using a model of unilateral alveolar hypoxia in anaesthetized rabbits. To prevent pulmonary vasoconstriction seen with the systemic administration of inhibitors of nitric oxide synthesis, selective pulmonary vasodilatation through the combined use of nitric oxide inhalation, may

be an elegant solution.[37]

In conclusion prolonged inhibition of nitric oxide synthesis may help to maintain vascular tone and blood pressure in patients with severe sepsis and give us valuable time to start therapy directed at the cause of sepsis. However, at this time it is too early to introduce inhibitors of nitric oxide synthesis into routine clinical use. Despite the fact that blood pressure and vascular resistance were increased during the twelve hours of L-NAME infusion, the overall clinical condition was not improved and mortality rate remained high in these severely ill patients. This finding could suggest that there are only limited effects of L-NAME on outcome, and more randomised clinical studies will be required to further investigate this question.

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Chapter 5

Effect of L-NAME, an inhibitor of nitric oxide synthesis, on cardiopulmonary function in human septic shock

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Abstract

In this prospective clinical study we tested the effects of continuous infusion of N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) synthesis on cardiovascular performance and pulmonary gas exchange in eleven patients with hyperdynamic septic shock. Standard hemodynamic measurements were made and blood samples taken before, during and after 12 hours of continuous infusion of 1 mg/kg/h of L-NAME.

Continuous infusion of L-NAME increased mean arterial pressure (MAP) from 65±3 (SEM) to 93±4 mmHg and systemic vascular resistance (SVR) from 962±121 to 1563±173 dyne·s·cm^{-5}/m^2. Parallel to this cardiac index (CI) decreased from 4.8±0.4 to 3.9±0.4 L/min/m^2 and myocardial stroke volume (SV) was reduced from 43±3 to 34±3 ml/m^2. Left ventricular stroke work was increased in the first hour of L-NAME infusion from 31±3 to 43±4 gm·m/m^2 (all $p<0.01$ compared to baseline). Heart rate, cardiac filling pressures and right ventricular stroke work did not change significantly ($p>0.05$). L-NAME increased the ratio of arterial pO_2 to the fraction of inspired oxygen (PaO_2/FiO_2) from 167±23 to 212±27 mmHg ($p<0.05$). Venous admixture (Q_{VA}/Q_T) was reduced from 19.4±2.6 to 14.2±2.1 % ($p<0.05$) and oxygen extraction ratio increased from 21.1±2.4 to 25.3±2.7 % ($p<0.05$). Oxygen delivery (DO_2) was reduced following L-NAME, whereas oxygen uptake and arterial lactate and pH were unchanged.

Prolonged inhibition of NO synthesis with L-NAME can restore MAP and SVR in patients with severe septic shock. Myocardial SV and CI decrease, probably as a result of increased afterload since heart rate and stroke work were not reduced. L-NAME can improve pulmonary gas exchange with a concomitant reduction in Q_{VA}/Q_T. L-NAME did not promote anaerobe metabolism despite a reduction in DO_2.

Introduction

Sepsis and septic shock are characterised by massive systemic vasodilatation with low vascular resistance, increased cardiac output despite myocardial depression, decreased sensitivity to catecholamines and high mortality rate.[1,2] Recent evidence suggests that pathologic overproduction of the nitric oxide (NO) radical, a potent vasodilator formerly known as endothelium derived relaxing factor (EDRF), is at least in part responsible for the cardiovascular dysfunction seen in sepsis and endotoxemia.[3,4]

Under normal conditions small amounts of NO are formed from L-arginine by the constitutive NO synthase present in the vascular endothelium. This results in a constant vasodilatory tone maintaining adequate tissue perfusion. Upon stimulation by endotoxins (LPS) and cytokines such as TNF and IL-1, an inducible calcium independent isoform of NO synthase is formed in various cell types. This inducible enzyme differs from the constitutive isoform in that it releases massive amounts of NO over long periods of time resulting in profound vasodilatation, hypotension and resistance to catecholamines.[3] Furthermore, the early hypotension and hyporeactivity to constrictors seen after exposure to endotoxins may result from increased activity of the constitutive enzyme.[5] High levels of nitrite and nitrate, the stable end product of NO metabolism, are found in patients with severe sepsis and these levels may correlate with vasodilation.[6] Recently NO has been incriminated in cascade leading to the myocardial depression during sepsis, as suggested by *in vitro* studies.[7,8]

Both the constitutive and inducible isoform of NO synthase are competitively inhibited by N^G-substituted analogues of L-arginine, such as N^G-monomethyl-arginine (L-NMMA), N^G-nitro-L-arginine (L-NA) and N^G-nitro-L-arginine methyl ester (L-NAME).[9,10] Infusion of these analogues of L-arginine can reverse endotoxin or cytokine induced hypotension and can restore reactivity to catecholamines in animals.[11-13] In patients with septic shock, short-term administration of these inhibitors of NO synthesis have been shown to increase blood pressure and systemic vascular resistance.[14-16] Furthermore, methylene blue, an inhibitor of soluble guanylate cyclase which is the effector enzyme of NO, has been shown to temporarily raise blood pressure in septic patients.[17] Therefore, inhibitors of NO synthesis have been suggested to be of value in the treatment of hypotension during human septic shock. However, limited data are

present about the effects of continued inhibition of NO synthesis on cardiovascular performance in human sepsis and the effect on pulmonary function remains to be determined.[18] Since inhalation of NO can improve oxygenation in patients with ARDS one could hypothesize that systemic inhibition of NO synthesis could compromise pulmonary gas exchange through pulmonary vasoconstriction.[19]

The present study was to designed to asses the effects of prolonged inhibition of NO synthesis on cardiovascular performance and pulmonary gas exchange during continuous infusion of N^G-nitro-L-arginine methyl ester (L-NAME) for 12 hours in patients with severe septic shock. To study the effect of L-NAME on NO production measurements were made of serum nitrite and nitrate levels.

Materials and Methods

Subjects. The hospital's Medical and Ethics Committee approved the study. First degree relatives were informed of the nature of the study and gave informed consent. Eleven adult critically ill patients of the intensive care unit of our hospital were included in the study.

All patients met the criteria of sepsis as described by Bone et al.[20] These criteria include evidence of infection, tachycardia (>90 beats/min in the absence of β-adrenergic receptor blockade), tachypnea (respiratory rate >20 breaths/min or the requirement of mechanical ventilation), fever or hypothermia (temperature >38.3°C or <35.6°C), plus at least one of the following signs of inadequate organ perfusion: Pao_2/Fio_2<280 (without other pulmonary or cardiovascular disease as the cause), increased blood lactate levels (>2 mmol/L), and oliguria (<0.5 mL/kg/h) for at least 1 hr. All patients were in shock (systolic BP <90 mmHg or a decrease >40 mmHg from baseline unresponsive to fluid challenge) on admission requiring therapy with pressor agents. At the time of the study all patients were receiving dopamine >15 μg/kg/min and/or noradrenaline >0.1 μg/kg/min. All patients had respiratory failure and required mechanical ventilation. Patients received antibiotic therapy based on culture results. Four patients required continuous hemodialysis because of renal failure. Only patients with cardiac index >3.0 L/min/m^2 were included since earlier reports have shown reductions of cardiac output during inhibition of NO synthesis.[14-16] We hypothe-

sized that a further reduction in cardiac output would be undesirable and even dangerous in a state where cardiac output is reduced. Exclusion criteria for the study were severe coronary artery stenosis (angina pectoris grade III according to NYHA classification), pregnancy and cardiac index <3.0 L/min/m^2.

Study Protocol. All patients underwent continuous electrocardiographic monitoring and had indwelling radial artery and pulmonary artery catheter (Criticath, Ohmeda, Singapore). Mean systemic arterial blood pressure (MAP), central venous pressure (CVP) and mean pulmonary artery pressure (PAP) were continuously measured. Triplicate measurements of cardiac output (CO) were made according to the thermodilution method, and the mean value reported. Data were recorded on a computerised data system (Mennen Medical Systems, USA). Cardiac output and pulmonary artery occlusion pressure (PAOP) were measured three and six hours before L-NAME infusion (T= -6 and -3 h), at baseline (T= 0), during L-NAME infusion (T= 0.5, 1, 3, 6, and 12 h) and three and six hours following L-NAME infusion (T= 15 and 18 h). L-NAME was obtained from Sigma Chemicals (St. Louis, MO, USA). The hospital pharmacy prepared a sterile and pyrogen-free solution of L-NAME 10 mg/mL, ready for infusion. Within six hours after inclusion, baseline measurements were made and L-NAME 1 mg/kg/h infusion was started and continued for 12 hours. If mean arterial blood pressure increased above 100 mmHg, the administration of vasopressors was gradually reduced and the dosage noted. Concomitant therapy was at the discretion of the clinician managing the patient. Continuous infusion of L-NAME was chosen since the effect of a bolus injection of 0.15 mg/kg L-NAME lasted only 5-10 minutes (unpublished observations). Before and after administration of L-NAME, blood was withdrawn for routine measurement of serum electrolytes, and creatinine, liver function tests, lactate, hemoglobin, platelets and leucocytes. Arterial and central venous blood was withdrawn at T= 0, 1, 3, 6, 12 and 15 h and blood gases, pH and hemoglobin content were analyzed (ABL505, Radiometer, Copenhagen) for determination of pulmonary gas exchange parameters, oxygen delivery and oxygen consumption. Since central venous blood gas measurements were incomplete in three patients, mean values of gas exchange parameters and related calculations are reported for only eight patients. In all patients blood was withdrawn at T= 0, 1, 3, 6, 12, 15, 18 and 24 h for determination of nitrite and nitrate levels, the stable end products of NO metabolism, and

analyzed using an automated procedure based on the Griess reaction.[21] Serum of eight postoperative intensive care patients served as control for nitrite and nitrate levels.

Calculations and statistical analysis. Calculations were performed according to standard formulas. Recorded variables were compared with baseline values using Student's paired *t*-test with Bonferroni correction. Nitrite/nitrate levels were compared with control using Student's unpaired *t*-test. A p-value <0.05 was considered statistically significant. Data are expressed as mean ± SEM.

Table 1. Patient characteristics

Age, yr	51.7±3.9
Sex, M/F	10/1
APACHE II score*	20.7±1.0
Sepsis Severity Score‡	20.8±0.9
Source of sepsis	peritonitis (n=5) pneumonia (n=2) pancreatitis (n=2) mediastinitis (n=1) peritonitis + pneumonia (n=1)
Bacteria	
Local culture	*Enterococcus faecalis* (n=6) *Staphylococcus epidermidis* (n=3) *Klebsiella pneumoniae* (n=1) *Streptococcus pneumoniae* (n=1) *Escherichia coli* (n=1) *Clostridium difficile* (n=1) *Bacteroides species* (n=1)
Blood culture	*Enterococcus faecalis* (n=1) *Pseudomonas aeruginosa* (n=1) *Staphylococcus aureus* (n=1) *Proteus vulgaris* (n=1) *Klebsiella pneumoniae* (n=1)
Mechanical ventilation	11
PEEP (cm H_2O)	5.7±2.0
Mortality (at day 28)	6/11

Values are displayed as mean±SEM of eleven patients. *APACHE II = Acute Physiology and Chronic Health Evaluation, Knaus et al. [22], ‡ Sepsis Severity Score as described by Elebute and Stoner [23], PEEP = positive end-expiratory pressure.

Results

Patient characteristics are shown in Table I. The majority of patients had an intra-abdominal infection as the cause for sepsis. Cultures of the infective focus revealed both gram negative and gram positive bacteria with positive blood cultures in 45% of patients. Mortality at 28 days was 55% and one patient died at day 34 when treatment was discontinued because of inoperable surgical problems. No obvious link was noted between deaths and infusion of L-NAME.

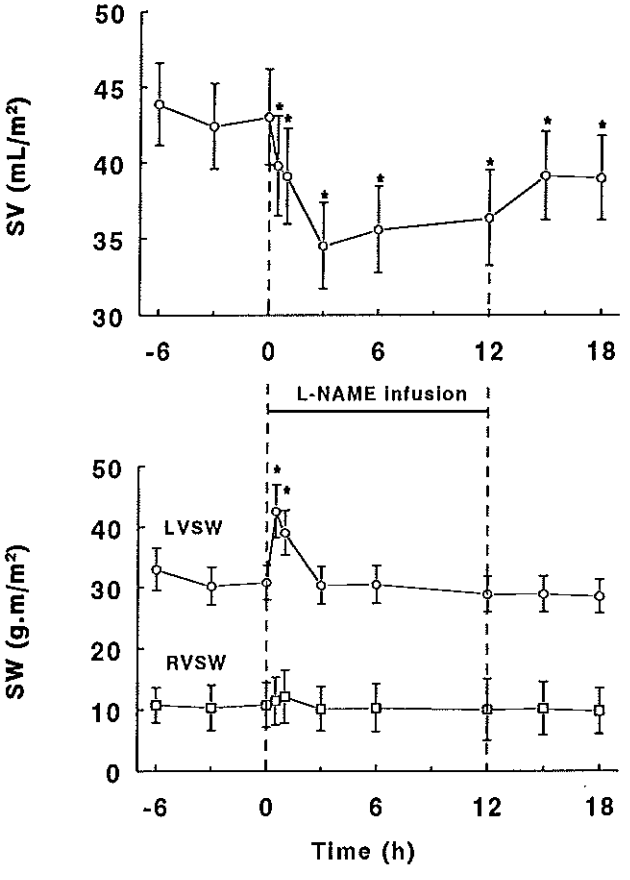

Figure 1. Effects of continuous infusion of 1 mg/kg/h of N^G-nitro-L-arginine methyl ester (L-NAME) on cardiac performance in eleven patients with severe septic shock. SV = stroke volume, SW = stroke work, LVSW = left ventricular stroke work, RVSW = right ventricular stroke work. Time of L-NAME infusion is from t=0 to t=12 hours. Values are displayed as mean±SEM, *$p<0.05$ for comparison with the baseline value.

Continuous infusion of L-NAME resulted in an increase in blood pressure (MAP), systemic vascular resistance (SVR), pulmonary artery pressure (PAP) and pulmonary vascular resistance (PVR) which was direct in onset (Table 2; $p<0.01$). Parallel to these changes cardiac index (CI) and myocardial stroke volume (SV) were reduced (Table 2 and Figure 1; $p<0.01$). Left ventricular stroke work (LVSW) was increased only in the first hour of L-NAME infusion whereas right ventricular stroke work (RVSW) was not significantly changed (Figure 1). Heart rate and cardiac filling pressures did not change significantly (Table 2). L-NAME infusion resulted in improved arterial oxygenation (Figure 2 and Table 3) with a maximum increase in the ratio of arterial PO_2 to the fraction of inspired O_2 (PaO_2/FiO_2) from 167±23 to 212±27 mmHg after 3 h of L-NAME infusion ($p<0.05$). Venous admixture (Q_{VA}/Q_T) was reduced from 19.4±2.6 to a minimum of 14.2±2.1 % ($p<0.05$) after 6 h of L-NAME infusion (Figure 2) and oxygen extraction ratio (O_2ER) increased from 21.1±2.4 to 25.3±2.7 % ($p<0.05$). No significant changes were seen in partial pressure of arterial carbon dioxide ($PaCO_2$) and alveolar-arterial oxygen pressure difference ($P(A\text{-}a)O_2$) (Table 3). The PEEP level was not significantly changed during L-NAME infusion (5.7±2.0 versus 4.7-±2.0 cm H_2O at the end of L-NAME infusion, $p>0.05$). Other ventilatory parameters such as mean airway pressure, peak inspiratory pressure and minute volume were unchanged (not shown). Oxygen consumption (VO_2) was unchanged despite a reduction in oxygen delivery (DO_2) (Table 3). Vasopressor support was significantly reduced during L-NAME administration (dopamine from 1920±170 to 1630±200 μg/min and noradrenaline from 8.3±2.1 to 7.5±2.3 μg/min at the end of L-NAME infusion, both $p<0.05$). During continuous infusion of L-NAME the hemodynamic effects persisted, although the effects on MAP were most pronounced in the early stages of L-NAME administration. CI and SV remained depressed throughout the whole 12 hour infusion period. At baseline, nitrite/nitrate levels ranged from 13 to 86 (median 37) μmol/L in the study group which was increased compared to control (range 5 to 17 (median 11) μmol/L, $p<0.05$). Serum nitrate and nitrite showed no significant changes during L-NAME infusion (Figure 3). Urine output increased from 92±27 to a maximum of 157±59 mL/h during L-NAME infusion which was not statistically significant ($n=7$; $p=0.09$). Arterial lactate (from 3.7±1.2 to 3.0±0.7 mmol/L) and arterial pH (from 7.36±0.02 to 7.36±0.02) were not significantly changed after the 12 h of L-NAME infusion.

Table 2. Effect of L-NAME infusion on hemodynamic variables in patients with severe sepsis

	continuous L-NAME infusion								
	baseline	0.5 h	1 h	3 h	6 h	12 h	15 h	18 h	24 h
CI, L/min/m^2	4.8±0.4	4.4±0.4*	4.3±0.4*	3.9±0.4*	4.0±0.4*	4.1±0.4*	4.2±0.4*	4.2±0.3*	4.4±0.4
MAP, mmHg	65±3	93±4*	87±4*	80±4*	75±3*	71±3*	66±3	66±3	68±3
SVR, dyne·s·cm^{-5}/m^2	962±121	1563±173*	1531±183*	1483±172*	1410±173*	1206±137*	1088±127	1092±124	1096±131
PAP, mmHg	31±2	35±2*	36±2*	36±2*	33±2	31±2	30±2	30±2	30±2
PVR, dyne·s·cm^{-5}/m^2	329±31	432±51*	463±51*	475±53*	469±57*	418±49	380±38	376±43	374±41
HR, beats/min	112±4	110±4	108±4	112±4	110±4	110±4	107±5	108±5	108±5
CVD, mmHg	12.9±1.0	14.1±1.1	13.5±1.2	14.1±1.3	11.8±1.0	11.7±1.3	11.7±0.9	11.9±0.7	11.6±1.3
PAOP, mmHg	12.7±0.9	14.1±0.9	13.9±0.9	14.4±1.2	12.7±0.9	12.3±1.1	12.2±0.8	12.6±0.8	12.9±1.0

L-NAME infusion = 1 mg/kg/h of N^G-nitro-L-arginine methyl ester. CI = cardiac index, MAP = mean arterial pressure, SVR = systemic vascular resistance, PAP = pulmonary artery pressure, PVR = pulmonary vascular resistance, HR = heart rate, CVD = central venous pressure, PAOP = pulmonary artery occlusion pressure. Values are displayed as mean±SEM of eleven patients, *$p<0.05$ for comparison with the baseline value .

Table 3. Effect of L-NAME infusion on pulmonary and metabolic variables in patients with severe sepsis.

	continuous L-NAME infusion						
	baseline	1 h	3 h	6 h	12 h	15 h	18 h
FIO_2, %	57±6	57±6	56±6	51±5	50±5	51±5	50±5
PaO_2, mmHg	81.0±5.4	99.4±10.6*	107.6±13.5*	93.1±7.9*	84.8±7.5	81.5±4.6	77.6±4.8
$PaCO_2$, mmHg	34.9±1.3	35.5±1.3	36.2±1.4	35.5±1.1	35.4±1.4	34.7±1.5	36.1±1.5
$P(A\text{-}a)O_2$, mmHg	292±48	273±48	258±42*	234±40*	236±39*	246±46	242±46
PvO_2, mmHg‡	41.3±1.8	41.8±1.7	40.7±2.0	39.3±1.9	40.3±1.6	40.0±1.4	39.6±1.6
Q_{VA}/Q_T, %‡	19.4±2.6	17.1±2.9*	14.9±2.6*	14.2±2.1*	14.3±2.3*	14.1±2.2*	13.9±2.2*
O_2ER, %‡	21.1±2.4	23.3±2.7	24.7±2.6*	25.3±2.7*	24.1±2.1	24.4±2.5	23.5±2.1
DO_2, mL/min/m²‡	713±68	658±64*	600±51*	621±55*	644±60	673±57	653±49
VO_2, mL/min/m²‡	158±12	153±13	151±10	162±12	156±16	167±19	159±19

PaO_2 = partial pressure of arterial oxygen, $PaCO_2$ = partial pressure of arterial carbon dioxide, $P(A\text{-}a)O_2$ = alveolar-arterial oxygen difference, PvO_2 = partial pressure of venous oxygen, Q_{VA}/Q_T = venous admixture, O_2ER = oxygen extraction ratio, DO_2 = oxygen delivery, VO_2 = oxygen consumption, FIO_2 = fraction of inspired oxygen. Values are displayed as mean±SEM of eleven (‡or eight) patients, *$p<0.05$ for comparison with the baseline value.

Also serum electrolytes, urea and creatinine, liver function tests, platelets and leucocytes were not significantly changed at the end of L-NAME infusion (results not shown). During administration of L-NAME no changes were seen in the electrocardiogram. No side effects were seen during administration of L-NAME.

Discussion

The mortality rate from septic shock remains high despite progress in antibiotic and

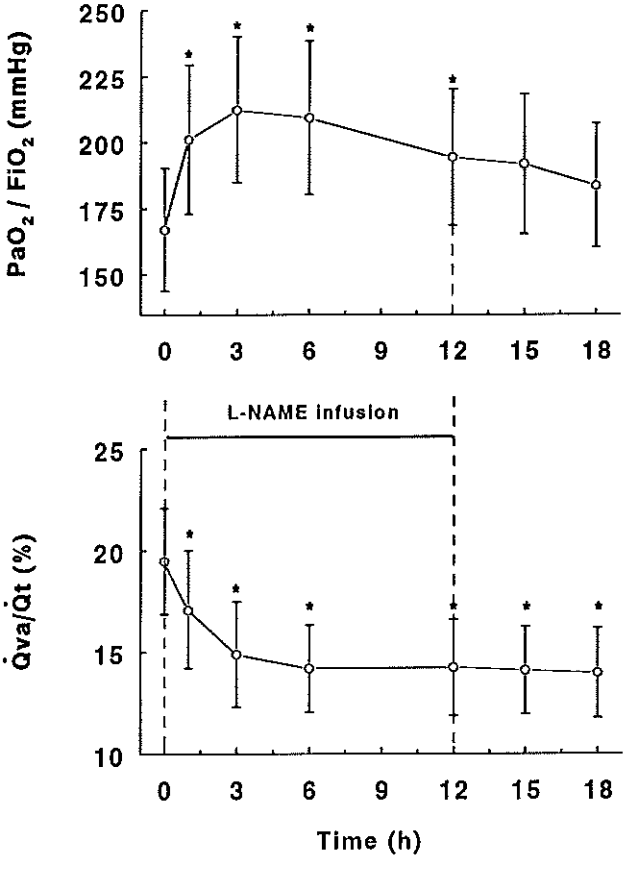

Figure 2. Effect of continuous infusion of 1 mg/kg/h of N^G-nitro-L-arginine methyl ester (L-NAME) on pulmonary gas exchange in patients with severe septic shock. PaO_2/FiO_2 = ratio of arterial pO_2 to the fraction of inspired oxygen (n=11), Q_{VA}/Q_T = venous admixture (n=8). Time of L-NAME infusion is from t=0 to t=12 hours. Values are displayed as mean±SEM, $*p<0.05$ for comparison with the baseline value.

vasopressor therapy and therefore new treatment modalities are warranted.[1] Increased production of nitric oxide (NO) by the inducible NO synthase has been implicated as a major contributor to the cardiovascular dysfunction of endotoxin and cytokine induced shock in animals.[11-13] The present study shows that NO is at least in part responsible for the hemodynamic derangements in patients with severe sepsis. Prolonged inhibition of NO synthesis with N^G-nitro-L-arginine methyl ester (L-NAME) can improve cardiovascular status and pulmonary gas exchange in these severely ill patients and may provide a new treatment modality in the management of septic shock.

Although no conclusions can be made regarding mortality in this non-randomised study with only eleven patients, the mortality rate was high (over 60%) which could raise questions regarding safety of L-NAME. However, no obvious deterioration of condition was noted during L-NAME infusion and mortality was not different from predicted death rate as computed by the APACHE II and Sepsis Severity scores.[22,23] Some animal studies have shown detrimental effects and increased mortality following L-NAME and other analogues of L-arginine during septic shock.[24,25] The detrimental effects of these NO synthase inhibitors have been contributed to their non-specifity towards the constitutive isoform of NO synthase and selective inhibition of the inducible enzyme has been suggested to be more effective.[25,26] Recent reports however, suggest that the constitutive form of NO synthase disappears in endotoxemia and other forms of sepsis.[27] If this is true, then the use of a non-specific inhibitor of NO synthase would be as effective as an inhibitor specific to the inducible isoform. In addition, the inhibition of the inducible isotype could hinder immune defense and bactericidal mechanisms which are important in sepsis.[28] Furthermore the detrimental effects described in many of these studies may be explained by administration of the inhibitor in the early phase of sepsis. In this situation the inducible NO synthase will not be yet fully expressed since this takes a lag-phase of several hours[2] and only the constitutive NOS will be blocked, resulting in severe and detrimental vasoconstriction. In addition the detrimental effects are dose-dependent and mostly seen in studies using extremely high doses of the NO synthase inhibitor.[29]

In our study patients received L-NAME 1 mg/kg/h for 12 hours resulting in a total dose of 12 mg/kg of L-NAME which is relatively low compared to the doses of NO inhibitors

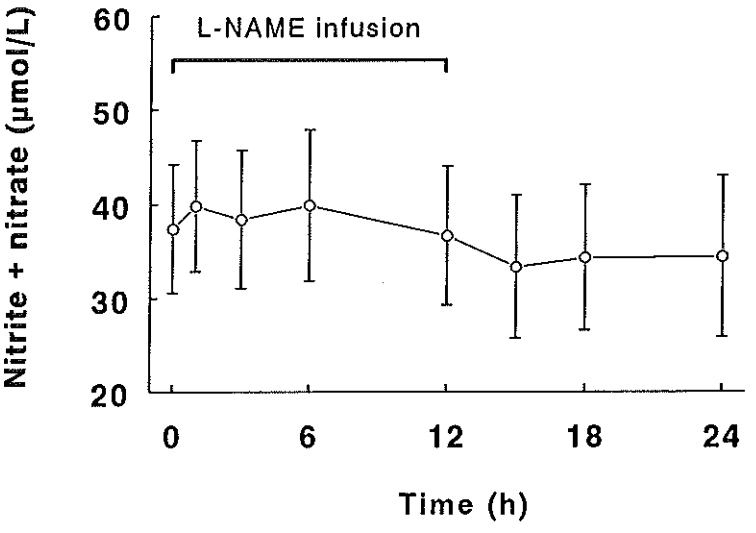

Figure 3. Effect of continuous infusion of 1 mg/kg/h of N^G-nitro-L-arginine methyl ester (L-NAME) on plasma levels of nitrite and nitrate, the stable end-products of nitric oxide metabolism, in patients with severe septic shock. Time of L-NAME infusion is from t=0 to t=12 hours. Values are displayed as mean±SEM, for all values $p>0.05$ compared to the baseline value.

used in most animal studies[12,13] and comparable to the doses used in studies in humans.[14-16,30] Animal studies have shown that L-NAME is probably a pro-drug that is converted to the active L-NA, which is much more stable and has a high plasma half-life.[31] However, little is known about pharmacokinetics and metabolism of L-NAME in humans. To minimize the potential chance of developing toxicity from high serum levels of L-NAME or L-NA we did not continue L-NAME infusion for a longer period than 12 hours and we did not restart L-NAME when patients redeveloped hypotension. At this time we do not know whether the optimum therapeutic dose of L-NAME was used and possibly the 12 hour administration period of L-NAME was too short for clinical improvement. Further pharmacodynamic studies will be necessary to find the optimum dose and schedule for L-NAME administration.

Our findings show that L-NAME can raise blood pressure in severe septic shock through preventing excess vasodilatation. Similar results have been found in previous studies using analogues of L-arginine in human sepsis,[14-16] although most reports only describe initial effects. The hemodynamic effects of L-NAME were direct in onset and persisted during the 12 hours of infusion. However, the effect on blood pressure was most pronounced in the first

hour of infusion and tended back towards baseline after one hour of L-NAME infusion. Since SVR remained elevated, the decline in blood pressure was probably caused by a concomitant decrease in CI and SV although some form of tolerance or counter-regulation may have played a role.

The reduction in CI has been reported with other inhibitors of NO synthesis in both animals and humans.[12-16] So far the precise mechanism underlying this reduction in cardiac output remains unclear. In our study it could partially have resulted from a reflex change due to the increased systemic vascular resistance and afterload, or a reduction in vasopressor support. Since LVSW was improved during L-NAME infusion and heart rate and RVSW were not significantly changed, direct cardiac depression by L-NAME seems unlikely. Myocardial depression secondary to myocardial ischemia[32] could have played a role although no changes on the electrocardiogram were observed during L-NAME infusion. The reduction in CI and concomitantly DO_2, may be harmful since tissue perfusion may be reduced. Therefore we only included patients with hyperdynamic sepsis as characterized by increased CI. The observations that VO_2 was unaffected and arterial lactate and pH were unchanged suggest that L-NAME did not promote anaerobe metabolism and that L-NAME preferentially caused vasoconstriction in metabolic inactive tissues. However, these observations only include indirect parameters that do not necessarily reflect what happens at capillary or cellular level. Thus, despite these findings, local areas of tissue underperfusion could have existed during L-NAME infusion.

Nitric oxide contributes to pulmonary vascular tone and inhaled nitric oxide can be used to reduce pulmonary hypertension and improve oxygenation in patients with Adult Respiratory Distress Syndrome (ARDS).[19,33] That NO vasodilates the pulmonary vasculature is underscored by the finding that L-NAME increased PAP and PVR. Pulmonary vasoconstriction is an undesirable effect that may lead to pulmonary hypertension and related deleterious effects on circulation, particularly on right sided cardiac function. Indeed in prior pilot experiments we found that higher doses of L-NAME resulted in severe pulmonary hypertension. The pulmonary vasoconstriction seen with L-NAME and other inhibitors of NO synthase,[12-15] may limit their clinical application especially in patients with high PAP. Since NO inhalation may improve oxygenation in patients with ARDS we hypothesized that inhibition of NO synthesis might lead to problems in pulmonary gas exchange. However, we

found that arterial oxygenation was improved during L-NAME infusion despite pulmonary vasoconstriction. Parallel to this there was a reduction in Q_{VA}/Q_T which implicates a decrease of the pulmonary right-to-left shunt. This may suggest that L-NAME caused a redistribution of intrapulmonary blood to well ventilated alveoli, improving ventilation-perfusion mismatch. Similar effects of L-NAME on arterial oxygenation were described by Sprague et al.[34] using a model of unilateral alveolar hypoxia in anaesthetized rabbits. Furthermore in a study by Radermacher et al.[35] it was found that the increase in cardiac output by systemic administration of nitrovasodilators worsened ventilation-perfusion mismatching and gas exchange in patients with ARDS. The combined use of NO inhalation with systemic inhibition of NO synthesis may prove effective therapy in the future.[36]

Septic patients had increased levels of nitrite and nitrate in the serum as compared to control. Although this could reflect increased systemic NO production,[6] some reservations must be made since NO is but one of the ways that nitrite and nitrates are formed. L-NAME infusion did not result in reduced serum levels of nitrite and nitrate, despite profound vasoconstriction. At this time we cannot explain this finding but it could suggest that L-NAME is not an effective inhibitor of NO synthesis and other mechanisms, independent of systemic inhibition of NO synthesis, may play a role in the increase in MAP and SVR by L-NAME.[25] Furthermore serum levels of nitrite and nitrate may not directly reflect the local amount of NO released or inhibited since active excretion through kidneys and gastro-intestinal tract takes place.[37]

In conclusion prolonged inhibition of NO synthesis with L-NAME may improve cardiovascular status with improvement of pulmonary gas exchange in severe septic shock. Mortality rate was high which could suggest only limited effects of L-NAME on outcome. Furthermore, the increase in PAP and the reduction in cardiac output are potentially harmful effects that may hinder the clinical utility of L-NAME and other NO synthase inhibitors. More randomised clinical studies will be required to further investigate whether inhibition of NO synthesis is beneficial in human septic shock.

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Chapter 6

Pulmonary hypertension and reduced cardiac output during inhibition of nitric oxide synthesis in human septic shock

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Shock 1998;9:451-454

Abstract

It has been suggested that inhibitors of nitric oxide synthesis are of value in the treatment of hypotension during sepsis. In this pilot study we examined the effects of inhibition of nitric oxide synthesis by continuous infusion of N^G-nitro-L-arginine methyl ester (L-NAME) 1.5 mg/kg/h in a patient with severe septic shock.

L-NAME produced a rise in mean arterial blood pressure and systemic vascular resistance; catecholamine infusion could be reduced. Parallel to these findings there was a 50% reduction in cardiac output and a five-fold rise in pulmonary vascular resistance which resulted in severe pulmonary hypertension after three hours of L-NAME infusion for which infusion had to be stopped. Following stop of L-NAME infusion pulmonary artery pressure and blood pressure returned to baseline values, although pulmonary and systemic vascular resistance remained elevated for several hours.

We conclude that nitric oxide appears to play a role in the cardiovascular derangements during human sepsis. Inhibition of nitric oxide synthesis with L-NAME can increase blood pressure and systemic vascular resistance. However, reduced cardiac output and pulmonary hypertension are possible side effects of continuous NO synthase inhibition. These side effects necessitate careful monitoring and may hinder the clinical application of NO synthase inhibitors.

Introduction

Human septic shock is characterised by massive systemic vasodilatation with low vascular resistance, increased cardiac output, hypotension and high mortality rate.[1] Increased synthesis of the potent vasodilator nitric oxide (NO) has been incriminated in the hypotension and vasodilatation that occurs during sepsis and endotoxemia.[2] Under normal conditions small amounts of NO are formed from L-arginine by the constitutive NO synthase (eNOS) present in the vascular endothelium.[3] This endothelium derived NO (EDNO) plays a role in the regulation of blood pressure and tissue perfusion by maintaining vasodilatory tone. Upon stimulation by endotoxins and cytokines such as tumor necrosis factor and interleukine-1, an inducible form of NO synthase (iNOS) is formed in macrophages and vascular smooth muscle cells.[2] This inducible enzyme differs from the constitutive isoform in that it releases massive amounts of NO resulting in profound vasodilatation with hypotension; dysfunction of vascular autoregulation, resistance to catecholamines and tissue damage.[2,4]

Analogues of L-arginine, such as N^G-mono-methyl-arginine (L-NMMA), N^G-nitro-L-arginine (L-NA) and N^G-nitro-L-arginine methyl ester (L-NAME), competitively inhibit the production of NO from L-arginine by both isoforms of nitric oxide synthase.[5] Animal studies have shown that these inhibitors of NO synthesis can prevent or reverse endotoxin or cytokine induced hypotension.[6] In human sepsis these inhibitors of NO synthesis can increase blood pressure and systemic vascular resistance.[7,8] Therefore inhibitors of NO synthesis have been suggested to be of value in the treatment of hypotension during human septic shock.

The present study tested the effects of continuous infusion of the NO synthase inhibitor L-NAME in a patient with severe septic shock.

Methods

The study was approved by the hospital's Medical and Ethics Committee. Direct family members were informed of the nature of the study and gave informed consent. The criteria used to define sepsis were described by Bone et al.[9] These criteria include evidence of infection, tachycardia (>90 beats/min in the absence of β-adrenergic receptor blockade),

tachypnea (respiratory rate >20 breaths/min or the requirement of mechanical ventilation), fever or hypothermia (temperature >38.3°C or <35.6°C), plus at least one of the following signs of inadequate organ perfusion: $PaO_2/FiO_2 < 280$ (without other pulmonary or cardiovascular disease as the cause), increased blood lactate levels (>2 mmol/L), and oliguria (<0.5 mL/kg/h) for at least 1 h. The patient had a pulmonary artery catheter and cardiac output was measured by the thermodilution method. Hemodynamic calculations were performed according to standard formulas and variables were indexed for body surface area (BSA). During L-NAME infusion, a 12-lead ECG was made at 30 min intervals. Blood was collected at intervals in heparin-coated tubes and immediately centrifuged; plasma was stored at -80°C. For pharmacokinetic analysis, plasma L-NAME and L-NA (the degradation product of L-NAME) were determined using high performance liquid chromatography (HPLC) as described by Kreycy et al.[10] For indirect determination of global NO release, the sum of plasma nitrite/nitrate was determined by an automated procedure based on the Griess reaction.[11] Normal values for plasma nitrite/nitrate were determined in ten healthy volunteers. L-NAME was purchased from Sigma Chemical (St. Louis, MO, USA). The hospital pharmacy prepared a sterile and pyrogen-free solution of 10 mg/mL L-NAME, ready for infusion.

Results

A 68-year old male (body weight 73 kg, body surface area 1.9 m^2) was admitted to the intensive care unit after esophagectomy for esophageal carcinoma with gastric tube reconstruction. On the second day following the operation, the patient developed sepsis of unknown origin with fever, shock (MAP = 56 mmHg, CI = 4.1 L/min/m^2 and SVRI = 935 dyne·s·cm^{-5}/m^2) and respiratory failure (FiO_2 100%, PEEP 10 cm H_2O). Anastomotic leakage of the gastrostomy was suspected, but no signs of this were found during endoscopy. Despite antibiotics, and vasopressor support with dopamine at 15 μg/kg/min and noradrenaline 0.4 μg/kg/min the circulatory status of the patient worsened. Then, continuous infusion of L-NAME was started in a dose of 1.5 mg/kg/h. Continuous infusion was chosen because recent reports suggest its superiority to high-dose bolus administration and we found that the effect of a low-dose bolus injection of 0.15 mg/kg L-NAME lasted only 5-10 minutes (our

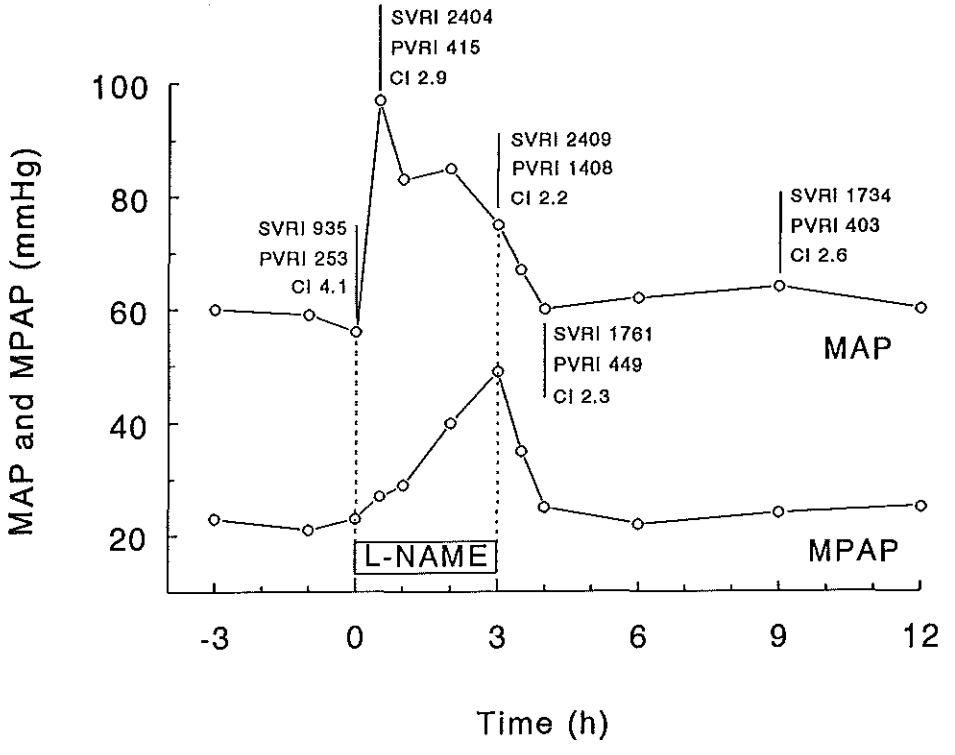

Figure 1. Effect of continuous infusion of 1.5 mg/kg/h of N^G-nitro-L-arginine methyl ester (L-NAME) on hemodynamics in a patient with severe septic shock. Time of L-NAME infusion is from t=0 to t=3 hours. L-NAME infusion was stopped because of severe pulmonary hypertension. Note the reduction in cardiac index during L-NAME infusion. MAP = mean arterial pressure; MPAP = mean pulmonary artery pressure; SVRI = systemic vascular resistance index; PVRI = pulmonary vascular resistance index; CI = cardiac index.

unpublished observations). L-NAME infusion resulted in a direct increase (within 5 min) in blood pressure, systemic vascular resistance, pulmonary artery pressure and pulmonary vascular resistance (Figure 1). Parallel to these changes cardiac index decreased and heart rate was reduced from 115 to 100 beats/min. Central venous pressure and pulmonary artery occlusion pressure were only slightly changed (from 8 to 10 mmHg and 10 to 13 mmHg, respectively). Vasopressors could be reduced during L-NAME administration, dopamine from 15 to 7 μg/kg/min and noradrenaline from 0.4 to 0.02 μg/kg/min. After three hours of L-NAME infusion, pulmonary artery pressure had risen to a mean value of 50 mmHg (systolic 65 mmHg, diastolic 45 mmHg) with a five-fold rise in pulmonary vascular resistance index

to 1408 dyne·s·cm^{-5}/m^2 and almost 50% reduction in cardiac index. Since these values were considered unacceptably high, L-NAME infusion was stopped. This resulted in a reduction in pulmonary and systemic blood pressure to pre-infusion values within one hour (Figure 1). However, pulmonary and systemic vascular resistance remained elevated and cardiac output depressed for twelve hours. Arterial oxygenation was not impaired during L-NAME infusion (p_aO_2= 63 mmHg before and 83 mmHg during L-NAME infusion). Urine output was increased from 48 mL/h to 67 mL/h during L-NAME infusion. Arterial lactate was 1.6 mmol/L before L-NAME and 1.2 mmol/L at the end of L-NAME infusion. During administration of L-NAME no signs of myocardial ischemia were seen on the electrocardiogram. Plasma nitrite/nitrate concentration was 31 μmol/L before L-NAME infusion which is increased as compared to normal (12$\pm$5 μmol/L). Plasma nitrite/nitrate concentration slowly declined in the hours after L-NAME infusion (Figure 2). Pharmacokinetic analysis revealed a plasma level of 1.4 μg/mL L-NAME and 4.8 μg/mL L-NA at the end of the 3-h infusion period.

Since the patient remained hypotensive and vasopressor dependent, one day later the patient was reoperated; failure of the intra-thoracic anastomosis was found and the anastomosis was disconnected. In the next three days the hemodynamic parameters of the patient improved and catecholamines were stopped. Six weeks later the patient left the hospital in good health.

Discussion

This study shows that pulmonary hypertension and reduced cardiac output can be major side effects of continuous NO synthase inhibition. Pulmonary vasoconstriction is undesirable because it may compromise pulmonary gas exchange and because it increases the workload on the right ventricle. In cases where strain already exists on the right ventricle (e.g. sepsis or PEEP ventilation) or in cases where right sided cardiac reserve is minimal, such increase in workload may lead to right ventricular failure, reduced cardiac output and compromised tissue perfusion.

Recently inhibitors of NO synthase have been presented as new therapeutic tools in the management of hypotension and vasodilatation in sepsis.[7,8] In the present study it was

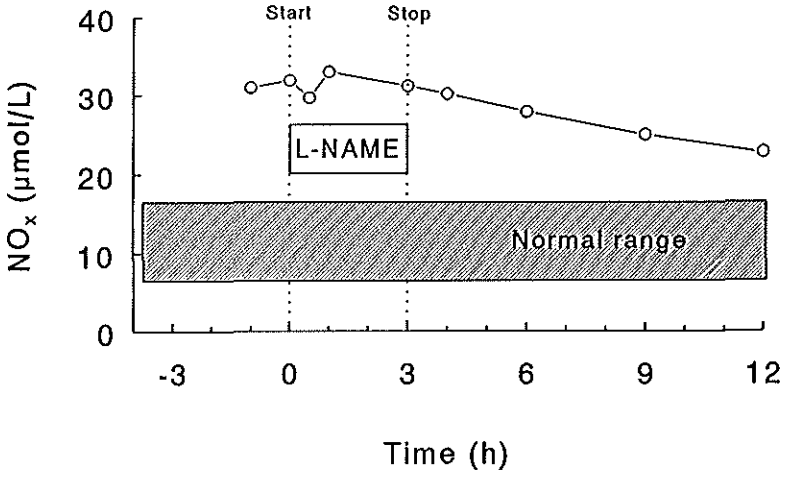

Figure 2. Effect of continuous infusion of 1.5 mg/kg/h of N^G-nitro-L-arginine methyl ester (L-NAME) on plasma nitrite/nitrate (NO_x) concentration in a patient with severe septic shock. Time of L-NAME infusion is from t=0 to t=3 hours. Normal values were determined in ten healthy volunteers. Values are displayed as the sum of plasma nitrite and nitrate (NO_x).

demonstrated that blood pressure and systemic vascular resistance increased during infusion of the NO synthase inhibitor L-NAME, and the dosage of catecholamines was reduced. The vasoconstrictive response to L-NAME most likely was the result of blocking the NO system as supported by the finding that the supranormal plasma levels of nitrite and nitrate, the stable endproducts of NO metabolism, slowly declined following L-NAME infusion. However, NO is but one of the ways which nitrite and nitrate are formed and nitrite/nitrate levels may not directly reflect the local amount of NO released.

In addition to the systemic effects of L-NAME, severe pulmonary vasoconstriction was observed with L-NAME. Analogous to these findings, in patients with Adult Respiratory Distress Syndrome (ARDS), inhalation of NO is reported to be beneficial by causing local vasodilation in bronchial and pulmonary circulation which results in reduced pulmonary vascular resistance and improved oxygenation.[12,13] This suggests that the pulmonary circulation is sensitive to the vasodilating effects of both endogenous and exogenous NO. Pulmonary vasoconstriction is not, therefore, unexpected with systemic inhibition of NO synthesis. In the endotoxemic pig, the pulmonary circulation is even more sensitive to the inhibition of NO synthase than the systemic circulation probably through the enhanced release of cyclo-

oxygenase products.[14] In the present study, which used a continuous infusion of a high dose of L-NAME, pulmonary vascular resistance increased five-fold, whereas systemic vascular resistance "only" doubled. Whether this difference in response reflects differences in sensitivity to L-NAME, the release of cyclo-oxygenase products, the tumor associated changes in vascular responsiveness, or the distribution of the different iso-enzymes of NO synthase or whether this difference can be explained by the pharmacokinetic distribution of the drug, cannot be deduced from our results. In the present study pulmonary hypertension was reversible after stopping L-NAME infusion. In prior experiments with a lower dose of L-NAME, pulmonary vasoconstriction was less pronounced and did not result in pulmonary hypertension.[8] Thus, pulmonary hypertension is a dose-related effect of L-NAME that can probably be attributed to overdosing of the drug, although the currently measured plasma levels of the drug were not high.[6,7] The presence of L-NA in the plasma during L-NAME infusion suggests conversion of L-NAME to L-NA in humans, as was reported in animals.[10]

The reduction in cardiac output during inhibition of NO synthesis has been described by others.[6-8] So far the precise mechanism underlying this reduction in cardiac output remains unclear. In the present study reduced cardiac output may have directly resulted from the extreme increase in pulmonary vascular resistance compromising venous return and left ventricular preload and/or a reflex reduction in heart rate by the increase in vascular resistance and blood pressure. Also the reduction in catecholamine dosage may have played a role. In addition L-NAME may have influenced myocardial perfusion,[15] although no evidence of myocardial ischemia was noted on the ECG. The reduction in cardiac output may be deleterious since tissue perfusion is reduced although arterial lactate was not increased in the present study. Several studies have shown harmful effects of nitric oxide synthase inhibitors on organ function and outcome in experimental sepsis and have related these detrimental effects to the inhibition of the constitutive NO synthase.[16] Most analogues of L-arginine inhibit both iso-types of NO synthase. L-NA and its precursor L-NAME even show preference towards the constitutive enzyme.[17] Therefore too much inhibition of the constitutive NO synthase by L-NAME may have played an additional role in the pulmonary hypertension and reduction in cardiac output in the present study. Whether selective inhibitors of the inducible enzyme are effective in human septic shock remains to be determined.

We conclude that pulmonary hypertension and reduced cardiac output are unwanted side effects of continuous NO synthase inhibition with L-NAME. In clinical situations, such as severe septic shock refractory to conventional therapy, blocking the NO system must be done with caution and at least under careful cardiovascular monitoring.

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Chapter 7

Effect of L-NAME, an inhibitor of nitric oxide synthesis, on plasma levels of IL-6, IL-8, TNF-α and nitrite/nitrate in human septic shock

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Abstract

In this prospective clinical study we tested the effects of N^G-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) synthesis, on plasma levels of IL-6, IL-8, TNF-α and nitrite/nitrate (NO_2^-/NO_3^-) in eleven consecutive patients with severe septic shock. Standard hemodynamic measurements were made and blood samples taken at intervals before, during and after a 12 h infusion period of L-NAME 1 mg/kg/h for determination of plasma IL-6, IL-8, TNF-α and NO_2^-/NO_3^- concentration.

Septic patients had increased plasma levels of IL-6, IL-8, TNF-α and NO_2^-/NO_3^- ($p<0.05$). Plasma levels of IL-6, IL-8 and NO_2^-/NO_3^- were negatively correlated with systemic vascular resistance (r=-0.62, r=-0.65 and r=-0.78 respectively, all $p<0.05$). Continuous infusion of L-NAME increased mean arterial pressure and systemic vascular resistance with a concomitant reduction in cardiac output (all $p<0.01$). No significant changes were seen in levels of plasma IL-6, IL-8 and NO_2^-/NO_3^- during the 24 h observation period. Plasma levels of TNF-α were significantly reduced during L-NAME infusion as compared to baseline ($p<0.05$).

We conclude that nitric oxide plays a role in the cardiovascular derangements of human septic shock. Inhibition of nitric oxide synthesis with L-NAME does not promote excessive cytokine release in patients with severe sepsis.

Introduction

Human septic shock is often characterised by massive systemic vasodilatation with low vascular resistance, increased cardiac output and high mortality rate.[1,2] The overproduction of nitric oxide (NO), a potent vasodilator formerly known as endothelium derived relaxing factor (EDRF), has recently been held responsible for the hemodynamic and metabolic consequences of sepsis and endotoxemia.[3,4] High levels of nitrite and nitrate, the stable end product of NO metabolism, are found in patients with severe sepsis and these levels may correlate with vasodilation.[5,6]

Under normal conditions small amounts of NO are formed from L-arginine by the constitutive NO synthase present in the vascular endothelium resulting in a constant vasodilatory tone maintaining adequate tissue perfusion. Upon stimulation by endotoxin (LPS) and cytokines, an inducible calcium independent isoform of NO synthase is formed in various cell types. This inducible enzyme differs from the constitutive isoform in that it releases massive amounts of NO over long periods of time resulting in profound vasodilatation, hypotension and resistance to catecholamines.[3]

Analogues of L-arginine competitively inhibit NO synthesis and can reverse endotoxin or cytokine induced hypotension and restore reactivity to catecholamines in animals.[7-9] In patients with septic shock, administration of these inhibitors of NO synthesis have been shown to increase blood pressure and systemic vascular resistance for prolonged periods of time.[10-12] Therefore inhibitors of NO synthesis have been suggested to be of value in the treatment of hypotension during human septic shock. However, several animal studies have shown excessive production of pro-inflammatory cytokines during inhibition of NO synthesis with detrimental effects.[13,14] These pro-inflammatory cytokines such as interleukin-6 (IL-6), IL-8 and tumor necrosis factor alpha (TNF-α) are involved in the pathogenesis of the sepsis syndrome and high levels of these cytokines may correlate with poor outcome.[15-18] Furthermore, several *in vitro* studies have shown that nitric oxide itself may have both pro- and anti-inflammatory effects and plays a role in modulation of the immune response.[19-21] At present no data are available about the effects of inhibition of NO synthesis on cytokine production in human sepsis.

The present study was designed to asses the effects of prolonged inhibition of NO synthesis with L-NAME on plasma levels of IL-6, IL-8 and TNF-α in patients with severe septic shock. To study the effect of L-NAME on NO production, measurements were made of plasma nitrite/nitrate (NO_2^-/NO_3^-) levels.

Materials and Methods

Subjects. The study was conducted according to the principles established in Helsinki and approved by the hospital's Medical and Ethics Committee. First degree relatives were informed of the nature of the study and gave informed consent. Eleven patients with septic shock in the surgical intensive care unit of our hospital were included in the study.

All patients met the criteria of sepsis as described by Bone et al.[22] All patients were in shock (systolic BP <90 mmHg or a decrease >40 mmHg from baseline unresponsive to fluid challenge) on admission requiring therapy with pressor agents. At the time of the study all patients were receiving dopamine >15 μg/kg/min and/or noradrenaline >0.1 μg/kg/min. All patients had respiratory failure and required mechanical ventilation. Patients received antibiotic therapy based on culture results. Four patients (two survivors and two non-survivors) required continuous hemodialysis because of renal failure. Seven patients were operated before the onset of septic shock with a time interval between surgery and the onset of sepsis ranging from 4 to 56 days (median 10 days). Only patients with a cardiac index >3.0 l/min/m^2 were included since earlier reports have shown reductions of cardiac output during inhibition of NO synthesis.[10-12] We hypothesized that a further reduction in cardiac output would be undesirable and even dangerous in a state where cardiac output is reduced. Exclusion criteria for the study were severe coronary artery stenosis (angina pectoris grade III according to NYHA classification), pregnancy and a cardiac index <3.0 l/min/m^2.

Study Protocol. All patients underwent continuous electrocardiographic monitoring and had indwelling radial artery and pulmonary artery catheters (Criticath, Ohmeda, Singapore) for hemodynamic measurements and data were recorded on a computerised data system (Mennen Medical Systems, USA). Survival was noted at 28 days.

L-NAME was obtained from Sigma Chemicals (St. Louis, MO, USA). The hospital

pharmacy prepared a sterile and pyrogen-free solution of L-NAME 10 mg/ml, ready for infusion. Following baseline measurements L-NAME 1 mg/kg/h was infused for 12 h. To minimize the potential chance of developing toxicity from high serum levels of L-NAME, infusion was not continued for longer periods. If mean arterial blood pressure increased above 100 mmHg, the administration of vasopressors was gradually reduced and the dosage noted. Time between onset of septic shock and initiation of L-NAME infusion ranged from 24 h to 21 days (median 6 days). Concomitant therapy was at the discretion of the clinician managing the patient.

Blood samples. For determination of plasma IL-6, IL-8, TNF-α and nitrite/nitrate blood samples (10 ml) were obtained in heparin coated blood collection tubes (Vacutainer, France) 3 h before L-NAME infusion (T=-3), at baseline (T=0), during L-NAME infusion (T= 1, 3, 6 and 12) and 3, 6 and 12 h after L-NAME infusion had stopped (T= 15, 18 and 24). Control values were established in 10 healthy volunteers among the laboratory personnel.

Table 1. Patient characteristics in survivors and non-survivors before L-NAME infusion.

	Survivors	Non-survivors	Normal range
Gender (M/F)	5/0	5/1	-
APACHE II score*	19.2±1.2	22.0±1.4	0-6
Source of sepsis	peritonitis (n=4) pancreatitis (n=1)	pneumonia (n=2) peritonitis (n=1) pancreatitis (n=1) mediastinitis (n=1) peritonitis + pneumonia (n=1)	
MAP, mmHg	68±4	62±4	70-110
SVR, dyne·s·cm^{-5}/m^2	1082±225	859±114	1900-2400
CI, l/min/m^2	5.1±0.5	4.6±0.6	2.5-3.6
Leucocytes, x10^9/l	19.3±3.0	14.8±1.7	4.0-10.0
Platelets, x10^9/l	169±33	116±30	140-320
Lactate, mmol/l	1.6±0.2	5.4±1.9	0.5-1.6

*APACHE Acute physiological and chronic health evaluation [25], MAP = mean arterial pressure, SVR = systemic vascular resistance, CI = cardiac index. Values are displayed as mean±SEM.

Blood samples were immediately centrifuged (1500 g, 10 min, 4 °C), plasma was collected and stored at -80 °C until tested. In addition blood was withdrawn for routine biochemical and hematological measurements and for blood gas analysis (ABL505, Radiometer, Copenhagen).

Cytokine assays. Levels of IL-6, IL-8 and TNF-α were measured using enzyme-linked immunosorbent assays (ELISAs) as described earlier.[23] Each ELISA was specific for the relevant cytokine and cytokine levels were expressed in pg/ml.

For IL-6 determination, flat-bottomed microtitre plates were coated overnight with purified monoclonal antibody (MAb) against IL-6 (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, CLB, Amsterdam, The Netherlands). After washing, serial dilutions of IL-6-containing samples were added. Bound IL-6 was detected by biotinylated affinity-purified polyclonal sheep anti-IL-6. The lower detection level of this assay was 1 pg/ml and normal healthy control subjects were below 10 pg/ml.

For IL-8 determination, a coat of IL-8 MAb (CLB) was applied overnight at a concentration of 1 μg/ml, and bound IL-8 was detected by biotinylated affinity-purified polyclonal sheep anti-IL-8, also at 1 μg/ml. The lower detection limit of this ELISA was about 5 pg per ml of serum. Values of IL-8 for healthy control subjects determined with this ELISA were below 20 pg/ml.

For TNF-α determination, flat-bottomed 96-well plates were coated with MAb (18b) anti-hu-TNF-α (Hoffman-La Roche, Basle, Switzerland). Biotinylated sheep anti-TNF-α was used as a second step. The detection limit of the assay was 10 pg/ml and normal healthy control subjects were below the detection limit.

Determination of nitrite/nitrate concentration. Total nitrite plus nitrate concentration was assayed as previously described by Phizackerley et al.[24] Briefly, serum was deproteinized with alkali and zinc sulphate and centrifugated for 3 min at 24000 g. To reduce nitrate to nitrite, volumes of 100 μl of supernatant or standards were incubated at room temperature for 30 min in the presence of *Klebsiella pneumoniae* under anaerobic conditions. Following centrifugation (3 min, 24000 g) 100 μl of total nitrite in the supernatant was subjected to the Griess reaction an assayed spectrophotometrically. Data are reported as nitrite plus nitrate values in μmol/l of plasma.

Calculations and statistical analysis. Hemodynamic calculations were performed

according to standard formulas. Data are expressed as mean ± SEM. Statistical analyses was performed using analysis of variance (ANOVA) for repeated measurements followed by *post hoc* Dunnett's test for comparison with the baseline value. Student's t-test was used to compare differences between groups. Pearson correlation and linear regression were used for analysis of correlations between variables. A p-value <0.05 was considered statistically significant. Statistical analysis was performed on a computer using the Statistical Package for the Social Sciences (SPSS) software for Windows.

Results

The causes for sepsis are displayed in Table 1. Cultures of the infective focus revealed both gram negative and gram positive bacteria with positive blood cultures in 45% of patients. Mortality at 28 days was 55% whereas expected mortality rate as calculated by APACHE II scores[25] was between 52 and 64% (95% confidence interval). Two patients died 2 days following L-NAME infusion, the others at 3, 5, 8 and 22 days respectively, because of a combination of persistent septic shock and multiple organ failure. No obvious link was noted between deaths and L-NAME infusion.

As displayed in Table 1 patients had increased APACHE II scores, hypotension, low systemic vascular resistance and increased levels of arterial lactate. Furthermore patients had increased plasma levels of IL-6, IL-8, TNF-α and NO_2^-/NO_3^- as compared to normal (all

Table 2. Cytokine and nitrite/nitrate concentrations in survivors and non-survivors before infusion of L-NAME.

	Survivors (n=5)	Non-survivors (n=6)	Control (n=10)
IL-6, pg/ml	357±213*	1412±679*	4±3
IL-8, pg/ml	384±258*	1090±653*	10±5
TNF-α, pg/ml	324±210*	674±318*	Undetectable
NO_x, μmol/l	44±12*	34±7*	12±5

IL = interleukin, TNF-α = tumor necrosis factor alpha, NO_x = nitrite plus nitrate. Values are displayed as mean±SEM. *$p<0.05$ compared to control.

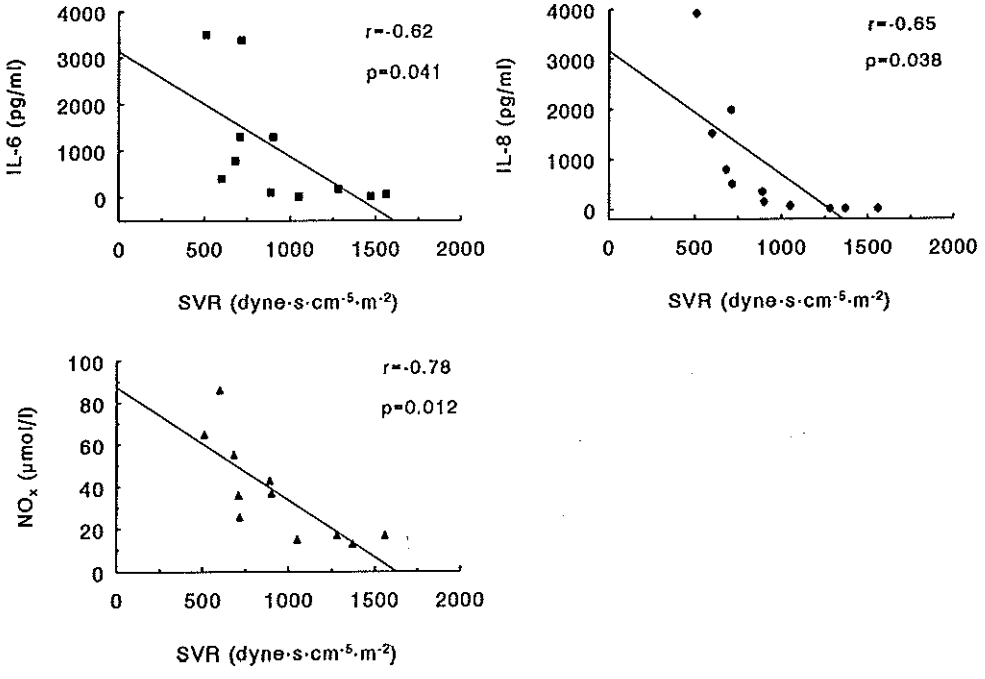

Figure 1. Scatterplot showing relation of IL-6, IL-8 and nitrite/nitrate (NO_x) with systemic vascular resistance (SVR). Measurements were made at baseline in each of eleven patients. Levels of IL-6, IL-8 and nitrite/nitrate correlated negatively with systemic vascular resistance ($p<0.05$).

$p<0.05$, Table 2). All patients had detectable levels of IL-6 and IL-8 whereas two patients, both survivors, had undetectable levels of TNF-α during the course of the study. Non-survivors (n=6) tended to have higher plasma levels of lactate, IL-6, IL-8 and TNF-α as compared to survivors although the differences were not statistically significant ($p=0.11$, $p=0.16$, $p=0.18$ and $p=0.33$ respectively, Table 1 and 2). Plasma levels of IL-6, IL-8 and NO_2^-/NO_3^- were negatively correlated with systemic vascular resistance ($p=0.041$, $p=0.038$ and $p=0.012$ respectively; Figure 1). Furthermore a positive correlation existed between plasma levels of IL-6 and IL-8 (r=0.67, $p=0.027$), arterial lactate and IL-8 (r=0.66, $p=0.026$) and IL-8 and NO_2^-/NO_3^- (r=0.71, $p=0.015$). Plasma NO_2^-/NO_3^- concentration was 43 ± 8 µmol/l in hemodialysed patients versus 35 ± 10 µmol/l in patients without hemodialysis ($p=0.315$). Cytokine levels in hemodialysed patients were not significantly different from patients without hemodialysis (data not shown).

L-NAME infusion resulted in profound hemodynamic changes as shown in Figure 2. Blood pressure and vascular resistance increased with a reduction in cardiac output and oxygen delivery ($p<0.01$). Heart rate and oxygen consumption were not significantly changed. There was a concomitant reduction in vasopressor requirement.

L-NAME infusion did not result in significant changes in the plasma levels of IL-6 and IL-8 in both survivors and non-survivors (Figure 3). For the whole group plasma IL-6 levels were 1112±443 pg/ml at baseline vs. 1268±459 pg/ml after 12 h of L-NAME infusion and IL-8 levels 911±421 vs. 772±373 pg/ml, respectively (both $p>0.05$, n=11). However, there was a significant reduction in TNF-α levels during L-NAME infusion for the whole group (515±207 pg/ml at baseline vs. 20±9 pg/ml after 12 h of L-NAME infusion, n=11; $p=0.042$) (Figure 3). After 12 h of L-NAME infusion only one of eleven patients had a plasma TNF-α concentration above the detection limit of 10 pg/ml versus seven patients at baseline. No significant changes were seen in plasma levels of NO_2^-/NO_3^- as a result of L-NAME infusion ($p>0.05$) (Figure 3). Leucocytes (17.0±2.3 $x10^9$/l before and 19.1±2.6 $x10^9$/l after 12 h; $p=0.56$) and platelets (139±25 $x10^9$/l before and 129±23 $x10^9$/l after 12 h; $p=0.52$) were not significantly changed by L-NAME.

Discussion

Cytokines play a central role in the pathophysiology of sepsis.[15-18] Recently, the NO radical has been incriminated in the cardiovascular derangements of sepsis[3,4] and inhibitors of NO synthesis have been proposed as a new therapeutic tools in the management of hypotension in sepsis and cytokine induced shock.[7-12] To our knowledge, no data are available on the effect of inhibition of nitric oxide synthesis on cytokine release in human sepsis. This is the first report that describes nitrite/nitrate and cytokine levels during inhibition of NO synthesis in septic shock patients.

Septic patients had increased levels of nitrite and nitrate as compared to control and levels of nitrite/nitrate correlated negatively with systemic vascular resistance. These findings are consistent with previous studies and probably reflect increased systemic NO production during severe sepsis.[5,6] Unexpectedly, serum levels of nitrite and nitrate were not significantly

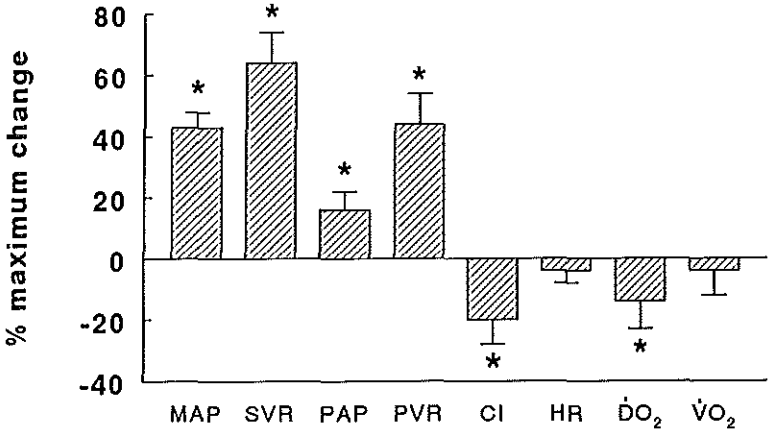

Figure 2. Effect of L-NAME infusion on hemodynamic and metabolic variables in severe sepsis. MAP = mean arterial pressure; SVR = systemic vascular resistance; PAP = pulmonary artery pressure; PVR = pulmonary vascular resistance; CI = cardiac index; HR = heart rate; DO_2 = oxygen delivery; VO_2 = oxygen consumption. Values are displayed as mean±SEM. *$p < 0.05$ as compared to baseline.

reduced during L-NAME infusion. Unchanged nitrite/nitrate levels may indicate that NO synthase activity was only partially blocked despite the significant hemodynamic effects. At present we do not know if the distribution of NO synthase inhibition with L-NAME was uniform throughout the body or among the different isoenzymes. Possibly the profound vasoconstrictive effects with L-NAME were the result of inhibition of the constitutive NO synthase.[26] However, some reservation must be made to draw conclusions from nitrite/nitrate levels since NO degradation is but one of the ways that nitrite and nitrate are formed.[27] Possibly plasma levels of nitrite and nitrate do not reflect the local amount of NO released or inhibited and local concentrations of nitric oxide may have been reduced without producing a demonstrable reduction in the plasma. Perhaps measurement of plasma nitrite/nitrate concentration in combination with other parameters such as urinary excretion of nitrate,[27] NO concentrations in exhaled air,[28] or direct NO measurement in the blood,[29] could help to further explore this in future studies.

The present study shows that TNF-α plasma concentration was reduced as compared to baseline during inhibition of NO synthesis with L-NAME. TNF-α is released early in the course of sepsis and appears to be a key mediator in the acute phase response in septic

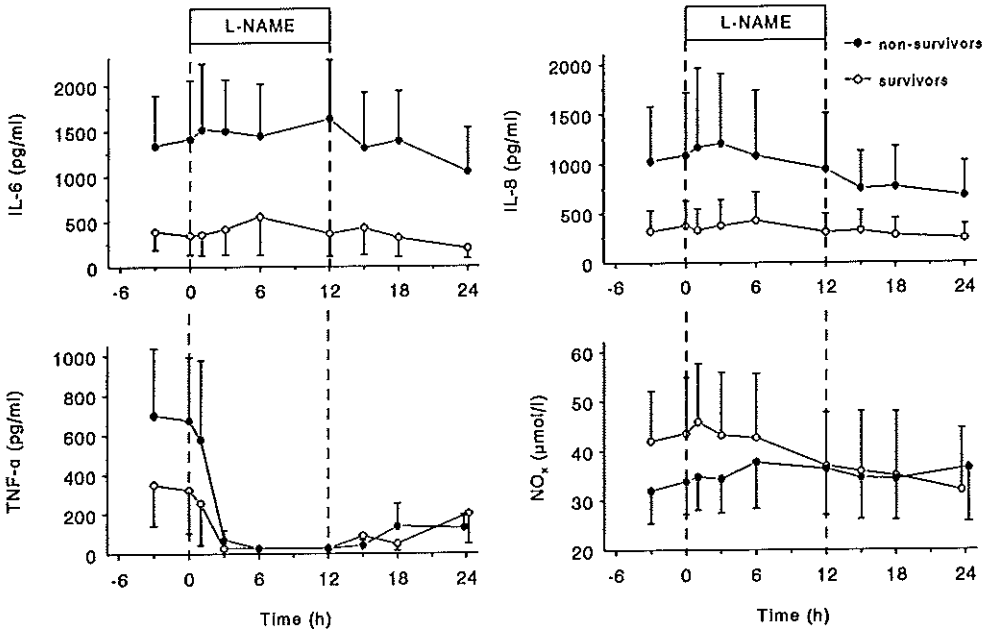

Figure 3. Effect of L-NAME infusion plasma levels of IL-6 (*left upper panel*), IL-8 (*right upper panel*) and TNF-α (*left lower panel*) and the sum of nitrite and nitrate (NO_x) (*right lower panel*) in severe sepsis in survivors (n=5, *open symbols*) and non-survivors (n=6, *closed symbols*). Values are displayed as mean±SEM. Note the reduction in TNF-α plasma level during L-NAME infusion as compared to baseline, which was significant for the whole group (p=0.042). No significant changes in time were seen of IL-6, IL-8 and NO_x plasma levels (all p>0.05).

shock.[17,30] Increased levels of TNF-α correlate with poor outcome in human septic shock.[15-18] However, there is also clinical and experimental evidence that TNF-α may be useful in some circumstances. For instance, TNF-α has been shown to contribute to antibacterial resistance against infections.[31] Numerous studies have shown that TNF-α stimulates NO release through the induction of the inducible NO synthase.[3,7] However, not much is known about the influence of NO on TNF-α release. Previous animal studies that examined the effect of NO synthase inhibition on TNF-α levels in septic animals, have yielded contradictory results. Increased serum TNF-α levels have been reported after L-NAME pre-treatment in septic mice and after administration of N^G-nitro-L-arginine (L-NA), another inhibitor of NO synthesis, in endotoxemic rats.[13,14] These results may indicate a negative feedback mechanism exhibited by NO on TNF-α synthesis.[32] In contrast to these findings, in another study in endotoxemic rats,

a reduction in TNF-α levels was reported during inhibition of NO synthesis with the NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA).[33] Aminoguanidine, a more specific inhibitor of the inducible NO synthase, did not modify serum TNF-α in this animal model of endotoxemia. Therefore it was suggested that NO synthesised by the constitutive isoform of the NO synthase positively modulates TNF-α synthesis. These findings are further supported by *in vitro* data suggesting that TNF-α synthesis may be up regulated by cGMP,[34] the conversion product of soluble gyanylate-cyclase that is the target enzyme of NO.[3] Since in the present study with L-NAME, which is an inhibitor of both the constitutive and inducible isoform of NO synthase, TNF-α levels were reduced as compared to baseline, one could suggest that NO positively modulates TNF-α release in human sepsis. However, these are speculations since spontaneous changes in TNF-α levels may have occurred despite the relatively short time interval,[16-18] and the present study did not include a control group for comparison. Therefore, it cannot be excluded that the reduction in TNF-α levels may have represented the natural course of the disease.

Levels of IL-6 and IL-8 were not changed during the 12 h period of L-NAME infusion. As compared to TNF-α, both IL-6 and IL-8 are secreted later in the inflammatory response and for longer periods of time.[17] IL-6 is a potent stimulator of liver synthesis of acute-phase proteins,[35] whereas IL-8 is the most important inducer of neutrophil chemotaxis and activation.[36] IL-6 and IL-8 levels were increased in this group of patients with severe sepsis.[15,16] Furthermore a positive correlation was found between IL-6 and IL-8 levels. Only few studies are present about the effect of NO on IL-6 and IL-8 release and these have shown contradicting results. NO can inhibit LPS-induced IL-6 production in cultured enterocytes.[20] In addition, *in vivo* inhibition of NO synthesis with L-NA can increase IL-6 levels and mortality in endotoxemic rats.[14] In contrast, in a recent *in vitro* study in human whole blood, L-NAME inhibited IL-6 and IL-8 release following stimulation with lipopolysaccharides.[21] L-NAME inhibition of IL-8 release was also observed at the mRNA level. Furthermore in the same study, direct exposure of whole blood to an exogenous NO donor resulted in increased production of IL-8, whereas no effect on IL-6 release was noted. Although the results from previous studies may indicate a complex interaction between NO and cytokine release, in the present study no significant changes in plasma IL-6 and IL-8 concentrations were seen.

However, some reservations must be made regarding interpretation of these findings. Cytokine levels in the plasma do not necessarily reflect the local synthesis of cytokines by cells. Many cells have surface receptors for these cytokines with high binding properties and cytokines are trapped by target cells and soluble receptors.[17] Thus, many of the cytokines released at the local level may have remained undetected in the plasma. Furthermore, cytokine concentrations were only measured during a 24 h observation period and changes in cytokine levels may have occurred later. Also, our study did not include a placebo group and spontaneous changes in cytokine levels may have occurred over time.[18]

The exact role of NO in modulating cytokine production in human sepsis has yet to be fully explored. In contrast to what has been reported in animal studies, no evidence of excessive pro-inflammatory cytokine production was found during 12 h of L-NAME infusion in septic shock patients. More randomized clinical studies will be necessary to determine as to whether inhibition of NO synthesis modulates cytokine release in human septic shock.

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Chapter 8

Endothelin-1 and blood pressure after inhibition of nitric oxide synthesis in human septic shock

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Abstract

The systemic hypotension during human sepsis has been ascribed to increased production of nitric oxide (NO). Therefore inhibitors of NO synthesis have been used in the treatment of hypotension in septic patients. In addition, NO production may inhibit the synthesis and vasoconstrictor effects of endothelin-1 (ET-1). In this study we tested whether ET-1 contributed to the vasopressor action of the NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) in patients with severe septic shock.

As compared to healthy volunteers, septic patients had increased plasma levels of nitrite/nitrate (37 ± 5 (SEM) versus 12 ± 5 μmol/L, $p < 0.01$), the stable end products of NO metabolism, and ET-1 (45 ± 7 versus 3 ± 2 pg/mL, $p < 0.001$). Plasma ET-1 concentration was not related to plasma nitrite/nitrate concentration and/or blood pressure. Continuous infusion of L-NAME (1 mg/kg/h IV) for 12 hours increased mean arterial pressure by $43 \pm 5\%$ and systemic vascular resistance by $64 \pm 10\%$ (both $p < 0.01$). The increase in blood pressure and systemic vascular resistance correlated positively with the level of ET-1 (both $p < 0.005$), but not with plasma nitrite/nitrate level. L-NAME infusion did not result in significant changes in the plasma concentrations of ET-1 or nitrite/nitrate.

We conclude that NO and ET-1 may both play a role in the cardiovascular derangements of human sepsis. Although L-NAME does not increase ET-1 concentration in patients with septic shock, the vasopressor response induced by L-NAME depends on the plasma level of ET-1. These findings may suggest that inhibitors of NO synthesis unmask a tonic pressor response of endothelin-1 in human septic shock.

Introduction

Recent evidence suggests a complex interaction of endothelium derived factors, including endothelin-1 (ET-1) and nitric oxide (NO), in the control of vascular smooth muscle tone in health and disease.[1] ET-1 is a 21-aminoacid peptide with potent vasoconstrictor actions in animals and man.[2,3] NO is a short-lived radical, derived from the amino acid L-arginine by the enzyme NO synthase, with direct vasodilatory action.[4,5] *In vitro* experiments show that continuous NO production inhibits the synthesis[6] and/or the vasoconstrictor action of ET-1.[7] Furthermore, endothelin receptor antagonists fail to affect blood pressure in normal animals.[8] This apparent absence of vascular effects of ET-1 has been attributed to continuous NO production. This is further supported by the finding that inhibition of NO synthesis in rats can unmask a tonic pressor response of endothelin and results in increased plasma levels of ET-1.[9,10] So far the precise interaction of ET-1 and NO in humans remains to be determined.[11,12]

Human sepsis is characterized by massive vasodilatation with low systemic vascular resistance, high cardiac output and severe hypotension.[13] Increased production of NO by an inducible isotype of NO synthase, has been held responsible for the cardiovascular derangements during sepsis.[5,14] High levels of nitrite and nitrate, the stable end products of NO metabolism, are found in patients with severe sepsis and these levels may correlate with vasodilation.[15] Analogues of L-arginine competitively inhibit the production of NO from L-arginine[16] and can reverse hypotension in endotoxin and cytokine induced shock in animals.[5,17] More recently these inhibitors of nitric oxide synthesis have been used to increase blood pressure in human sepsis.[18-20] At present it is unknown whether inhibition of NO synthesis modulates endothelin release in septic shock. Since high levels of ET-1 have been found in septic patients,[21,22] we hypothesized that part of the vasoconstrictive response after inhibition of NO synthesis may result from increased production of ET-1.

The purpose of the present study was to examine whether ET-1 plays a role in the increase in blood pressure after inhibition of NO synthesis in patients with severe sepsis. Plasma concentrations of ET-1 and nitrite/nitrate, as an indirect measure of NO production, were measured during continuous infusion of the L-arginine analogue N^G-nitro-L-arginine methyl ester (L-NAME) in patients with severe septic shock.

Methods

Subjects The hospital's Medical and Ethics Committee approved the study. First degree relatives were informed of the nature of the study and gave informed consent. Eleven adult critically ill patients of the intensive care unit of our hospital were included in the study. All patients met the criteria of sepsis as described by Bone et al.[23] The source for sepsis was peritonitis (n=5), pneumonia (n=2), pancreatitis (n=2), mediastinitis (n=1) and peritonitis with pneumonia (n=1). Patients were in shock (systolic BP <90 mmHg or a decrease >40 mmHg from baseline unresponsive to fluid challenge) on admission requiring therapy with pressor agents. At the time of the study all patients were receiving dopamine >15 μg/kg/min and/or noradrenaline >0.1 μg/kg/min. All patients required mechanical ventilation because of respiratory failure. Four patients required continuous hemodialysis because of renal failure. Only patients with cardiac index >3.0 L/min/m^2 were included since earlier reports have shown reductions of cardiac output during inhibition of NO synthesis. Exclusion criteria for the study were severe coronary artery stenosis (angina pectoris grade III according to NYHA classification), pregnancy and cardiac index <3.0 L/min/m^2.

Study Protocol All patients underwent continuous electrocardiographic monitoring and had indwelling radial artery and pulmonary artery catheters (Criticath, Ohmeda, Singapore). Measurements of systemic arterial blood pressure, central venous pressure, pulmonary artery pressure and cardiac output (thermodilution method) were made at baseline and at 0.5, 1, 3 and 6 h intervals, for a total period of 24 hours. Systemic and pulmonary vascular resistance were calculated according to standard formula. Following baseline measurements L-NAME 1 mg/kg/h infusion was started and continued for 12 hours. To minimize the potential chance of developing toxicity from high serum levels of L-NAME, infusion was not continued for longer periods. Maximum changes in blood pressure and systemic vascular resistance were noted after 30 minutes of L-NAME[20] and hemodynamic variables at this time point were used to compare with the baseline value. Concomitant therapy was at the discretion of the clinician managing the patient.

Blood samples. For later determination of plasma ET-1, nitrite/nitrate and cortisol, arterial blood samples (10 ml) were obtained in heparin coated blood collection tubes

(Vacutainer, France) at baseline (T=0), during L-NAME infusion (T= 0.5, 1, 3, 6 and 12) and 3, 6 and 12 h after L-NAME infusion had stopped (T= 15, 18 and 24). Control values were established in 10 healthy volunteers among the laboratory personnel. Blood samples were immediately centrifuged (1500 g, 10 min, 4 °C), plasma was collected and stored at -80° C until tested. Creatinine concentration was determined by a standard method.

Determination of Endothelin-1. Endothelin-1 was determined, after Sep-Pak extraction, with a commercially available radioimmunoassay kit (Nichols Institute, Wijchen, The Netherlands), as previously described by Smits et al.[24] Normal values in our laboratory were 1-5 pg/mL. The limit of detection was 1 pg/mL.

Determination of nitrite/nitrate concentration. Total nitrite plus nitrate concentration was assayed as described by Phizackerley et al.[25] To reduce nitrate to nitrite, supernatant or standards were incubated at room temperature in the presence of *Klebsiella pneumoniae* under anaerobic conditions. Total nitrite in the supernatant was subjected to the Griess reaction and

Table 1. Patient characteristics, plasma endothelin, nitrite/nitrate and cortisol concentration in severe septic shock.

	Septic patients	Control
Age, yr	52±4	
Sex, M/F	10/1	
MAP, mmHg	65±3	[70-110]
SVR, dyne·s·cm^{-5}/m^2	962±121	[1900-2400]
CI, L/min/m^2	4.8±0.4	[2.5-3.6]
Creatinine, μmol/L	283±56	[60-110]
ET-1, pg/mL	45±7***	3±2
NO_x, μmol/L	37±5**	12±5
Cortisol, nmol/L	753±87*	521±62
Mortality (at day 28)	6/11	

Values are displayed as mean±SEM. MAP = mean arterial pressure, SVR = systemic vascular resistance, CI = cardiac index, ET-1 = endothelin-1, NO_x = nitrite plus nitrate. ***$p<0.001$, **$p<0.01$ and *$p<0.05$ as compared to healthy volunteers. Normal range indicated between brackets.

assayed spectrophotometrically. Data are reported as the sum of nitrite plus nitrate. Normal values in our laboratory were 5-15 μmol/L. The limit of detection was 0.1 μmol/L.

Determination of cortisol. Cortisol concentrations were determined with a commercially available radioimmunoassay kit (DPC, Los Angeles, CA, USA) as described previously.[26] Normal values were 400-800 nmol/L (15-30 μg/dL). The limit of detection was 28 nmol/L.

Drugs L-NAME was obtained from Sigma Chemicals (St. Louis, MO, USA). The hospital pharmacy prepared a sterile and pyrogen-free solution of L-NAME 10 mg/mL, ready for IV infusion.

Data analysis All results are expressed as mean ± SEM. Changes over time were compared with baseline values by repeated measures ANOVA. Differences between groups were compared by Student's *t*-test. Pearson correlation and scatter plots were used for analysis of correlation between variables. A p-value <0.05 was considered statistically significant.

Results

Patient characteristics are shown in Table 1. All patients had hypotension, low systemic vascular resistance, high cardiac output and increased serum creatinine due to septic shock (Table 1). As compared to healthy volunteers, plasma levels of ET-1, nitrate/nitrate and

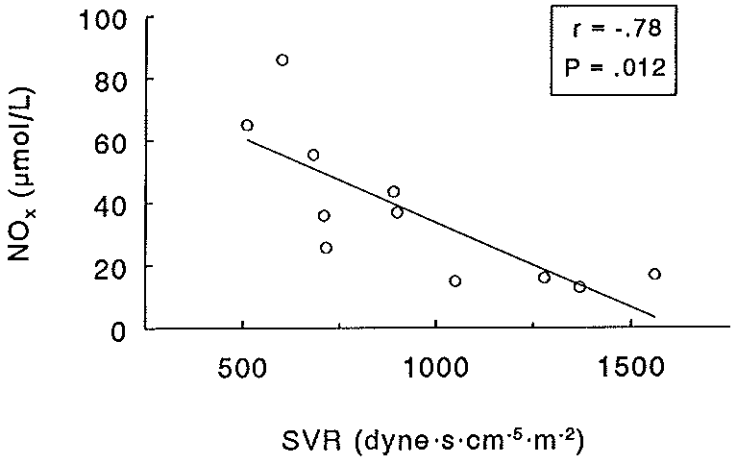

Figure 1. Scatter plot showing relation between plasma nitrite/nitrate (NO_x) concentration and systemic vascular resistance in patients with severe septic shock.

cortisol were significantly increased in septic patients (all $p<0.05$, Table 1).

No correlation was found between plasma ET-1 concentration and plasma nitrite/nitrate concentration (r=0.07, $p>0.80$), blood pressure (r=-0.34, $p=0.30$), systemic vascular resistance (r=-0.41, $p=0.22$) or creatinine concentration (r=-0.13, $p=0.69$). ET-1 concentration was 46±5 pg/mL in survivors and 43±12 pg/mL in non-survivors ($p>0.05$). Plasma nitrite/nitrate concentration was negatively correlated with systemic vascular resistance (r=-0.78, $p=0.012$) (Figure 1), but not with creatinine concentration (r=-0.03, $p=0.93$).

Continuous infusion of L-NAME (1 mg/kg/h IV) resulted in a maximum increase of mean arterial pressure by 43±5% and systemic vascular resistance by 64±10% (Figure 2). There was a concomitant reduction in cardiac output by 20±4%. The increase in blood pressure (r=0.84, $p=0.001$) and systemic vascular resistance (r=0.81, $p=0.002$) during L-NAME infusion correlated positively with the baseline plasma concentration of ET-1 (Figure 3). The increase in blood pressure with L-NAME did not correlate with plasma nitrite/nitrate level (r=0.12, $p=0.89$). L-NAME infusion for a period of 12 hours did not result in changes in the plasma concentration levels of ET-1 (from 45±7 at baseline to 43±6 pg/mL after 12 h of L-NAME, $p>0.05$) and/or cortisol (from 753±86 at baseline to 682±73 nmol/L after 12 h of L-NAME, $p>0.05$; Figure 4). Plasma nitrite/nitrate also did not change during L-NAME (from 37±5 at baseline to 36±5 μmol/L after 12 h of L-NAME, $p>0.05$).

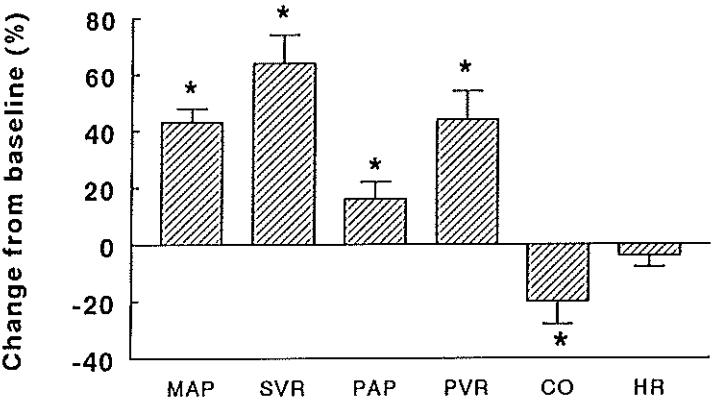

Figure 2. Effect of continuous infusion of N^G-nitro-L-arginine methyl ester (L-NAME, 1 mg/kg/h IV) on hemodynamic variables in patients with severe septic shock. MAP = mean arterial pressure; SVR = systemic vascular resistance; MPAP = mean pulmonary artery pressure; PVR = pulmonary vascular resistance; CO = cardiac output; HR = heart rate. Values are maximum changes as compared to baseline and displayed as mean±SEM.

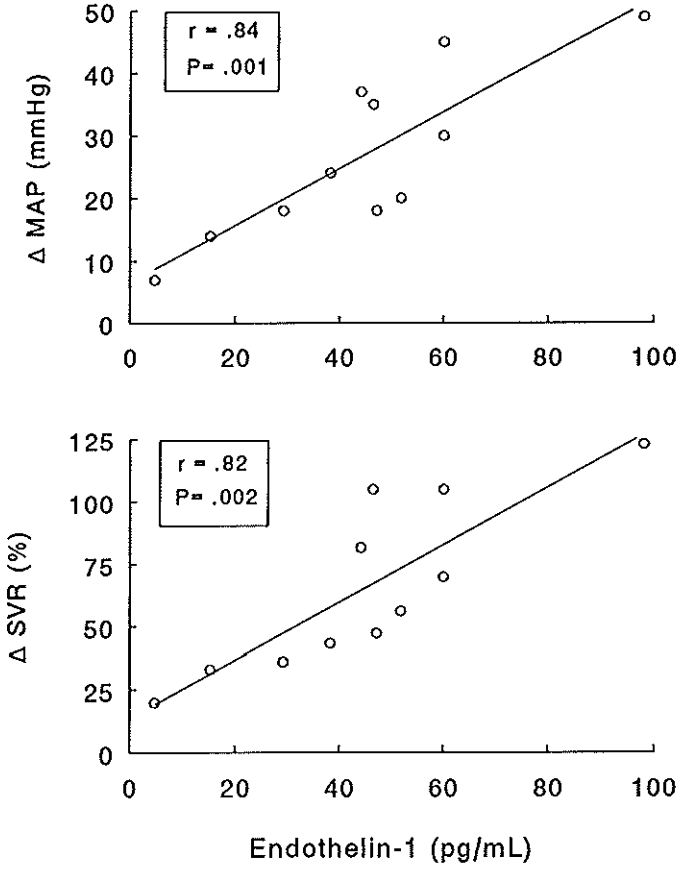

Figure 3. Scatter plots showing relation between plasma endothelin-1 (ET-1) concentration and increase in blood pressure (*upper graph*) and systemic vascular resistance (*lower graph*) during continuous infusion of N^G-nitro-L-arginine methyl ester (L-NAME, 1 mg/kg/h IV) in patients with severe septic shock.

Discussion

Inhibitors of NO synthase have recently been presented as new therapeutic tools in the management of hypotension in human septic shock.[18-20] The present study demonstrates that the increase in blood pressure and the magnitude of the vasopressor response induced by the NO synthase inhibitor L-NAME is related to the plasma level of ET-1 suggesting that inhibitors of NO synthesis unmask a tonic pressor response of ET-1 in human septic shock. In addition this study shows that prolonged inhibition of NO synthesis with L-NAME does not

further increase the already elevated plasma ET-1 concentration.

In the present study elevated plasma levels of ET-1 were found in patients with severe sepsis, as has been reported by others.[21,22] The mechanism involved in the increase of ET-1 concentration during sepsis is largely unknown. High ET-1 levels could have resulted from increased synthesis and/or diminished clearance of ET-1. Levels of circulating cytokines such as tumour necrosis factor (TNF) and interleukins are increased in septic shock and may stimulate ET-1 production in endothelial cells and macrophages.[27,28] Furthermore, intravascular leakage of ET-1 from damaged endothelial cells may have occurred.[13] Since most septic patients had impaired renal function, diminished renal clearance could have played a role.[29,30] However, no correlation was found between plasma ET-1 and creatinine in the present study. This supports the idea for the occurrence of increased generation of endothelin in human sepsis, although other vascular beds, including liver and skeletal muscles, may also contribute to the clearance of ET-1. The finding that high ET-1 levels coincided with high nitrite/nitrate levels as an indirect measure of increased NO production, are not in line with previous data that indicate an inhibitory action of NO on ET-1 release.[1,6] This suggests that the normal interaction between NO and ET-1 is disturbed during sepsis. Abnormalities in the balance between vasoconstricting and vasodilating factors have been reported during sepsis.[13,31] However, at present the exact interaction between ET-1 and NO in septic shock remains to be determined. We hypothesize that the high circulating ET-1 concentration helps to maintain vascular tone during sepsis and opposes the vascular smooth muscle relaxation induced by increased NO production. Indeed, the high circulating ET-1 levels in patients with sepsis, in the present study 45 pg/mL (≈18 pmol/L), are sufficient to induce relevant vasoconstriction.[32,33] The fact that the increase in blood pressure with L-NAME correlated with the level of ET-1 further supports the idea of a continuous vasopressor tone by ET-1 during human sepsis and that this vasopressor tone is unmasked by an inhibitor of NO synthesis. Our findings are consistent with prior reports in normal rats where the increase in blood pressure with L-NAME was reduced with the specific endothelin receptor antagonist bosentan.[9,10] In healthy humans[34] and humans with chronic heart failure,[35] endothelin receptor antagonists can reduce blood pressure. In the present study we did not test the effects of an endothelin receptor antagonist. We speculate that endothelin receptor antagonism during human septic shock,

where plasma levels of ET-1 are high, would be detrimental since it may result in severe hypotension. Whether endothelin receptor antagonists can prevent the increase in blood pressure of NO synthase inhibitors in humans, remains to be determined.

In the present study no increase was found in the plasma levels of ET-1 during 12 hours of inhibition of NO synthesis with L-NAME. These findings may suggest that ET-1 release itself is not modulated by L-NAME in septic patients. In normal rats acute inhibition of NO synthesis results in modest increases in ET-1 levels, but these ET-1 levels were measured only 15 and 35 min after L-NAME infusion.[9,10] In contrast to these findings chronic inhibition of NO synthesis with L-NAME (three weeks) in normal rats does not increase ET-1

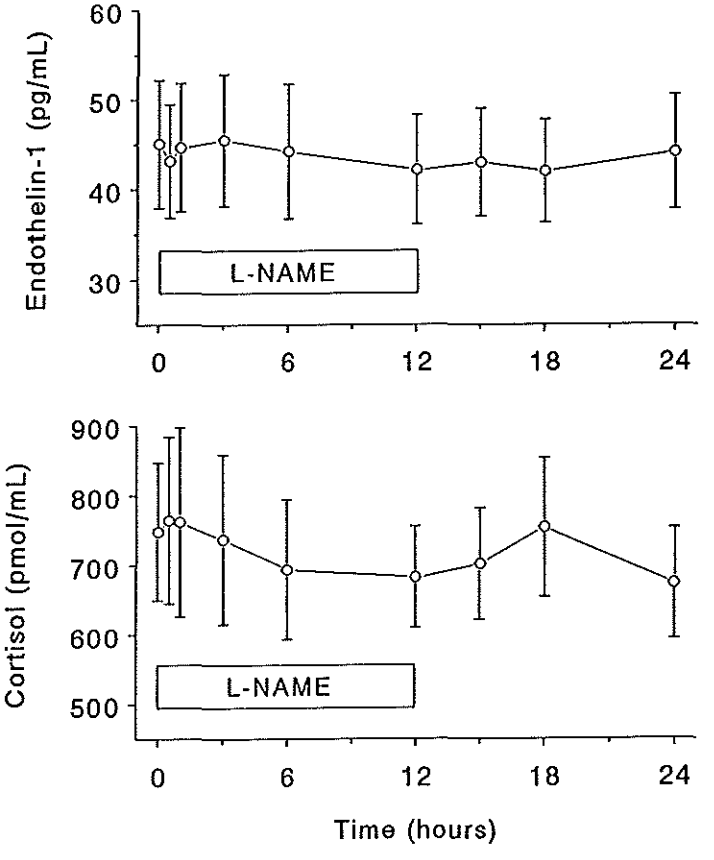

Figure 4. Effect of continuous infusion of N^G-nitro-L-arginine methyl ester (L-NAME, 1 mg/kg/h IV) on plasma concentrations of endothelin-1 (*upper graph*) and cortisol (*lower graph*) in patients with severe septic shock. Time of L-NAME infusion is from T=0 to T=12 hours. No significant changes over time as compared to baseline. Values are mean±SEM.

levels and/or gene expression.[36] Only a few reports are available on the NO-endothelin interaction in humans. Although nitrovasodilators can reduce ET-1 production by human endothelial cells in culture,[37] intravenous infusion of nitroglycerin, a NO donating drug, does not change plasma ET-1 levels in healthy subjects.[11] In a recent study in healthy men, the NO synthase inhibitor L-NMMA temporarily (only after 20 min) increased ET-1 levels from 7.6 to 9.6 pmol/L.[12] In our study we did not find a temporary increase in already elevated ET-1 concentration. One possible explanation is that the continuous high release of ET-1 during sepsis resulted in a depletion of storage vesicles with ET-1 (or its precursor).[38] The results from the present study and previous reports suggest that increased production of ET-1 does not play a major role in the rise in blood pressure during prolonged inhibition of NO synthesis.

However, some reservations must be made regarding interpretation of plasma ET-1 levels. Plasma levels of ET-1 may not correctly reflect production rate, and the local concentration of the peptide at the vascular smooth muscle binding sites is probably more essential. For instance, big endothelin, that needs conversion to endothelin by tissue endothelin-converting enzyme to gain activity, is able to cause vasoconstriction without notable increases in plasma endothelin.[39] Since main ET-1 release seems to occur abluminally, local concentrations of ET-1 are probably higher than in the plasma.[40] Therefore, in the present study increased local production of ET-1 may have occurred with L-NAME without detectable changes in plasma concentration. Similar reservations must be made regarding nitrite/nitrate levels. NO is but one of the ways that nitrite and nitrate are formed and L-NAME infusion did not result in reduced serum levels of nitrite and nitrate, despite increased vasoconstriction. Possibly plasma levels of nitrite and nitrate do not directly reflect the local amount of NO released and active excretion of nitrite/nitrate through kidneys and gastro-intestinal tract may have influenced the plasma levels currently measured.[41]

Cortisol has a vital role in the maintenance of vascular tone and endothelial function.[42] Furthermore cortisol potentiates the vasoconstrictor actions of catecholamines.[43] Thus, increases in plasma cortisol may indirectly increase vasopressor tone. Animal studies have shown that inhibition of NO synthesis with L-NAME stimulates adrenal steroidogenesis.[44,45] We hypothesized that increased cortisol production might be an additional mechanism by which L-NAME increases blood pressure. However, we found no evidence of changes in

cortisol levels during inhibition of NO synthesis in septic patients. We conclude that changes in cortisol levels do not contribute to the increase in blood pressure with L-NAME.

In conclusion, inhibitors of NO synthesis may unmask a tonic pressor response of ET-1 in humans with septic shock. Prolonged inhibition of NO synthesis does not influence the plasma levels of ET-1. These results suggest that ET-1 maintains blood pressure in human sepsis and plasma ET-1 concentration may determine the vasopressor response of NO synthase inhibitors in septic shock. These findings may provide further evidence of interaction between NO and ET-1 in pathological conditions of the cardiovascular system where an imbalance between endothelial-derived vasodilator and vasoconstrictor substances disturbs the normal regulation of vascular tone.

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Chapter 9

Distribution and metabolism of N^G-nitro-L-arginine methylester in patients with septic shock

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Abstract

The present study tested pharmacokinetics of N^G-nitro-L-arginine methylester (L-NAME), an inhibitor of nitric oxide (NO) synthesis, in patients with septic shock. Blood was sampled at intervals before, during and after a 12 h infusion period of L-NAME 1 mg/kg/h in nine septic shock patients for determination of plasma concentrations by high performance liquid chromatography. In three patients renal clearance of the drug was determined.

Incubation of L-NAME with plasma *in vitro* revealed hydrolysis to N^G-nitro-L-arginine (L-NA), an active inhibitor of NO synthesis. L-NA, however, did not undergo further degradation. Continuous intravenous infusion of 1 mg/kg/h of L-NAME for 12 h in patients with septic shock increased blood pressure and resulted in increasing plasma levels of L-NA (C_{max} 6.2±1.0 (SD) μg/ml at 12 h) whereas L-NAME concentrations reached a plateau within 1.5 h (C_{max} 1.0±0.2 μg/ml). After the infusion was stopped L-NAME disappeared from the plasma rapidly (half-life 19.2±2.4 min) whereas L-NA concentration only declined slowly (half-life 22.9±3.0 h). The calculated volume of distribution for L-NAME was 449±119 ml/kg body weight and 1957±270 ml/kg for L-NA. The renal clearance of L-NA was 3.5±0.6% of total body clearance for L-NA. L-NAME could not be detected in urine.

We conclude that inhibition of nitric oxide synthesis with L-NAME in septic patients may result (at least in part) from hydrolysis to L-NA. The high plasma half-life and volume of distribution for L-NA suggests extensive distribution to extravascular tissues. Since renal excretion is minimal, elimination of the metabolite L-NA follows other pathways. This pharmacokinetic information may be useful in future studies using L-NAME or L-NA in humans.

Introduction

Human septic shock is often characterised by massive systemic vasodilatation with low vascular resistance and hypotension unresponsive to treatment with vasopressors.[1] Nitric oxide (NO), a potent endogenous vasodilator formerly known as endothelium derived relaxing factor (EDRF), has recently been held responsible for the hemodynamic and metabolic consequences of sepsis and endotoxemia.[2,3]

Nitric oxide is a small molecule formed from the precursor amino acid L-arginine by two forms of the enzyme NO synthase, and stimulates the enzyme soluble guanylate-cyclase (GC) in the vascular smooth muscle cell which converts GTP to cGMP resulting in vasodilatation.[2] NO normally plays a key role in the regulation of vascular tone. During sepsis and endotoxemia overproduction of the nitric oxide radical results in massive vasodilatation. High concentrations of nitrite and nitrate, the stable end product of NO metabolism, are found in patients with sepsis and these levels may correlate with vasodilation.[4]

Analogues of L-arginine competitively inhibit NO production by NO synthase.[3] N^G-nitro-L-arginine methyl ester (L-NAME), is an analogue of L-arginine, that induces enantiomerically a specific reversible inhibition of NO synthase *in vitro* and *in vivo*.[5,6] L-NAME has been used to reverse endotoxin and cytokine induced hypotension and restore reactivity to catecholamines in animals.[7,8] Recently, L-NAME has been used to reverse hypotension in patients with septic shock.[9,10] Pharmacokinetic studies in rats have shown the conversion of L-NAME to N^G-nitro-L-arginine (L-NA), an active inhibitor of NO synthesis,[11] that has a long plasma half life of approximately 20 hours.[12,13] However, no pharmacokinetic information exists on the clearance, biodistribution or excretion of L-NAME in humans, and the rationale for dosage regimens in septic patients has been largely empirical.[9,10]

The present study assessed the pharmacokinetics of L-NAME *in vitro* and during continuous intravenous (IV) infusion for 12 hours in patients with severe septic shock. Such information about may be useful in further studies using L-NAME or L-NA to inhibit NO synthesis in humans.

Materials and Methods

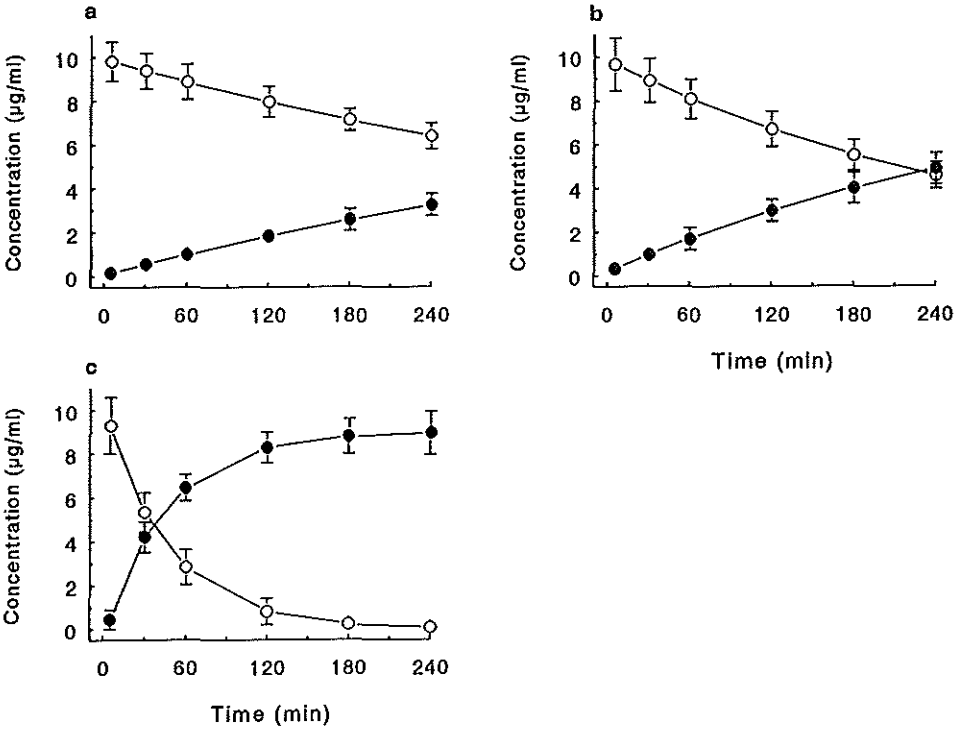

Figure 1. Hydrolysis of L-NAME to L-NA *in vitro*; in buffer (a), plasma (b) and whole blood (c) of septic patients. L-NAME (10 µg/ml) was incubated at 37 °C for the indicated time period, followed by determination of L-NAME (O) and L-NA (●) by h.p.l.c. as described in the Methods section. Samples were assayed in duplo. Data are mean ± SD of four experiments with plasma and blood from four different septic patients.

Subjects. The study was conducted according to the principles established in Helsinki and approved by the hospital Medical and Ethics Committee. First degree relatives were informed of the nature of the study and gave informed consent. In total eleven patients were given L-NAME, and in nine patients plasma samples were collected for pharmacokinetic analysis.

Patients met the criteria of sepsis as described by Bone et al.[14] and were receiving dopamine >15 µg/kg/min and/or noradrenaline >0.1 µg/kg/min. All patients had respiratory failure and required mechanical ventilation. Three patients of nine required continuous hemodialysis because of renal failure. Only patients with a cardiac index >3.0 l/min/m^2 were included since earlier reports have shown reductions of cardiac output during inhibition of NO

synthesis.[7,12] Exclusion criteria for the study were severe coronary artery stenosis (angina pectoris grade III according to NYHA classification), pregnancy and a cardiac index <3.0 l/min/m^2.

Study Protocol. All patients underwent continuous electrocardiographic monitoring and had indwelling radial and pulmonary artery catheters (Criticath, Ohmeda, Singapore) for hemodynamic measurements and data were recorded on a computerised data system (Mennen Medical Systems, USA).

L-NAME hydrochloride was obtained from Sigma Chemicals (St. Louis, MO, USA). The hospital pharmacy prepared a sterile and pyrogen-free solution of L-NAME 10 mg/ml (content L-NA <2% by h.p.l.c. analysis), for infusion. Following baseline measurements L-NAME 1 mg/kg/h was infused for 12 h. The rationale for the continuous infusion resulted from pilot experiments where the effect of a bolus injection of 0.15 mg/kg L-NAME lasted only 5-10 minutes. In prior dose-finding experiments, L-NAME at a rate of 1.5 mg/kg/h resulted in unacceptably high pulmonary artery pressure for which the infusion had to be stopped.[15] Therefore the present study used a lower dose of L-NAME 1 mg/kg/h which avoided in pulmonary hypertension. To minimize the potential chance of developing toxicity from high serum levels of L-NAME, infusion was not continued for longer periods. Concomitant therapy was at the discretion of the clinician managing the patient.

Blood samples. For pharmacokinetic analysis, blood samples (10 ml) were obtained in heparin coated blood collection tubes (Vacutainer, France) at baseline (T=0), during L-NAME infusion (T= 0.5, 1, 3, 6 and 12 h) and 1, 3, 6 and 12, 36 and 72 h after L-NAME infusion had stopped (T= 13, 15, 18, 24, 48, and 72 h). Blood samples were immediately centrifuged (1500 g, 10 min, 4 °C), and the plasma stored at -80° C until tested. In three patients with creatinine clearances above 90 ml/min urine samples were collected at T=12 h for determination of the renal clearances of L-NAME and L-NA. In four untreated septic patients blood (10 ml) was withdrawn for *in vitro* analysis of L-NAME hydrolysis.

In vitro hydrolysis of L-NAME. Aqueous solutions of L-NAME and L-NA (Sigma Chemicals, St. Louis, MO, USA) (10 mg/ml) were prepared and added to phosphate buffer (pH 7.4), plasma and whole blood of septic patients (n=4), to give a final concentration of 10 μg/ml. Samples were shaken in a waterbath at 37 °C over the whole observation period, and

at the each time point (after 5, 30, 60, 120, 180, 240 min and 12 h) aliquots were removed for h.p.l.c. analysis.

Analysis of L-NAME and L-NA. The h.p.l.c method used for quantitative analysis of L-NAME and L-NA has been described previously by Kreycy et al.[12] Blood samples of 200 μl were pipetted into a vial containing 40 μl perchloric acid (4.4 M). Plasma, buffer and urine samples of 100 μl were transferred into vials containing 10 μl perchloric acid (4.4 M). Samples were vortex mixed for 1 min and centrifuged at 16000 g for 10 min. Aliquots of the supernatant were diluted and analyzed in duplo by high performance liquid chromatography. The apparatus used included a gradient pump and a multiwavelength detector set at 268 nm (Kratos Analytical Instruments, Ramsey, New Yersey, USA). For chromatographic separation we used a Nucleosil 5 μm 5 SA 250·4 mm column (Machery Nagel, Düren, Germany). The mobile phase consisted of 100 mM NaH_2PO_4 + 10% methanol (Merck, Darmstadt, Germany), pH 2.3. The flow rate was 1.5 ml/min. Calibration of the method with authentic L-NAME and L-NA, freshly dissolved in 100 mM NaH_2PO_4 (pH 2.3), yielded linear responses of peak areas versus concentration in the range of 0.1 to 50 μg/mL. The detection limit for L-NAME and L-NA was 0.05 μg/mL.

Pharmacokinetic calculations and statistics. Data are expressed as mean $\pm$ SD. The half-life of L-NAME hydrolysis *in vitro* was calculated from linear regression analysis of log concentration versus time plots. In patients receiving L-NAME non-compartmental methods were used to determine pharmacokinetic parameters from the concentration to time data, using commercially available software (Mediware, Heereveen, The Netherlands). Pharmacokinetic parameters included area under the plasma concentrations vs. time curve (AUC), systemic clearance (Cl_s), apparent volume of distribution (V_d), plasma half-life ($t½$), mean residence time (MRT) and renal clearance (Cl_r). Hemodynamic changes were compared with baseline values using Student's paired *t*-test with Bonferroni correction. Pearson correlation was used for analysis of correlations between plasma concentrations and hemodynamic effects.

Results

The *in vitro* data on hydrolysis of L-NAME are displayed in Figure 1. Following

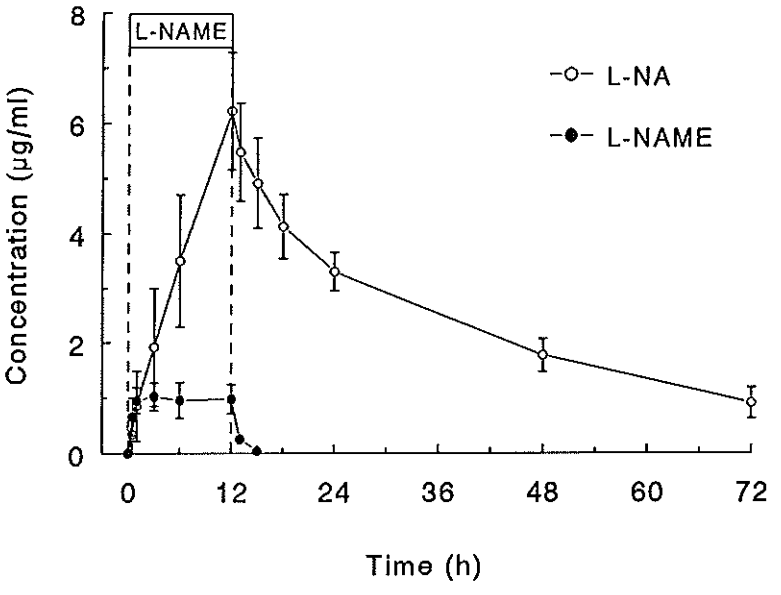

Figure 2. Hydrolysis of L-NAME to L-NA during continuous infusion of L-NAME (1 mg/kg/h IV) in patients with severe septic shock. Time of L-NAME infusion is from t=0 to t=12 h. Blood samples were taken at indicated time points, followed by determination of L-NAME (●) and L-NA (O) by h.p.l.c. as described in the Methods section. Samples were assayed in duplo. Data are mean±SD of plasma samples from nine different septic patients.

addition of L-NAME to buffer (pH 7.4) there was an apparently non-enzymatic hydrolysis to L-NA with a half-life of 383±24 min (Figure 1a). After 12 hours the concentration of L-NAME and L-NA were 2.7±0.3 and 6.6±0.6 μg/ml, respectively. Plasma incubation almost doubled the rate of L-NAME hydrolysis ($t_{½}$=218±7 min) (Figure 1b). After incubation for 12 h in plasma, the concentrations of L-NAME and L-NA were 1.0±0.2 and 8.1±0.7 μg/ml, respectively. In whole blood, L-NAME was metabolized to L-NA at half-life of 33±6 min, and after 3 hours of incubation hydrolysis was essentially complete (Figure 1c). L-NA was stable after incubation for 12 hours in buffer, plasma and whole blood (results not shown).

As shown in Figure 2, continuous intravenous infusion of 1 mg/kg/h of L-NAME for 12 h resulted in increasing plasma levels of L-NA (C_{max} 6.2±1.0 μg/ml) whereas L-NAME concentration reached a plateau within 1.5 h (C_{max} 1.0±0.2 μg/ml). After infusion was stopped L-NAME disappeared from the plasma with a half-life of 19.2±2.4 min whereas L-NA concentration only declined slowly (half-life 22.9±3.0 h). Pharmacokinetic parameters are

displayed in Table 1. L-NA had a high apparent volume of distribution and a relatively low total body clearance. The L-NA concentration in urine determined in three patients was 12±5 μg/ml, whereas L-NAME was undetectable in urine. The renal clearance of L-NA was 2.0±0.3 ml/h/kg and was 3.5±0.6% of total body clearance of L-NA.

Continuous infusion of L-NAME (1 mg/kg/h) increased mean arterial pressure and systemic vascular resistance (Figure 3). Although sustained hemodynamic effects were seen, L-NAME was most effective during the early stages of administration and the effect on blood pressure and vascular resistance tended to diminish during continued infusion. The increases in blood pressure and/or systemic vascular resistance were not significantly correlated with plasma concentrations of L-NAME ($r=0.54$ and $r=0.60$, respectively; both $p>0.05$) or plasma levels of L-NA ($r=-0.018$ and $r=-0.15$, respectively; both $p>0.05$).

Discussion

L-NAME and L-NA are inhibitors of NO synthesis that can be used therapeutically to reverse hypotension in patients with severe septic shock.[9,10] However, most studies in humans have related their dosage regimens on an empirical basis since little pharmacokinetic information on the clearance, biodistribution, and excretion is available.

Table 1. Pharmacokinetic parameters of L-NAME and the metabolite L-NA in patients with septic shock.

Parameter	L-NAME	L-NA
C_{max} (μg/ml)	1.0±0.2	6.2±1.0
$AUC_{0-\infty}$ (μg/ml·h)	12.8±2.5	208±27
CL_s (ml/h/kg)	961±161	59.8±7.0
V_d (ml/kg)	449±97	1957±120
$t_{½}$ (h)	0.32±0.03	22.9±3.0
MRT (h)	0.46±0.04	32.9±4.2

L-NAME (1 mg/kg/h IV) was infused for 12 hours in nine patients with severe septic shock. L-NAME = N^G-nitro-L-arginine methyl ester, L-NA = N^G-nitro-L-arginine, C_{max} = maximum plasma concentration, AUC = area under curve, CL_s = systemic clearance, V_d = volume of distribution, $t_{½}$ = plasma half-life, MRT = mean residence time. Values are mean±SD.

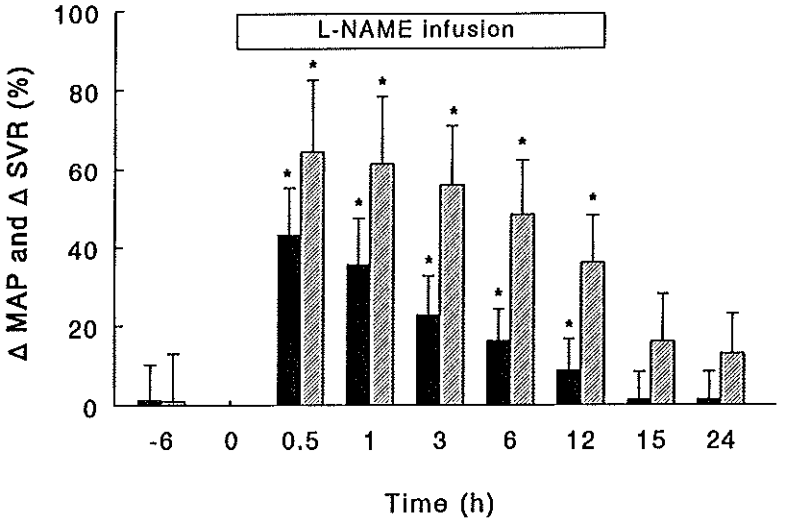

Figure 3. Effect in time of continuous infusion of L-NAME (1 mg/kg/h IV) on mean arterial blood pressure (MAP, *solid bars*) and systemic vascular resistance (SVR, *hatched bars*) in patients with severe septic shock. Data are displayed as % increase compared to baseline (T=0). Data are mean±SD of nine septic patients. $*p<0.05$ as compared to baseline.

The present study shows that in humans with sepsis L-NAME is rapidly hydrolysed to L-NA both *in vitro* and *in vivo*. Since hydrolysis was almost doubled in plasma as compared to buffer, plasma esterases may contribute to the apparently non-enzymatic hydrolysis in buffer as has been reported by others.[12,16] Metabolism of L-NAME was faster in whole blood which could suggest that L-NAME is mainly metabolised by blood cell esterases. Since the *in vivo* half-life of L-NAME in septic shock patients was even shorter ($t_{½}=19$ min), other cells, e.g. vascular endothelial cells or hepatic cells, may have contributed to intracellular conversion of the drug by esterases. In the anaesthetized rabbit the calculated half-life of L-NAME following bolus injection was only 7.5 min suggesting that species differences exist in esterase activity.[17]

The conversion of L-NAME to L-NA may be accompanied by the formation of neurotoxic methanol.[18,19] In the present study methanol was not measured and although no obvious signs of neurotoxicity were noticed in the present study, conversion to methanol may have occurred. If so, it may hinder the clinical application of L-NAME as an inhibitor of NO synthesis and the direct use of L-NA may be superior to the use of L-NAME.[20] However, maximum conversion of L-NAME (total dose 900 mg in 12 h, body weight 75 kg) would have

generated approximately 0.15 g of methanol, which is 30% of total daily endogenous methanol production in humans,[21] and unlikely to cause any toxic manifestations.

High concentrations of L-NA were found at the end of the L-NAME infusion and these only declined slowly after the L-NAME infusion was stopped. The high apparent volume of distribution (almost 2 l/kg) for L-NA together with relatively low plasma clearance are reflected by an extremely high plasma half-life of around 22 h. Similar findings were observed in pharmacokinetic studies in rats given L-NA. In a study by Tabrizi et al.[13] after a single intravenous bolus of L-NA 10 mg/kg, plasma volume of distribution was 2.5 l/kg which is comparable to that found in man in the present study. These findings indicate extensive extravascular distribution. In the same animal study L-NA was not detected in urine. In contrast, we found concentrations of L-NA up to 18 μg/ml in urine of patients. This accounted for less than 5% of the total clearance of L-NA, and renal excretion is clearly not a major pathway of L-NA excretion. L-NA may be cleared primarily by biliary excretion,[22] or further conversion to unknown metabolites.

The long half-life of L-NAME has important clinical implications. Recent animal studies on NO synthase inhibitors suggest that continuous infusions may be superior to high dose bolus administration.[23] In the present study a continuous infusion was used; to minimize the risk of toxicity it was not continued for more than 12 hours. Although a steady state concentration of L-NAME was reached during infusion, the long plasma half-life for L-NA will cause a continuous increase so that a steady state plasma concentration will not be reached before at least 60 hours of infusion. Therefore, with prolonged infusions of L-NAME or L-NA caution is warranted,[9,10] since L-NA will accumulate.

Prior *in vitro* studies suggest that L-NAME is an inactive pro-drug and that L-NA is the only active component.[16,24] Unexpectedly, in the present study no correlation was found between plasma concentrations of the active drug L-NA and increases in blood pressure and/or systemic vascular resistance. In addition, blood pressure and systemic vascular resistance tended to diminish during continued L-NAME infusion, despite increasing plasma concentrations of L-NA. At present we cannot explain these findings. Plasma concentrations of L-NAME and L-NA may not accurately reflect the local concentrations in effector cells, i.e. endothelial cells and vascular smooth muscle cells. Indeed, several in vitro studies have

suggested that L-NAME easily enters the intracellular compartment whereas L-NA does not.[12] Once inside the cell, L-NAME may be converted by esterases to L-NA which is then "trapped" in the cell. By this mechanism L-NA may accumulate intracellularly and only slowly redistribute to the plasma.[25] These speculations should be further investigated with measurement of the intracellular concentration of the drug.

In summary, we have characterized some of the fundamental pharmacokinetic characteristics of L-NAME and L-NA in patients with severe septic shock. These results may help to develop rational designs for further studies.

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Chapter 10

General discussion

General discussion

Septic shock is the most common cause of death in todays intensive care.[1,2] Despite progress in antibiotic therapy, new therapeutic interventions and better intensive care support, mortality from septic shock has remained high over the years. Therefore new treatment modalities are warranted. Endotoxin and cytokine stimulated overproduction of nitric oxide (NO) from L-arginine has been suggested to play a central role in the cardiovascular derangements of septic shock. Since NO has emerged as a final common mediator leading to vasodilatation in shock, pharmacologic manipulation of NO production may be used as a novel therapeutic approach in the treatment of hypotension in septic shock. However, the exact role of NO during sepsis is yet unclear and the consequences of inhibition of NO synthesis remain to be determined. The objectives of our studies were:

1. To investigate the pathophysiological role of NO in vasodilatation in an animal model of hyperdynamic sepsis.
2. To investigate the pathophysiological role of NO in patients with hyperdynamic sepsis.
3. To investigate the hemodynamic, metabolic and immunologic effects of L-NAME, an inhibitor of NO synthesis, in patients with septic shock.

A hyperdynamic model of endotoxemia in the rat was developed that used intraperitoneal injection of lipopolysaccharides (LPS). After 12 hours this model resulted in raised temperature, reduced blood pressure, increased levels of plasma lactate, and increased plasma concentration of nitrate and nitrite (NO_x), the stable end products of NO metabolism. These changes resemble much of the changes as found in patients with sepsis.[3,4] Furthermore this model has the advantage that allows induction of i-NOS which has been shown to take a lag-phase of at least 4 to 8 hours needed for *de novo* synthesis of protein.[5] Several others have used models of LPS and cytokine-induced shock where the time between induction of shock and initiation of experiments is too short for the inducible NO synthase to form. In endotoxemic dogs two hours following IV injection of LPS or TNF, inhibition of NO synthesis resulted in correction of hypotension.[6,7] However, shock in this animal model was probably not caused by increased production of NO by i-NOS but rather by other mechanisms. Direct cardiac depression or increased production of NO by the constitutive enzyme may have played

a role.[8] Although LPS injection in humans may result in many of the characteristics of sepsis, LPS injection is only a simplified model of the clinical syndrome of sepsis and septic shock.[9] Chronic animal models of sepsis such as prolonged infusion of live bacteria or slow release from an implanted, infected clot may better simulate the sequence of events associated with clinical sepsis.[10,11]

During isolated perfusion of the rat heart we observed increased coronary flow rates with dysfunction of coronary autoregulation. Similar inappropriate coronary flow rates can be seen in patients with sepsis and these changes resemble the changes in the peripheral vasculature.[12] The vasodilatation and dysfunction of vascular autoregulation, were normalised with an inhibitor of NO synthesis and methylene blue. These findings confirm the central role for NO in cardiovascular derangements during septic shock and the therapeutic potential of inhibitors of NO synthesis. However, endotoxemia promoted myocardial ischemia in vulnerable areas of the heart following inhibition of the NO pathway or direct vasoconstriction (Chapter 2). This local myocardial ischemia may have resulted from pathological heterogenous microcirculatory flow distribution as has been found by others during experimental sepsis.[13,14] Possibly increased nitric oxide production prevents these vulnerable areas from becoming hypoxic. This could suggest that part of increased NO release during sepsis may be beneficial and inhibition of NO synthesis could be detrimental. Indeed, many animal studies have shown controversial data on the use of pharmacological inhibitors of NO synthesis. Inhibition of NO synthesis can raise mortality[15] and increase damage to the liver,[16] gastrointestinal tract[17] and kidney[18] in animal models of shock. However, most of these studies use models of sepsis and endotoxemia that cause a hypodynamic circulation with reduced cardiac output. In this situation there is already an underperfusion of the organs in contrast to hyperdynamic septic shock. Additional vasoconstriction by inhibition of NO synthesis can, in this situation, lead to a further reduction in cardiac output and organ perfusion. Therefore inhibition of NO synthesis might be less effective in hypodynamic shock.

The presented *in vitro* studies were designed to investigate the role of NO in the pathophysiology of sepsis. Although the findings from *in vitro* and *in vivo* animal studies cannot be directly extrapolated to the clinical situation, their data can used in the design of clinical studies to test the therapeutic efficacy of NO synthase inhibition in human sepsis.

Prolonged inhibition of NO synthesis with L-NAME 1 mg/kg/h in hyperdynamic septic patients resulted in increased blood pressure and systemic vascular resistance with a concomitant reduction in cardiac output (Chapter 4 and 5). Reductions in cardiac output have been reported by others during inhibition of NO synthesis in sepsis.[19,20] Therefore, in the presented study we only included patients with hyperdynamic shock and increased cardiac output. So far the precise mechanism underlying this reduction in cardiac output remains unclear. In our study the reduction in cardiac output did not result from direct cardiac depression since cardiac work was not compromised and was probably related to a reflex change due to the increased systemic vascular resistance and afterload. The reduction in cardiac output may also have contributed to the reduction in blood pressure during continued L-NAME infusion. In a recent study by Kiehl et al.[21] in leucocytopenic septic patients, L-NAME in a lower dosage of 0.3 mg/kg/h did not result in significant changes in cardiac output and the increase in blood pressure was maintained for at least 24 h. Possibly the dosage we used in the present study was to high for optimum hemodynamic effects and a lower dose, with lesser effects on cardiac output, could be more effective. This could be further investigated in additional dose-response studies. To prevent the reduction in cardiac output and augment the anti-hypotensive effects of L-NAME and other inhibitors, co-infusion of the inotropic agent dobutamine may be a possible solution.[22]

Despite the reduction in cardiac output in our study, no detrimental effects on organ function indices were seen. Furthermore arterial lactate and oxygen consumption were not changed (Chapter 4 and 5). These findings may indicate that a reduction in cardiac output is not necessarily detrimental in hyperdynamic sepsis and suggest that L-NAME preferentially caused vasoconstriction in metabolic inactive tissues. However, these observations only include indirect parameters that do not directly reflect what happens at capillary or cellular level. However, in clinical practice these indirect macrovascular parameters are mostly the only parameters available. Microvascular parameters such as tissue pO_2, microvascular flow patterns and tissue redox state are not routine clinical measurements and not often measured in patient related studies because of the invasive nature of most techniques, the need for special equipment and the time consuming nature of the measurements. In future studies it would be interesting to investigate the effect of L-NAME and other inhibitors of nitric oxide synthesis,

on microvascular parameters of different organs in human sepsis or appropriate animal models of hyperdynamic sepsis. Microvascular parameters could be studied with oxygen electrodes to measure tissue pO_2, intravital microscopy to measure microvascular flow patterns and optical spectroscopy to measure tissue redox state.[23]

NO has a role as a physiologic mediator in the lung.[24] NO synthase is present in lung epithelium and other pulmonary cells and has been suggested to be a mediator of nerve-dependent bronchodilatation.[25] Furthermore hypoxic pulmonary vasoconstriction may result from reduced NO release.[26] Inhalation of NO can be used to improve oxygenation in patients with ARDS and reduce pulmonary artery pressure in patients with pulmonary hypertension.[27] In two patients a high continuous dose of L-NAME resulted in severe pulmonary hypertension for which infusion had to be stopped (Chapter 6). Pulmonary hypertension is dangerous since it may cause right sided heart failure and circulatory collapse. Although lower doses of L-NAME increased pulmonary vascular resistance, no signs of pulmonary hypertension were seen in these patients (Chapter 5). We conclude that pulmonary hypertension is a dose-related side effect of L-NAME infusion. Inspired NO gas acts locally without systemic effects and co-administration of inspired NO may be used to prevent the pulmonary hypertension during inhibition of NO synthesis.[28] Since NO inhalation may improve oxygenation in respiratory insufficiency we hypothesized that inhibition of NOS may compromise pulmonary gas exchange. Unexpectedly, we found that pulmonary gas exchange was improved during inhibition of NO synthesis probably through a reduction in pulmonary right-to-left shunt with L-NAME and an improvement of ventilation-perfusion mismatch.

Most inhibitors of NO synthase such as L-NA, L-NAME and L-NMMA are non-selective toward the different isotypes of NO synthase and inhibit both the constitutive and the inducible isoform. *In vitro* L-NAME even shows some preference towards the constitutive enzyme.[29] Some have been related negative side effects of NO synthase inhibition to inhibition of the constitutive isoform of NO synthase and selective inhibition of the inducible enzyme could be more effective. In theory the inhibition of only the i-NOS may attenuate the excessive vasodilation, correct hypotension and prevent the toxic effects of increased NO synthesis without interfering in physiologic NO mediated processes, whereas inhibition of the constitutive enzyme may lead to the disturbance of physiologic processes regulated by NO and

may lead to tissue hypoperfusion and ischemia. However, this hypothesis has never been tested in hyperdynamic models of sepsis. Recently identified, non-arginine based NOS inhibitors, such as aminoguanidine[30] and S-methyl isothiourea and related compounds[31] may show selectivity for the inducible enzyme. Aminoguanidine can inhibit delayed circulatory failure in endotoxic shock in the anaesthetized rat.[32] S-methyl-isothiourea sulphate, a novel potent inhibitor of i-NOS, can improve survival in a rodent model of septic shock.[31] Recent reports however, suggest that the constitutive form of nitric oxide synthase disappears in endotoxemia and other forms of sepsis.[33,34] If this is true, the use of an unselective inhibitor of NOS would be as effective as an inhibitor specific to the inducible isoform. In addition, inhibition of inducible NOS could be harmful to immune defense and bactericidal mechanisms which could be extra important in case of sepsis.[35] The theoretical benefit of selective inhibitors of the inducible NO synthase has not been established in human sepsis and this needs to be clarified in further studies. Furthermore these selective inhibitors may prove to be of help in other disease states where the inducible NO synthase plays a role (Chapter 1).

Not much attention has been given to the immunologic consequences of NO synthase inhibition during septic shock. Activated macrophages release NO for the killing of bacteria and fungi,[35] although it is not yet clear or the same mechanism is present in humans.[36] Nitric oxide may inhibit leucocyte adherence, regulate gene expression of certain cytokines such as TNF-α and IL-6, and inhibit or facilitate T-cel proliferation.[37,38] Induction of i-NOS may be associated with graft rejection.[39] Excessive production of pro-inflammatory cytokines have been found during inhibition of NO synthesis in septic animals.[40,41] These results may indicate a negative feedback mechanism exhibited by NO on cytokine production. In contrast to these findings, we found no evidence of excessive cytokine production during prolonged inhibition of NO synthesis in human sepsis (Chapter 7). Although TNF-α levels were reduced as compared to baseline this may have represented the natural course of the disease since no untreated control group was included for comparison.

Septic patients had increased plasma levels of nitrite and nitrate, as an indirect measure of increased NO production,[3] and these levels correlated with vasodilatation (Chapter 7). However, reservation must be made since NO is but one of the ways that nitrite and nitrate are formed and unexpectedly L-NAME infusion did not result in significant changes in

nitrite/nitrate levels. Probably plasma nitrite/nitrate levels do not accurately reflect the local production of NO. This has to be verified in further studies.

Besides nitric oxide, other vasoactive mediators are released during human septic shock.[4] In the present study endothelin-1 (ET-1), a potent endothelial derived vasoconstrictor substance, was increased in septic patients and ET-1 levels determined the extent of the anti-hypotensive response to L-NAME in these patients (Chapter 8). These findings may indicate that L-NAME unmasks a tonic pressor response to ET-1 in septic shock and that measurement of ET-1 levels before treatment may help to select these patients that benefit most from inhibition of NO synthesis in septic shock.

In the present study mortality rate was almost 60% in these patients with severe sepsis. This mortality rate was in line with predicted death rate as computed by APACHE II scores. Although hemodynamics were improved by L-NAME, this did not result in mortality lower or higher than the predicted death rate. We could speculate that L-NAME did not influence mortality in the present study. However these are only speculations since it is impossible to determine the impact on survival in an 11 patient study and this study was not designed to do so. Furthermore we did not include an untreated control group for comparison which is a major limitation of the studies. To determine the effect of L-NAME on mortality, a randomised placebo controlled (multicenter) study with much larger numbers of patients would be necessary. For L-NMMA such a study is currently undertaken. L-NMMA has the theoretical advantage that acts more on the inducible enzyme than L-NAME, although this may have immunologic disadvantages as discussed earlier. Preliminary data in 312 patients show that L-NMMA (546C88, Glaxo Wellcome) 0-20 mg/kg/h resulted in a significant increase in the proportion of patients with resolution of shock at 72 h (24% in the L-NMMA group compared to 39% in the placebo group).[42] Since this surrogate end point correlated with survival, it was suggested that L-NMMA may improve survival. However overall survival at 28 days was not significantly different between groups (83 survivors in the L-NMMA group compared 80 in the placebo group). The final results of the phase III clinical trial must be awaited to see whether inhibition of NO synthesis with L-NMMA can improve survival in patients with septic shock.

Increased synthesis of nitric oxide is only one of many factors contributing to the

derangements in the pathophysiological processes of septic shock (Chapter 1). Inhibition of nitric oxide synthesis is probably not the 'magic bullet' for single treatment of sepsis. In our opinion, inhibition of nitric oxide synthesis alone will most likely not be sufficient in stopping the cascade of events leading to overactivation of the immune system in this complicated disease process. In the last years, more than a dozen other magic bullet candidates have failed to improve outcome of sepsis in randomized placebo-controlled clinical trials.[43] However, a modest effect on outcome by a single agent may provide a piece of the puzzle and increase the depth of our understanding of this disease process. Besides adequate removal of the infective focus, ultimately a combination of approaches that attack vasodilatation, cardiac depression, cytokine imbalance, multiple organ damage and metabolic disturbances may be needed to treat septic shock. Further investigations should be performed to gather the pieces and eventually solve the puzzle.

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Summary

Chapter 1 is the general introduction of this thesis. The characteristics and pathophysiology of septic shock and the central role that nitric oxide (NO) may play are described. Sepsis is the systemic response to infection and characterised by the release of several inflammatory mediators leading to massive vasodilatation, shock and organ failure. Nitric oxide (NO), formerly known as Endothelium Derived Relaxing Factor (EDRF), a powerful endogenous vasodilator, is released in high quantities during sepsis. NO may be responsible for much of the vasodilatation and hypotension seen during sepsis and NO may be involved in myocardial depression during septic shock. The release or action of NO can be inhibited by pharmacologic agents that intervene with the L-arginine/NO/c-GMP pathway and these pharmacologic agents may be used to investigate the role of nitric oxide in the pathophysiology of sepsis and other diseases. Furthermore, these pharmacologic agents may offer new therapeutic potential in the treatment of hypotension in septic shock patients. At the end of Chapter 1 the aims of the study are presented. We investigated the pathophysiological role of NO and the effect of NO synthase inhibitors in experimental and clinical sepsis.

Chapter 2 shows the role of nitric oxide during hyperdynamic endotoxemia in the rat as a model for sepsis. Similar to human sepsis, this experimental model resulted in raised temperature, decreased mean arterial blood pressure, increased heart rate, hyperlactatemia and increased serum levels of nitrite+nitrate, as a sign of increased systemic release of NO. Isolated perfusion of the hearts showed increases in coronary flow and oxygen consumption following endotoxemia that resulted from increased NO production. N^G-Nitro-L-Arginine (L-NA), an inhibitor of NO synthesis, and methylene blue (MB), an inhibitor of soluble guanylate cyclase, could prevent coronary vasodilatation, although this resulted in local myocardial ischemia in endotoxin-treated hearts. These data suggest that vasodilatation in endotoxemia results from increased NO production which can be prevented by inhibitors of NO synthesis. However, vasoconstriction may result in myocardial ischemia in vulnerable areas of the endotoxic heart which warrants caution when using NO synthase inhibitors in clinical sepsis.

Chapter 3 describes coronary autoregulation in the mentioned model of hyperdynamic endotoxemia in the rat. Autoregulation was studied in the isolated rat heart by analyzing flow-

pressure relations and reactive hyperemia after occlusion of coronary flow. Endotoxemia resulted in dysfunction of coronary autoregulation and altered reactive hyperemia. The dysfunction in vascular autoregulation was corrected with L-NA and MB, but not with vasopressin. These findings suggest that endotoxemia results in dysfunction of vascular autoregulation by increased production of nitric oxide which can be corrected by inhibition of NO synthesis but not by direct vasoconstriction.

Chapter 4 shows the results of a prospective clinical study that evaluated the effects of prolonged inhibition of NO synthesis with the NO synthase inhibitor L-NAME in patients with severe septic shock. The 'L-NAME trial' was conducted in the intensive care units of the department of Surgery and the department of Internal Medicine of the University Hospital Rotterdam Dijkzigt. Patients were included in the period between January 1995 and February 1996. In this chapter the effects on hemodynamic, biochemical and metabolic parameters are presented during continuous L-NAME infusion in eleven patients with septic shock. L-NAME increased blood pressure and systemic vascular resistance with a concomitant reduction in cardiac output and oxygen supply. Despite the reduction in cardiac output, no negative effects on organ function indices were found. Although sustained hemodynamic effects were seen, L-NAME was most effective during the early stages of administration and the effect on blood pressure and vascular resistance tended to diminish during continued infusion.

Chapter 5 presents the effects of continuous L-NAME infusion on cardiac performance and pulmonary gas exchange. Despite the reduction in cardiac index no reduction was seen in left and right ventricular stroke work indices which makes direct cardiac depression unlikely. The increase in afterload is probably the main reason for the reduction in cardiac output. Analogous to the effects on the peripheral vasculature, L-NAME resulted in pulmonary vasoconstriction as indicated by increased pulmonary vascular resistance. However, this did not result in pulmonary hypertension and unexpectedly pulmonary gas exchange was improved during L-NAME infusion. The reason for this was a reduction in pulmonary right-to-left shunt and improvement of ventilation-perfusion mismatch. Nitric oxide appears to play a role in the cardiovascular derangements during human sepsis and inhibitors of NO synthesis may provide new therapeutic tools for the treatment of unresponsive hypotension in septic shock.

Chapter 6 describes the dose related side effects of L-NAME in a postoperative patient

with severe septic shock. High dose continuous infusion of L-NAME resulted in severe pulmonary hypertension and an extreme reduction in cardiac output. For these reasons L-NAME was stopped after three hours of infusion. We conclude that pulmonary hypertension and reduced cardiac output are major dose related side effects of NO synthase inhibition. In clinical situations, such as severe septic shock refractory to conventional therapy, blocking the NO system must be done with caution and at least under careful cardiovascular monitoring.

Chapter 7 analyzes some of the immunologic consequences of NO synthase inhibition in human sepsis. Septic patients had increased levels of IL-6, IL-8 and TNF-α. During continuous L-NAME infusion no signs of detrimental increases in plasma cytokine levels were found. Plasma IL-6 and IL-8 levels were not significantly changed, whereas plasma TNF-α concentration was reduced as compared to baseline. However, these observations may have been confounded by spontaneous changes over time. Plasma nitrite+nitrate (NO_x) levels, as an indirect measure of NO production, were increased in septic patients and these levels correlated with vascular resistance. However, NO_x levels were not significantly reduced during L-NAME infusion which may indicate that NO_x does not directly reflect local NO production.

Chapter 8 shows that part of the vasoconstriction with L-NAME in human septic shock results from increased plasma levels of endothelin-1 (ET-1). Septic patients had increased plasma levels of ET-1. No significant changes in plasma ET-1 concentration were observed during L-NAME infusion. However, the increase in blood pressure and systemic vascular resistance was significantly correlated with the plasma level of ET-1, but not with plasma nitrite+nitrate concentration. These findings may suggest that inhibitors of NO synthesis unmask a tonic pressor response of endothelin-1 in human septic shock.

Chapter 9 evaluates pharmacokinetic information on the clearance, biodistribution, and excretion of L-NAME in septic patients. Analysis of samples was done using high performance liquid chromatography (HPLC). L-NAME was converted to L-NA both *in vitro* and *in vivo*. A high plasma half-life and volume of distribution was found for L-NA *in vivo* suggesting extensive distribution to extravascular tissues. Since renal excretion was minimal, elimination of the metabolite L-NA follows other pathways. We conclude that inhibition of NO synthesis with L-NAME results at least in part from hydrolysis to L-NA. This pharmacokinetic information may be useful in future studies using L-NAME or L-NA.

Samenvatting

Hoofdstuk 1 is de algemene inleiding van dit proefschrift. In dit hoofdstuk worden de kenmerken en pathofysiologie van septische shock beschreven en de centrale rol die stikstofoxide hierin speelt. Sepsis is de reactie van het lichaam op infectie en wordt gekenmerkt door het vrijkomen van verschillende ontstekingsmediatoren die leiden tot massale vaatverwijding, shock en orgaan falen. Stikstofoxide (NO), eerder bekend als Endothelium Derived Relaxing Factor, is een krachtige endogene vaatverwijder, die tijdens sepsis in grote hoeveelheden vrijkomt. NO is mogelijk de oorzaak voor de vaatverwijding en hypotensie tijdens sepsis en speelt mogelijk een rol in de verminderde pompfunktie van het hart tijdens septische shock. De produktie en het effect van NO kan geremd worden door farmaca die het L-arginine/NO/c-GMP pad beïnvloeden en deze stoffen kunnen gebruikt worden om de rol van NO in het onstaan van sepsis en andere ziektebeelden te onderzoeken. Tevens zouden deze farmaca als geneesmiddel gebruikt kunnen worden in de behandeling van hypotensie bij patiënten met septische shock. Aan het eind van hoofdstuk 1 worden de doelstellingen van het onderzoek beschreven. Wij onderzochten de pathofysiologische rol van NO en het effect van NO synthese remming in experimentele en klinische sepsis.

Hoofdstuk 2 beschrijft de rol van NO tijdens hyperdynamische endotoxemie in de rat als model voor sepsis. Zoals bij humane sepsis, resulteerde dit diermodel in koorts, verminderde arteriële bloeddruk, verhoogd serum lactaat en verhoogd serum nitraat+nitriet gehalte, een indirecte maat voor verhoogde systemische produktie van NO. Geïsoleerde perfusie van het rattenhart liet een verhoogde coronaire doorstroming en zuurstof consumptie zien na endotoxemie die het gevolg was van verhoogde NO produktie. N^G-Nitro-L-Arginine (L-NA), een NO synthese remmer, en methyleen blauw (MB), een remmer van guanylate cyclase, voorkwamen de coronaire vaatverwijding, hoewel hierbij lokale myocardiale ischemie optrad in endotoxine behandelde harten. Deze bevindingen laten zien dat vaatverwijding tijdens endotoxemie wordt veroorzaakt door verhoogde NO produktie hetgeen voorkomen kan worden met NO synthese remmers. Echter, vasoconstrictie kan leiden tot lokale myocard ischemie in kwetsbare gebieden in het endotoxine hart en voorzichtigheid is derhalve geboden indien NO synthese remmers tijdens klinische sepsis worden gebruikt.

Hoofdstuk 3 gaat in op de coronaire autoregulatie in het beschreven endotoxemie model in de rat. Autoregulatie werd bestudeerd in het geïsoleerde rattenhart door de relatie tussen flow en perfusiedruk en door de reactieve hyperemie na occlusie van flow te analyseren. Endotoxemie resulteerde in een verstoorde coronaire autoregulatie en veranderde reactieve hyperemie. De verstoorde vasculaire autoregulatie kon gecorrigeerd worden met L-NA en MB, maar niet met de directe constrictor vasopressine. De bevindingen laten zien dat endotoxemie leidt tot een verstoorde vasculaire autoregulatie als gevolg van verhoogde NO produktie welke gecorrigeerd kan worden door NO synthese remmers.

Hoofdstuk 4 toont de resultaten van een klinische studie die de effecten onderzocht van langdurige NO synthese remming met de NO synthase remmer L-NAME in patiënten met ernstige septische shock. De 'L-NAME trial' werd uitgevoerd op de intensive care van de afdelingen chirurgie en interne geneeskunde in het Academisch Ziekenhuis Rotterdam Dijkzigt in de periode tussen januari 1995 en februari 1996. In dit hoofdstuk worden de effecten van continue L-NAME toediening op hemodynamische, biochemische en metabole parameters beschreven in elf patiënten met septische shock. L-NAME verhoogde bloeddruk en systemische vaatweerstand met een gelijktijdige reductie in cardiac output en zuurstoftoevoer. Ondanks deze reductie in cardiac output werden geen negatieve effecten gezien op orgaanfunctie gerelateerde parameters. Hoewel langdurige hemodynamische effecten gezien werden, was L-NAME het meest effectief in de begin periode van toediening en het effect op bloeddruk en vaatweerstand werd geleidelijk minder.

Hoofdstuk 5 laat de effecten zien van continue L-NAME toediening op myocardfunctie en pulmonale gasuitwisseling. Ondanks de vermindering in cardiac output werd geen vermindering gezien in slagarbeid van linker en rechter ventrikel hetgeen directe cardiale depressie onwaarschijnlijk maakt. De toename in afterload is waarschijnlijk de belangrijkste reden voor de afname in cardiac output. Analoog aan het effect op de perifere vasculatuur, was er sprake van pulmonale vasoconstrictie met verhoging van de pulmonale vaatweerstand, echter zonder pulmonale hypertensie. Tevens was er een onverwachte verbetering in pulmonale gas uitwisseling. De reden voor de verbeterde arteriële oxygenatie was een vermindering in de pulmonale rechts-naar-links shunt. Wij concluderen dat NO een rol speelt in de cardiovasculaire stoornissen tijdens humane sepsis. Remmers van NO synthese kunnen mogelijk als

nieuwe therapie gebruikt worden in de behandeling van hypotensie tijdens septische shock.

Hoofdstuk 6 beschrijft de dosis gerelateerde bijwerkingen van L-NAME in een post-operatieve patiënt met septische shock. Tijdens continue infusie met een hoge dosis L-NAME ontstond ernstige pulmonale hypertensie met een extreme reductie van cardiac output. Om deze redenen werd L-NAME na 3 uur toediening gestaakt. Pulmonale hypertensie en reductie in cardiac output zijn twee belangrijke dosis-afhankelijke bijwerkingen van NO synthese remming in de klinische situatie. Het gebruik van NO synthese remmers in patiënten moet daarom met voorzichtigheid gebeuren en tenminste onder cardiovasculaire bewaking op een intensive care.

Hoofdstuk 7 analyseert de mogelijk immunologische consequenties van NO synthese remming in humane sepsis. Septische patiënten hadden verhoogde plasma concentraties van interleukine-6 (IL-6), IL-8 en tumor necrosis factor-α (TNF-α). Tijdens continue L-NAME infusie werden geen tekenen gevonden van verhoogde cytokine produktie. Plasma IL-6 en IL-8 waarden veranderden niet significant tijdens L-NAME en plasma TNF-α concentratie was verminderd vergeleken de uitgangswaarde. Echter deze metingen kunnen beïnvloed zijn door spontane veranderingen van cytokine concentraties in de tijd. Plasma nitraat+nitriet concentratie, als een indirecte maat voor NO synthese, was verhoogd in patiënten met sepsis en deze concentratie correleerde met de systemische vaatweerstand. Nitraat+nitriet concentratie verminderde echter niet tijdens L-NAME infusie hetgeen kan betekenen dat plasma nitraat+nitriet gehalte mogelijk geen goede maat is voor lokale NO produktie.

Hoofdstuk 8 laat zien dat een deel van de vasoconstrictie door L-NAME in humane septische shock wordt veroorzaakt door verhoogde plasma concentraties van endotheline-1 (ET-1). Sepsis patiënten hadden hoge ET-1 spiegels in het bloed. Tijdens L-NAME toediening werden geen veranderingen gezien in plasma concentratie ET-1. De toename in bloeddruk en systemische vaatweerstand waren duidelijk gecorreleerd met de hoogte van het plasma ET-1 niveau, echter niet met het nitraat+nitriet gehalte. Deze bevindingen laten zien dat de bloeddruk stijging door NO synthese remming in humane septische shock gedeeltelijk verloopt via een continue vaattonus door ET-1.

Hoofdstuk 9 bestudeert de farmacokinetiek van L-NAME in septische shock. Voor analyse van de monsters werd gebruik gemaakt van high performance liquid chromatography (HPLC). L-NAME bleek omgezet te worden naar L-NA, waarschijnlijk door esterases, zowel

in vitro als *in vivo*. Een hoge plasma halfwaardetijd en distributievolume werden gevonden voor L-NA in sepsis patiënten hetgeen uitgebreide verdeling over de extravasculaire weefsels waarschijnlijk maakt. Aangezien renale excretie minimaal was, volgt de eliminatie van de metaboliet L-NA andere paden. Wij concluderen dat NO synthese remming met L-NAME tenminste gedeeltelijk het gevolg is van hydrolyse naar L-NA. Deze farmacokinetische informatie kan gebruikt worden in toekomstige klinische studies met L-NAME of L-NA.

List of Abbreviations

ADP	adenosine diphosphate
APACHE	acute physiology and chronic health evaluation
ARDS	acute respiratory distress syndrome
ArI	autoregulation index
AUC	area under curve
BH_4	tetrahydrobiopterin
c-GMP	cyclic guanyl monophosphate
CI	cardiac index
Cl_s	systemic clearance
CO	cardiac output
c-NOS	constitutive nitric oxide synthase
DIC	disseminated intravascular coagulation
DO_2	oxygen delivery
EDRF	endothelium derived relaxing factor
ELISA	enzyme linked immunosorbent assay
ET-1	endothelin-1
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
FiO_2	fraction of inspired oxygen
GTP	guanyl triphosphate
HPLC	high performance liquid chromatography
ICU	intensive care unit
IL	interleukine
IFN	interferon
i-NOS	inducible nitric oxide synthase
LPS	lipopolysaccharide
L-NA	N^G-nitro-L-arginine
L-NAME	N^G-nitro-L-arginine methyl ester

L-NMMA	N^G-monomethyl-L-arginine
LVSW	left ventricular stroke work
MAb	monoclonal antibody
MAP	mean systemic arterial pressure
MB	methylene blue
MDF	myocardial depressant factor
MRT	mean residence time
MOF	multiple organ failure
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NNLA	N^G-nitro-L-arginine
NO	nitric oxide
NP	nitroprusside
NS	not significant
O_2ER	oxygen extraction ratio
PAF	platelet activating factor
PaO_2	partial pressure of arterial oxygen
$PaCO_2$	partial pressure of carbon dioxide
$P(A\text{-}a)O_2$	alveolar-arterial oxygen pressure difference
PEEP	positive end-expiratory pressure
PVR	pulmonary vascular resistance
Q_{VA}/Q_T	venous admixture, pulmonary shunt
r	correlation coefficient
RVSW	right ventricular stroke work
SOD	superoxide dismutase
SVR	systemic vascular resistance
SV	stroke volume
$t_{½}$	half-life
VO_2	oxygen uptake

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