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Review

Nitric oxide in septic shock

Michael A. Titheradge *

School of Biological Sciences, University of Sussex, Falmer, Brighton BN1 9QG, UK

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Abstract

Septic shock is a major cause of death following trauma and is a persistent problem in surgical patients throughout the world. It is characterised by hypotension and vascular collapse, with a failure of the major organs within the body. The role of excessive nitric oxide (NO) production, following the cytokine-dependent induction of the inducible nitric oxide synthase (iNOS), in the development of septic shock is discussed. Emphasis is placed upon the signal-transduction process by which iNOS is induced and the role of NO in cellular energy dysfunction and the abnormal function of the cardiovascular system and liver during septic shock.

Keywords: Septic shock; Endotoxin; Nitric oxide; Cardiovascular; Liver

Contents

* Fax: +44 (1273) 678433; E-mail: m.a.titheradge@sussex.ac.uk

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1. Sepsis and septic shock

Sepsis is defined as the systemic response to infection, the most common cause being the contamination of the blood with bacteria [1]. Septic shock (sepsis with hypotension) develops in almost half of the patients as a complication, with a mortality rate of 40–60%, despite treatment [2–5]. The development of shock results in a progressive failure of the circulation to provide blood and oxygen to vital organs of the body resulting in impaired tissue perfusion and oxygen extraction [4]. The key symptoms include a severe fall in blood pressure (hypotension) with hyporeactivity to vasoconstrictor agents (vasoplegia) which may lead to the dysfunction or failure of major organs including lungs, liver, kidneys and brain (multiple organ dysfunction, MODS) and ultimately death [2–5].

Although the most common cause of septic shock is secondary to Gram-negative infections (endotoxic shock), invasion of Gram-positive organisms accounts for an increasing percentage of cases (1/3 to 1/2 of all incidences) with viruses, fungi and parasites accounting for a small number of cases [1]. Individuals may be predisposed to septic shock following impairment of the host defence mechanisms, for example, following trauma, burn injuries, diabetes, cirrhosis and treatment with immunosuppressive and chemotherapeutic agents [1,2].

Septic patients are in a hyperdynamic state characterised by tachycardia, high cardiac output, low systemic vascular resistance, hypoxaemia, oliguria and lactic acidosis [4,5]. Following infection there is an increase in the circulating concentrations of catecholamines, cortisol and glucagon [2] resulting in tachycardia and peripheral vasoconstriction. This phase is followed by a progressive vasodilation associated with high cardiac output and decreased vascular resistance and in some cases vasoplegia. Subsequent to this, cardiac failure develops with a progressive fall in cardiac output and marked disturbances in tissue perfusion and oxygenation occur [4]. Hyperglycaemia is commonly present and represents the influence of circulating cytokines and glucoregulatory hormones; however, in advanced stages hypoglycaemia may occur due to depletion of hepatic glycogen and inhibition of gluconeogenesis ([6] and references therein). There are also associated marked increases in plasma transaminase activity, urea and bilirubin concentrations, indicative of hepatic and renal injury, while the development of lactic acidosis reflects inadequate tissue perfusion and anaerobic metabolism [2].

It is now clearly established that bacterial endotoxin, a lipopolysaccharide (LPS) component of the outer membrane of Gram-negative bacteria, is the major mediator of the high morbidity and mortality rates characteristic of Gram-negative septic shock. Administration of endotoxin experimentally to animals mimics the symptoms of septic shock, while infusion in man results in a hyperdynamic, hypermetabolic response similar to that of shock due to Gram-negative bacteraemia. The morbidity and mortality in septic patients and animals can be decreased by treatment with antibodies to endotoxin (reviewed in [2]). The structure of the endotoxin complex is shown in Fig. 1. In Gram-positive shock, a variety of mediators have been proposed [2]; however, it is evident that the peptidoglycan and lipoteichoic acids (LTA) of the cell wall (Fig. 1) are important mediators (reviewed in [3]). Both the peptidoglycan and LTA synergise to produce the characteristic features of septic shock, MODS and ultimately death in rodent models [3,7]. These effects are not observed with either LTA or peptidoglycan alone, although high doses of LTA can cause circulatory failure but not MODS [7,8]. Gram-positive bacteria may also release other enterotoxins and exotoxins, for example toxic shock syndrome toxin 1, which are involved in the pathogenesis of sepsis (reviewed in [1]).

The presence of products of bacterial origin in the bloodstream leads to the release of endogenous cytokines which are responsible for the development of septic shock. Patients with septic shock display increased levels of TNF-α, IL-1β, IFN-γ and IL-6 in their serum, the latter showing a positive correlation with fatal outcome [5]. Administration of LPS to rabbits triggers a sequential release of TNF-α, IL-1β and IL-6 [9], while peptidoglycan and LTA synergise to cause release of TNF-α and IFN-γ in the rat [7] and TNF-α and IL-6 production by monocytes in vitro [10]. TNF-α and IL-1β alone reproduce the systemic and pathological effects of septic shock in animals and man [11,12], while animals pretreated with anti-TNF-α antibodies or IL-1β receptor antagonists are protected against shock, although they are

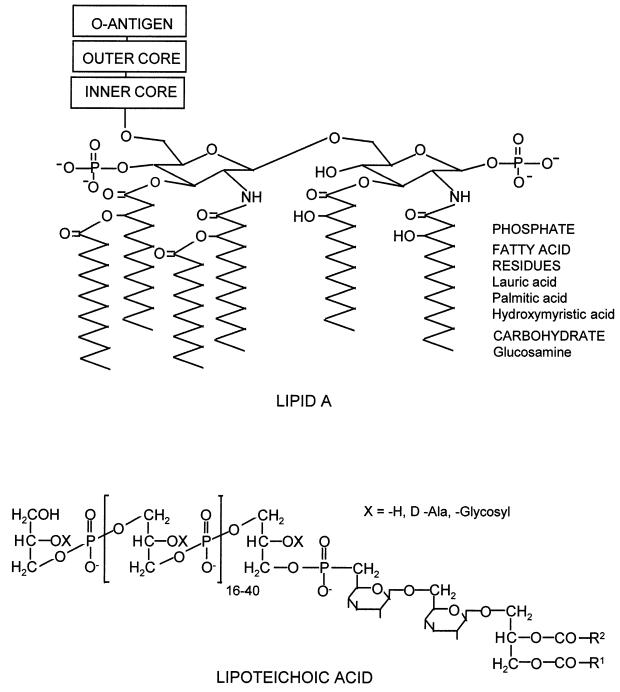

Fig. 1. The structure of LPS and lipoteichoic acid. The endotoxin (LPS) complex is composed of three functionally distinct regions: the outermost region consisting of multiple polysaccharide units that form the specific bacterial O-antigens; the inner and outer core region; and the lipid A component linking the endotoxin molecule to the bacterial outer membrane. The majority of the toxicity is considered to reside in the lipid A component. The lipoteichoic acids are poly(glycerophosphate) chains with position 2 of the glycerophosphate substituted in part with either D-alanine ester or D-alanine ester and glycosyl residues such as D-glucose, D-galactose and *N*-acetyl-D-glucosamine. It is anchored in the plasma membrane and extends to the surface of the cell wall.

without effect once shock has developed [5,13]. Mice in which the gene for the TNF-α receptor, TNF-αRp55, has been disrupted are also resistant to endotoxic shock, although TNF-α does not induce lethality by itself. LPS-induced lethal toxicity requires the synergistic effect of TNF-α, IL-1β, IFN-γ and NO ([13] and references therein).

2. Nitric oxide and septic shock

Nitric oxide (NO) is a free radical generated by a family of enzymes, the NO synthases (NOS), by the oxidation of one of the guanidino nitrogen atoms of L-arginine to form NO plus citrulline. This is associated with the oxidation of 1.5 molecules of NADPH per molecule of NO produced and the reduction of molecular oxygen. The reaction sequence involves the generation of an N^{ω}-hydroxy-L-arginine intermediate and water, followed by the oxidation of N^{ω}-hydroxy-L-arginine in presence of molecular oxygen to form NO, L-citrulline and water [14,15]. There are at least three different isoforms of NO synthase in mammalian cells: endothelial NOS (eNOS or NOS III) found in endothelial cells, epithelial cells and cardiac myocytes; neuronal NOS (nNOS or NOS I) found in neuronal cells and skeletal muscle, and inducible NOS (iNOS or NOS II) found in macrophages, hepatocytes, smooth muscle and a variety of other tissues. All NOS enzymes contain several cofactors in their active form – calmodulin, flavin adenine dinucleotide, flavin mononucleotide and tetrahydrobiopterin [14,16]. eNOS and nNOS are expressed constitutively and are activated by an increase in intracellular Ca^{2+} and are involved in regulating vascular tone [17,18] and neurotransmission [17] respectively, whereas iNOS is functionally Ca^{2+}-independent and involved in the immune defence [16,17], although it is expressed in many cell types following endotoxin or pro-inflammatory cytokine treatment [14–16,19]. nNOS and eNOS are thought to produce small Ca^{2+}-dependent bursts of NO formation, whereas expression of iNOS results in sustained production of large quantities of NO which are postulated to mediate the alterations in the vascular system and the tissue damage apparent in septic shock and MODS.

In recent years there has been a plethora of publications implicating the overproduction of NO in the pathophysiology of septic shock and MODS and the induction of NO synthase, with the consequent excessive formation of NO, has been proposed to be a major factor involved in the pathologic vasodilatation and tissue damage observed [3–6,18]. It is evident that nitrate production is increased in endotoxaemia and that plasma and urinary nitrite and nitrate concentrations are elevated in patients with sepsis and septic shock, the level increasing with the presence of shock and in patients with MODS ([20] and references therein). The only known source of nitrite and nitrate production in mammalian tissues is through the conversion of L-arginine to NO by the NO synthases and subsequent degradation of NO into nitrite and nitrate, the stable end products of

NO metabolism [17]. There is a quantitative and qualitative clinical relationship between NO production, endotoxaemia, haemodynamic dysfunction and MODS in human septic shock [20].

iNOS is induced in a variety of tissues in endotoxaemia [17,19] (see Fig. 2) and is proposed to be part of an adaptive response of the host defence mechanism directed towards limiting tissue injury and infections. Once expressed, the inducible enzyme of activated macrophages is capable of producing high concentrations of NO over long periods which has both bactericidal and tumouricidal effects [17].

During endotoxaemia iNOS expression and increases in iNOS mRNA and protein have been observed in a variety of cell types including endothelial cells, macrophages, Kupffer cells, hepatocytes, vascular smooth muscle cells, kidney cells, chondrocytes, cardiac myocytes, pancreatic islets and fibroblasts [15–17,19]. Similarly in the Gram-positive model of sepsis, LTA alone or in combination with peptidoglycan induces iNOS activity and protein in vascular smooth muscle cells and macrophages in vitro and the lung, aorta, heart, liver, pancreas and kidney in vivo [7,21,22]. Although peptidoglycan does not induce iNOS in cultured macrophages, it synergises with LTA in causing the expression of iNOS protein and activity in vitro and in the rat in vivo [7].

The specific factors which induce iNOS in septic shock vary between tissues; however, they include microbes, microbial products and proinflammatory cytokines and there is strong synergy between agents. For example, in murine macrophages LPS induces iNOS and strongly synergises with IFN-γ, whereas in cultured hepatocytes LPS alone has no effect, but enhances the induction by IL-1β, TNF-α and IFN-γ, with a maximal response being observed with a combination of IL-1β, TNF-α and IFN-γ plus LPS [23,24]. Similarly, while IL-1β enhances iNOS expression in chondrocytes, smooth muscle cells, hepatocytes and islets of Langerhans, it has no effect in macrophages [25].

The relevance of overproduction of NO to the development of endotoxic shock has been demonstrated by the use of NO synthase inhibitors and agents which block the expression of iNOS. Administration of N^{ω}-methyl-L-arginine (L-NMMA) in both animal models and man with septic shock has been demonstrated to restore blood pressure and reverse the endotoxin-induced hypotension and hyporesponsiveness to vasoconstrictor agents [5]. In addition NOS inhibitors have been shown to improve renal and hepatic function following either endotoxin treatment or treatment with LTA and peptidoglycan [26–28] and increase survival rates in animal models of septic shock [5] (but see later). The hypotension and early mortality caused by endotoxin is also attenuated in iNOS-deficient mice, suggesting that while the enhanced formation of NO by iNOS defends the body against infectious agents and tumour cells, excessive induction of iNOS in other tissues (e.g. vasculature) may cause shock and ultimately tissue damage [29]. Further it is now well established that the expression of iNOS in response to LPS and LTA is negatively regulated by dexamethasone in vivo [30,31] and endogenous glucocorticoids and an increase in the plasma levels of steroids has been proposed to contribute to the development of cardiovascular tolerance to repetitive injections of LPS [31]. Similarly pretreatment of rats with dexamethasone prevents the LPS- and LTA-induced induction of iNOS and attenuates the circulatory failure and hyporeactivity to vasoconstrictor agents [8,31], further

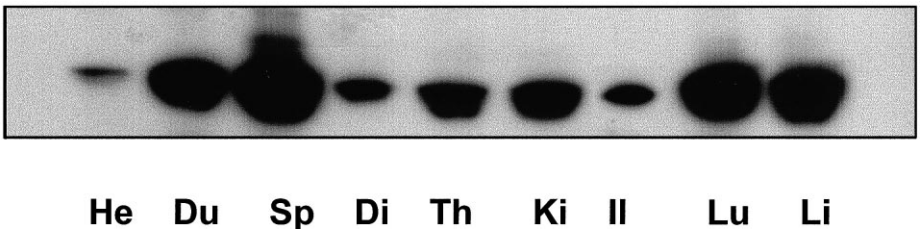

Fig. 2. Western blot analysis of iNOS in tissues in the endotoxin-treated rat. Rats were injected with LPS (4 mg/kg) and the tissues removed after 6 h. 40 μg of protein was used from each tissue and analysed for iNOS expression by Western blotting. Control rats showed no significant iNOS expression in any tissue. He, heart; Du, duodenum; Sp, spleen; Di, diaphragm, Th, thymus; Ki, kidney; Il, ileum; Lu, lung; Li, liver.

supporting the concept that excess NO production accounts for circulatory failure in septic shock.

Experiments using agents directed against cytokines or knockout mice generally support a role for the involvement of iNOS in sepsis ([32] and references therein). Pretreatment of animals with the IL-1β receptor antagonist has been demonstrated to attenuate the induction of iNOS in rats and ameliorate the delayed hypotension and tachycardia in response to endotoxin and the hyporeactivity to norepinephrine in aorta ex vivo [25,32]. Similarly administration of anti-TNF-α antibodies prevents the onset of septic shock during lethal bacteraemia and anti-TNF-α antibody or soluble TNF-αR antagonist is able to partially block LPS-induced pulmonary and hepatic NOS expression [32]. Experiments using TNF-αR1 knockout mice have confirmed that TNF-α is involved in iNOS expression; however, this may not be the case for all tissues as it is evident in liver but not spleen [32]. The use of monoclonal antibodies to IFN-γ also partially protects against iNOS expression and mortality, while disruption of the IFN-γ gene indicates that endogenous IFN-γ is required for both the LPS-induced iNOS expression and increase in serum nitrate plus nitrite, although again the extent of the involvement of IFN-γ in the induction of iNOS is tissue dependent [32].

Recent work indicates that not only is iNOS mRNA induced in liver after endotoxin or LTA treatment of the rat, but eNOS (NOSIII) mRNA expression is also increased to an even larger extent [22]. The significance of this during septic shock remains to be established as it is not evident in kidney [22] and eNOS is down-regulated in vascular epithelium under these conditions [5].

3. The mechanism of iNOS induction in septic shock

The expression of iNOS by LPS, LTA and cytokines results from the interaction of LPS, LTA, peptidoglycan, IL-1β and TNF-α with receptors on the plasma membrane, resulting in a signal being transferred to the gene. LPS interacts with a specific LPS binding protein (LBP) and this complex interacts with cell surface molecule CD14 [33]. Recent evidence suggests that the signal may be transduced across the membrane via the Toll-like receptor-2 [34]. Signalling via peptidoglycan has also been suggested to occur through CD14 [35]. IL-1 and TNF each occur in two forms, α and β. TNF-α and both forms of IL-1 are made by activated monocytes and macrophages, whereas TNF-β is made by activated T lymphocytes (reviewed in [36]). There are two receptors for both IL-1 and TNF and the two forms of each cytokine interact with the same receptors. IL-1α and β interact with the type 1 IL-1 receptor for signal transduction, whereas type II does not appear to transmit any signal and functions as an inhibitor of IL-1 action [36]. The two types of TNF receptor, p55 (type I) and p75 (type II) have different end effects, the p55 receptor signals the inflammatory response and apoptosis, whereas p75 mediates the proliferative actions of TNF-α [36].

Although the precise signal transduction pathway for iNOS induction and the development of septic shock and MODS remains to be established, it is evident that tyrosine kinase activation is involved in both the secretion of the cytokines and the signal transduction pathway following interaction of the cytokine with its receptor at the target cell. The tyrosine kinase inhibitors, tyrphostin AG 126, genistein and erbstatin, can prevent LPS-induced lethal toxicity in mice [13] and have also been reported to prevent the circulatory failure (hypotension and vascular hyporeactivity) caused by endotoxin in vivo [37]. Liver injury, pancreatic injury, lactic acidosis and hypoglycaemia are also attenuated by preventing tyrosine phosphorylation in the rat model of endotoxic shock, although the renal failure is not reversed [37]. Both tyrphostins and genistein prevent the rise in serum TNF-α [37] and TNF-α production in macrophages [13], together with the expression of iNOS in response to endotoxin and cytokines in a variety of tissues [13,37]. These agents therefore have a dual role in preventing both cytokine release and blocking the cytokine signalling pathway. The signal transduction pathway leading to the LTA-dependent iNOS expression in J774.2 macrophages is also dependent on tyrosine kinases activity [21].

Fig. 3 shows the proposed mechanism by which these cytokines may act. It is evident that the major response is via activation of the mitogen-activated protein kinase (MAPK) family of proteins with subsequent activation of transcription factors required for the expression of iNOS and other genes [36,41–

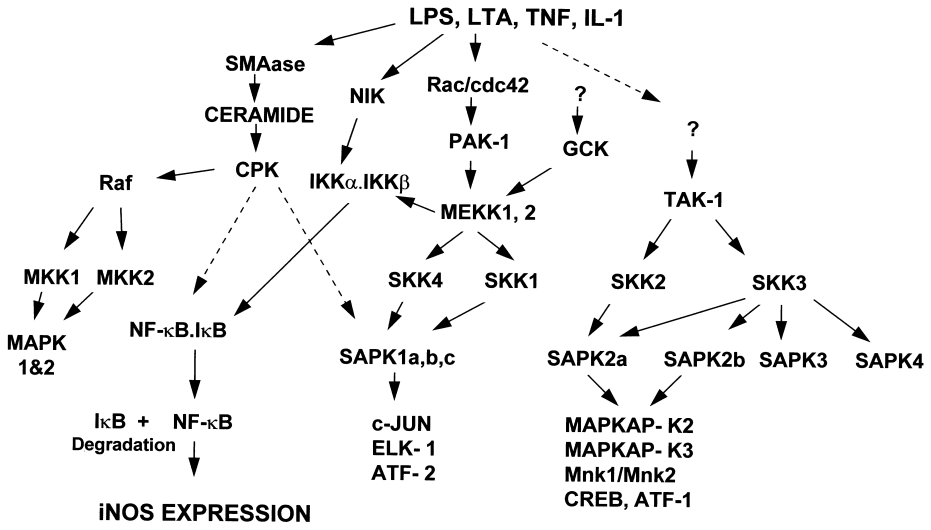

Fig. 3. Signal transduction pathway for LPS, LTA, TNF-α and IL-1β. The nomenclature is based upon that of Cohen [44]. MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; MKK, MAPK kinase; SKK, SAPK kinase; Mnk, MAPK-interacting kinase; MAPKAP-K, MAPK-activated protein kinase; NIK, NF-κB inducing kinase; IKK, IκB kinase; CPK, ceramide-dependent protein kinase; GCK, germinal centre kinase; SMAase, sphingomyelinase.

44]. There are a number of groups of MAP kinases in mammalian cells which have been postulated to be activated by cytokines: the extracellular signal regulated kinases (MAPK1 and 2/ERK1 and 2); the c-Jun NH_2-terminal kinases (SAPK1/JNKs) and the p38 MAP kinase family (SAPK2, 3 and 4). The activity of these kinases is regulated by dual phosphorylation within the protein kinase subdomain VIII by a kinase cascade consisting of a MAP kinase kinase and a MAP kinase kinase kinase (Fig. 3). The importance of the extracellular signal regulated kinase cascade in septic shock is currently unclear, as although IL-1β and TNF-α can activate MAPK1 and MAPK2 in a variety of cells via activation of MKK1 and c-Raf (reviewed in [36]) and LPS and peptidoglycan can strongly activate MAPK1 and MAPK2 in RAW264.7 macrophages, this is not universally found [36] and the relevance of this pathway may be tissue dependent. In contrast, the stress activated protein kinases appear to be the major pathway for stimulation [42].

TNF-α and IL-1β are potent activators of the proline-directed Ser/Thre SAPK1 class, a family of protein kinases which are potent c-Jun kinases [41,43]. For example, IL-1β and TNF-α activate SAPK1a (JNK2) and SAPK1c (JNK1) in human glomerular mesangial cells, under conditions where no effects on MAPK1 or 2 are observed and LPS alone is ineffective [45]. SAPK1 is activated under cell stress conditions by phosphorylation on tyrosine and threonine residues by two kinase kinases, SKK1 (SEK1/MKK4/SAPKK1) and SKK4 (MKK7/SAPKK4/5) [44,49]. SKK4 appears to be specific for SAPK1, and in epithelial Kb cells at least, is the major mediator in the response to IL-1β and TNF-α, SKK1 being unresponsive to proinflammatory cytokines but not other forms of cell stress [47]. In vitro SKK1 functions as a dual functional kinase activating both SAPK1 and SAPK2; however, it appears to be ineffective in activating SAPK2 in vivo [42]. Both SKK1 and SKK4 are subject to phosphorylation and activation in situ and in vitro by the serine/threonine kinase MEKK1, although two further MEKK activities have been described which activate both the SAPK1 and MAPK pathways, MEKK2 and MEKK3 [41–44]. MEKK2 preferentially activates SKK1 and the SAPK1 family, while MEKK3 shows preference for MKK1 and the MAPK pathway [49]. Other MEKK-like activities have been found such as MUK which can also act as regulators of the SAPK1 pathway; however, the relative importance of these different components is unclear and may well vary from tissue to tissue, cellular distribution and activating agent. The SAPK1 pathway can also be activated in an SKK1-dependent manner by TNF-α activating the germinal centre kinase (GCK) independently of

any activation of SAPK2 or MAPK [41,43]. SAPK1 activation results in phosphorylation and *trans*-activation of the transcription factors c-Jun, Elk-1, and ATF-2 [41,42].

The second stress activated protein kinase group, SAPK2a (p38) and 2b (p38β), has also been shown to be phosphorylated on tyrosine and activated in response to LPS in macrophages and other tissues, while the novel inflammatory drugs, CSAIDS (cytokine-suppressive anti-inflammatory drug), have been shown to have a protective role in the endotoxic shock model and inhibit LPS-induced TNF-α and IL-1β production by inhibiting SAPK2a and SAPK2b activity [36,42], suggesting the involvement of SAPK2 in cytokine release. SAPK2 has also been implicated in IL-6 production [46]. Like SAPK1, SAPK2a is activated by dual phosphorylation on Tyr and Thre residues by SKK2 (MKK3) and SKK3 (MKK6), while SAPK2b is activated only by SKK3 (reviewed in [41,43,44,49]). Activation of SKK2 and SKK3 has been suggested to occur via the protein kinase TAK-1, which may itself be regulated by two binding proteins, TAB1 and TAB2 [49]. Stimulation of the SAPK2 kinases culminates in phosphorylation of the serine kinases, MAPKAP kinase-2 and MAPKAP-kinase-3 leading to activation and HSP25/HSP27 phosphorylation. MAPKAP-kinase-2 also phosphorylates CREB and ATF-1 [41–43] and therefore SAPK2s are capable of switching on genes whose promoters contain CRE elements or are sensitive to homo/heterodimers of the CREB/ATF and AP-1 families. Other physiological substrates are Elk1, SAP1, CHOP, MEF2C, MAPK-interacting protein kinase-1 (Mnk1 and Mnk2) [41,43,44,47]. Recently a further two members of the SAPK family have been identified which are activated in response to IL-1β and TNF-α, SAPK3 and SAPK4 [44,47,48].

The upstream signalling mechanism for the activation of the stress activated kinase cascades in response to endotoxin and cytokines is still not established; however, the Rho family of GTPases, Rac1 and Cdc42Hs have been demonstrated to be involved in the activation of SAPK1 and 2 [43,47]. It is suggested that Rac/Cdc42Hs and SAPKs are coupled via the PAK family of proteins (p21 activated kinases) (PAKs 1–3). PAK-1 is activated by binding to the GTP bound form of Rac/Cdc42Hs and can induce activation of SAPKs [43]. How activation of these kinases leads to activation of stress activated kinases remains to be established; however, complexes of GTP-Cdc42Hs, PAK and MEKK1 have been demonstrated and in yeast, the PAK equivalent has been shown to activate the MEKK1 homologue. [49].

A further signalling mechanism proposed for both TNF-α and IL-1β is via the rapid breakdown of plasma membrane and subcellular membrane sphingomyelin by a variety of sphingomyelinases to generate ceramide [43,50–53]. Exogenously applied ceramide to cells or hydrolysis of sphingomyelin with sphingomyelinase results in activation of a ceramide-dependent serine/threonine kinase and a protein phosphatase 2A-like activity which mimics many of the effects of TNF-α and IL-1β, e.g. activation of stress activated kinases and MAPK activity, apoptosis, generation of ROIs, NF-κB translocation to the nucleus [50–52], and the dose-dependent relaxation of phenylephrine-contracted endothelium-denuded rat aortic rings [53]. It is suggested to couple to MAPK, SAPK1 and SAPK2 via activation of ceramide-dependent protein kinase.

The promoter-regulatory region of both the mouse and human iNOS genes contains a variety of possible regulatory sequences through which iNOS expression may be stimulated, including NF-κB, AP-1, NF-IL-6, interferon response elements, γ-activated sites and TNF-α-response elements. In macrophages, LPS inducibility of iNOS is dependent upon the NF-κB sequence and activation of NF-κB [16], while suppression of NF-κB activation in vivo prevents the induction of iNOS and the deleterious effects of endotoxin on blood pressure, responsiveness to vasoconstrictor agents, blood glucose and tissue damage [38]. The synergistic action of IFN-γ in iNOS induction depends upon the interaction of interferon regulatory factor-1 with the interferon-regulatory binding factor site (reviewed in [39]); however, this may be tissue dependent as in murine macrophages IFN-γ has been reported to cause accumulation of mRNA in the absence of an effect on the basal rate of iNOS transcription, suggesting it may stabilise the mRNA in addition to its transcriptional role [16]. Although activation of the NF-κB site is important for iNOS expression in other tissues, binding sites for other transcription factors in the downstream portion of

the iNOS 5′-flanking sequence are also critical for iNOS induction [40].

The mechanism by which iNOS expression through activation of NF-κB links to LPS and cytokines has recently been described (reviewed in [54]). In unstimulated cells, NF-κB is retained in the cytoplasm in an inactive complex with the inhibitory IκB proteins. TNF-α-induced trimerisation of the TNFR1 receptor results in recruitment of the TNFR-associated death-domain protein (TRADD), the TNFR-associated factor-2 (TRAF-2) and the serine/threonine kinase, receptor-interacting protein (RIP). TRAF-2 binds to a further serine/threonine MAP kinase kinase kinase family member, NF-κB inducing kinase (NIK), which itself associates with a complex of two further kinases, IκB kinase (IKK) α and β, initiating a kinase cascade leading to the phosphorylation of the IκB proteins. There is evidence to suggest that IKKα is preferentially phosphorylated and activated by NIK [55], whereas MEKK1 has been postulated to preferentially phosphorylate and stimulate IKKβ activity [56]. Phosphorylation of the IκB proteins by either of the IKKs leads to polyubiquitination and subsequent degradation of IκBs [54–56]. Degradation of IκB releases NF-κB and allows the NF-κB to translocate to the nucleus to activate its target genes [54]. Evidence suggests that IL-1 also activates NF-κB via a similar mechanism [54]. IL-1 induces the formation of a complex consisting of the IL-R1 receptor, an accessory protein, AcP, the serine/threonine kinase protein kinase IRAK, and a TRAF protein, TRAF-6. Like TRAF-2, TRAF-6 can also interact with NIK and activate the kinase cascade. TNF-α and IL-1β have also been proposed to activate NF-κB via stimulation of acidic sphingomyelinase and generation of ceramide [51,52]; however, the importance of this route has been questioned (reviewed in [52]).

Although it is evident that activation SAPK2 (p38) is involved in the LPS-induced release of TNF-α and IL-1β and both TNF-α and IL-1β activate SAPK2 (p38) and NF-κB, the role of the SAPK2 arm of the signal transduction pathway in NF-κB activation and iNOS expression is to be questioned. In addition to cytokines, arsenite and osmotic stress both potently activate SAPK2 in 293 cells; however, neither leads to NF-κB activation [57]. Similarly while numerous studies have demonstrated that antioxidants abolish iNOS expression and NF-κB activation ([57,58] and references therein), they are reported to activate SAPK2 and AP-1 [57]. Recent studies indicate that SAPK2 activation occurs via TRAF-2, but that it is independent of NIK and NF-κB activation[59]. It is suggested that while it is a crucial mediator in TNF-induced activation of NF-κB-dependent gene expression, it is not involved in increasing NF-κB activation and translocation to the nucleus, but is important for the transcriptional activation by modulating the transactivation capacity of the NF-κB p65 subunit [59,60]. In contrast to this, the work of Guan et al. has suggested that in rat glomerular mesangial cells, IL-1β activates iNOS expression and mRNA production, while at the same time it activates SAPK2, which acts as a negative regulator of iNOS expression [61], suggesting that the level of expression of iNOS may be dependent on the balance between the differential activation of the SAPK1 and 2 pathways within different tissues with different stimulants.

4. Inhibition of iNOS expression in endotoxic shock

While it is clear that IL-1β, IL-6, TNF-α and IFN-γ play a role in the induction of iNOS during septic shock, it is evident that a variety of anti-inflammatory cytokines and other agents are also expressed which are able to antagonise this induction process [4,15]. For example, glucocorticoids are known to be elevated during sepsis and these can almost completely inhibit iNOS expression in a wide variety of cell types exposed to LPS, LTA or cytokines [4,15]. Dexamethasone is suggested to inhibit iNOS expression by the activated glucocorticoid receptor preventing the binding of NF-κB, but not AP-1, to the NOS II promoter, thereby preventing transcription [62]. Blocking NF-κB activation and iNOS expression by dexamethasone ameliorates the hypotension, hyporesponsiveness to vasoconstrictor agents, hypoglycaemia, lactic acidemia and liver and pancreatic injury [38]. Similarly cytokines such as TGF-β, IL-4, IL-8, IL-10 and macrophage deactivating factor are capable of suppressing iNOS activity (reviewed in [4,5]). Evidence suggests that TGF-β reduces cytokine-induced iNOS activity by both inhibiting mRNA translation, decreasing its stability and in-

creasing iNOS protein degradation, whereas IL-4 interferes with iNOS transcription [15,16].

The interaction of hormones which act via cAMP and agents which elevate cAMP is complex and tissue dependent. In vascular smooth muscle cells, rat mesangial cells, peritoneal macrophages and cardiac myocytes, an elevation of cAMP synergises with cytokines to increase the induction of iNOS (see [63] for references) and indeed an activation of protein kinase A has been demonstrated to be essential for iNOS expression by IL-1β in neonatal cardiac myocytes [64]. In contrast, in astrocytes and hepatocytes [63,65] NO production is diminished by elevating cyclic AMP with glucagon or forskolin. The latter occurs via a reduction in the rate of transcription and translation of the iNOS gene [63,65]. The physiological significance of this remains to be established; however, it is evident that a major feature of both sepsis and endotoxin administration to animals is hyperglucagonaemia and a decrease in the plasma insulin:glucagon ratio [63]. A recent study has indicated that treatment of rats with dibutyryl cAMP can increase blood flow through the liver, reduce the serum concentration of TNF-α, prevent liver necrosis and acute hepatic failure and increase survival following endotoxin treatment of galactosamine-sensitised animals [66]. Preliminary experiments in our laboratory have also demonstrated that glucagon pretreatment of rats results in a reduction of the endotoxin-induced increases in serum $NO_2^-+NO_3^-$ concentrations, gives a partial inhibition of hepatic iNOS induction and completely eliminates hepatic damage as judged by release of lactate dehydrogenase and aspartate aminotransferase activity, suggesting that an elevation of circulating concentrations of glucagon and steroids may help to limit tissue damage during sepsis. Elevating cAMP by isoproterenol administration has similarly been shown to block LPS-induced TNF-α secretion, increase circulating concentrations of IL-10 and IL-6 and reduce NO formation in mice [67]. In the same study, treatment of anaesthetised rats with isoproterenol prevented the endotoxin-induced hyporeactivity to norepinephrine suggesting an elevation of cAMP may be beneficial at multiple sites within the pathway. The mechanism by which cAMP exerts this negative effect on iNOS expression has not been established; however, cAMP and forskolin have been reported to inhibit SAPK1c activity and c-Jun expression and AP-1 activation in vascular smooth muscle [68].

5. NO and energy transduction in septic shock

Severe sepsis is characterised by a progressive failure of energy metabolism and a marked decrease in tissue oxygen extraction, resulting in tissue hypoxia and increased venous oxygen concentration in both septic patients and animal models [69]. Taken together with the lactic acidosis and acidemia, this suggests an impairment of oxygen utilisation, although this may also be caused by tissue hypoperfusion [2,69]. A number of studies, particularly using the endotoxic model of shock, have demonstrated an inhibition of respiration in liver, heart, skeletal muscle, kidney and brain; however, this has not been universally found and mitochondrial respiration has been reported to be either inhibited, unchanged or even stimulated during sepsis (reviewed in [69–71]). It is likely that the predominant effect on mitochondrial metabolism depends upon a number of factors including the method of inducing shock, whether by endotoxin or caecal ligation and perforation, the time period over which sepsis is induced, the dose of the inducing agent and the species.

Numerous studies have indicated that authentic NO or induction of iNOS in tissues by LPS or cytokines is accompanied by an inhibition of respiratory chain activity and accumulation of lactate. This has been demonstrated in vascular smooth muscle cells, hepatocytes, macrophages, astrocytes and cardiac myocytes (reviewed in [72]). It is now evident from studies using intact cells and isolated mitochondria that physiological levels of NO interact competitively with oxygen at cytochrome oxidase (complex IV) reversibly inhibiting O_2 consumption [72]. The ability of NO to inhibit cytochrome oxidase and ATP synthesis therefore will be greater in areas of low oxygen tension (i.e. physiological O_2 conditions) and may be exacerbated in shock if there is redistribution of blood flow away from highly metabolic areas, compromising oxygen availability. An inhibition of cytochrome oxidase also increases the production of reactive oxygen intermediates (H_2O_2 and O_2^-) by the mitochondrial respiratory chain which may lead to irreversible damage as a result of the NO interacting

with the O_2^- to form peroxynitrite ($OONO^-$) [73]. Initial studies suggested that NO directly damages iron-sulphur centres in the mitochondrial electron transport chain; however, it is now evident that these centres are relatively insensitive to NO and that long term exposure to NO irreversibly inhibits complexes I, II, the ATPase and possibly III, but not complex IV as result formation of peroxynitrite (see [72] for references). Mitochondrial aconitase is also inhibited under these conditions resulting in an inhibition of both TCA cycle activity and respiration [74]. There is also potential for $OONO^-$ formation to be further enhanced by NO interacting with the haem of catalase, reversibly inactivating the enzyme and preventing H_2O_2 breakdown, while encouraging superoxide dismutase (SOD)-dependent $OONO^-$ formation [72]. Peroxynitrite has also been shown to cause nitration and inhibition of SOD, making more O_2^- potentially available and setting up an autocatalytic cycle [75]. Experiments employing cell permeable SOD mimetics in immunostimulated macrophages suggest a role for both peroxynitrite-dependent and direct NO-dependent mechanism in the suppression of mitochondrial respiration [76].

It is now established that endotoxic or septic shock leads to substantial increase in levels of NO and biologically active $OONO^-$. It has been demonstrated in the vicinity of large blood vessels such as thoracic aorta, in activated macrophages, endothelial cells, human neutrophils and in lungs of endotoxic animals [77,78]. The inhibition of respiration in macrophages and smooth muscle cells by $OONO^-$ is paralleled by DNA single strand breakage that triggers the activation of the nuclear repair enzyme, poly(ADP)ribosyltransferase (PARS) [78]. Activation of PARS results in ADP-ribosylation and depletion of intracellular NAD^+, which, together with the inhibition of the respiratory chain and TCA cycle activity, is proposed to result in decreased ATP formation and ultimately cell death (reviewed in [78]). Over-production of NO is also associated with inhibition of glyceraldehyde phosphate dehydrogenase (GAPDH) by ribosylation and nitrosylation [79,80] resulting in an inhibition of glycolytic flux, thus contributing to defective energy production. The latter has been proposed to be important to tissue O_2 metabolism during sepsis, and to contribute to the contractile suppressing effects of NO in the vascular system in endotoxic shock [5]. The use of the inhibitors, 3-aminobenzamide and nicotinamide, has demonstrated the importance of the PARS pathway in septic shock. They are protective in the rat model of endotoxic shock and ameliorate the defects in cellular energy metabolism, maintain blood pressure by preventing loss of contractility and reduce the degree of endothelial cell dysfunction [78]. Inhibition of PARS activation by preventing $OONO^-$ formation by SOD mimetics also suppresses the inhibition of mitochondrial respiration in macrophages and cultured smooth muscle cells after immunostimulation and ameliorates the vascular hyporeactivity and endothelial dysfunction in thoracic aorta ex vivo in endotoxin-treated rats [78].

$OONO^-$ has also been implicated in the oxidation of mitochondrial thiols, mixed disulphides and NAD(P)H [81,82]. The latter has been suggested to lead to permeability transitions and depolarisation of the mitochondrial membrane, further compromising ATP synthesis and ability to sequester Ca^{2+}, both of which are suggested to contribute to necrotic cell death or apoptotic cell death (reviewed in [81]). In addition, other cyclosporin-sensitive pathways have been suggested to account for alterations in Ca^{2+} release by $OONO^-$, possibly involving ADP-ribose, which do not involve changes in $\Delta\Psi$ [82].

Although the above suggests that alterations in mitochondrial function via NO or $OONO^-$ are important in the development of cellular dysfunction during septic or endotoxic shock, this may not be applicable to all tissues. The initial work of Stadler et al. indicated that exposure of hepatocytes to authentic NO leads to a decrease in aconitase and complex I and II activity (although not complex IV) [83]. However, in contrast, endogenously generated NO by exposure to cytokines plus LPS or activated Kupffer cell supernatants results in only a minor NO-dependent depression of mitochondrial aconitase and no significant change in the other complexes [83,84]. Similarly induction of septic shock and NO synthesis by *C. parvum* injection into rats results in virtually no inhibition of mitochondrial activity [84]. Subsequent studies have demonstrated that the major inhibition of respiration in hepatocytes following cytokine addition is due to TNF-α via an NO-independent mechanism, possibly involving activation of the sphingomyelinase pathway [51,85]. This suggests that the importance of physiological concentrations

of NO at the level of the mitochondria is tissue specific and may not be relevant, particularly in tissues such as liver which have a high capacity to metabolise the NO.

6. NO and the vascular system

Excessive NO synthesis and induction of iNOS has been implicated in the cardiovascular dysfunction observed in both patients with septic shock and animal models of endotoxaemia (reviewed in [4,5]). It is evident that elevated production of NO in response to LPS, LTA and cytokines is due to an initial activation of eNOS followed by induction of iNOS resulting in vasodilation and hyporeactivity to pressor agents (epinephrine, vasopressin, angiotensin II, serotonin, calcium, potassium) [4,5,18]. Interestingly during the late phase of shock there is an impairment of the biosynthesis of NO by eNOS in vascular endothelium, the constitutive form being down-regulated as the inducible form is expressed [5]. Although iNOS induction occurs in the endothelial cells, the most important source of NO within the vessel wall is probably the vascular smooth muscle and iNOS derived from the vasculature is responsible for the excessive vasodilatation and hyporeactivity which is characteristic of the hypotension in shock. The work of Li et al. suggests that the level of induction of iNOS and production of NO within the vascular system at different anatomical sites may be variable, resulting in different degrees of vasodilatation and catecholamine insensitivity, with areas of underperfusion of metabolically active tissues and areas of over-perfusion of areas that are not metabolically active [86]. This may contribute to the deficient oxygen extraction, cell hypoxia and development of lactic acidosis characteristic of shock. [2].

In vivo, prevention of induction of iNOS by anti-inflammatory cytokines, e.g. TGF-β, glucocorticoids or neutralisation of proinflammatory cytokines and lipid mediators of shock (e.g. TNF-α, IL-1β, PAF) improves haemodynamics in shock and reduces the hypotension and degree of vascular hyporeactivity [5]. Similarly the vasodilatation of the smooth muscles and the hyporeactivity can be reversed by both non-isoform selective inhibitors of NOS and inhibitors selective for iNOS [5] and are reduced in iNOS-deficient mice [29].

Induction of iNOS is also associated with time-dependent vascular permeability changes, indicative of endothelial cell injury, which can be inhibited by dexamethasone [87]. This may result from the down-regulation of eNOS expression by pro-inflammatory cytokines such as TNF-α, or endothelial cell damage due to cytotoxic effects of NO as a result of iNOS expression, $OONO^-$ or oxygen derived radicals [88]. Excessive production of NO by iNOS has been shown to be cytotoxic towards endothelial cells [88], while the increased blood flow may augment direct injurious action on microvascular endothelium. The increase in systemic microvascular permeability, with loss of intravascular blood volume, may be a major factor contributing to the decrease in cardiac output seen in severe sepsis [2]. Inhibitors of NOS have been demonstrated to reduce the degree of endothelial cell dysfunction in rodent models of endotoxic shock and can attenuate the changes in vascular permeability and oedema formation induced by endotoxin and proinflammatory cytokines in a variety of tissues including skin, jejunum, ileum and colon (reviewed in [88]).

Excessive vasodilation in response to bacterial products and cytokines is suggested to be due to NO acting via both cGMP-dependent and cGMP-independent mechanisms [5]. NO produced in both the vascular smooth muscle and endothelial cells activates the soluble guanylate cyclase by binding to the haem moiety, causing an increase in cGMP formation and cGMP-dependent reduction of intracellular Ca^{2+} and vascular relaxation [5,18]. Non-cGMP-dependent mechanisms are the result of direct effects of NO or $OONO^-$ on the vascular smooth muscle leading to reduced contractility by energy depletion, activation of Ca^{2+}-dependent K^+ channels and ATP-sensitive K^+ channels [5].

Reduced myocardial performance is an important contributing factor in shock and induction of NOS in the myocardium has been demonstrated and proposed to be responsible for cardiac dysfunction in endotoxaemia [89]. Although induction of iNOS activity in the heart of animals exposed to endotoxin is relatively small when compared to other organs [19] (Fig. 2), it is clearly established that the expression of

iNOS in isolated rat cardiac myocytes or papillary muscle cells exposed to TNF-α, IL-1β or macrophage-conditioned medium is associated with an L-NMMA-attenuated reduction in myocardial contractility, decreased beating rate of myocytes and reduced inotropic response to isoproterenol (reviewed in [5]). Infusion of isolated hearts with IL-1β and TNF-α induces iNOS and suppresses contraction [89], while cardiac myocytes isolated from endotoxic rats show impaired contractility which is ameliorated by dexamethasone and iNOS inhibitors [90]. In contrast, studies using Langendorff heart preparations prepared from endotoxic animals, rather than cardiac myocytes, failed to demonstrate an inhibition of the isoproterenol-inotropic response, while pretreatment of the animals with N^{ω}-nitro-L-arginine or aminoguanidine prior to the endotoxin failed to improve left ventricular function [91]. Despite the fact that iNOS inhibitors were ineffective in these studies, dexamethasone pretreatment did inhibit the endotoxin-induced cardiac depression, suggesting mechanisms other than NO may also be important in the cardiac dysfunction in the intact heart [91].

NO produced in the heart is derived from both the expression of iNOS in the vascular and endocardial endothelium, coronary smooth muscle and cardiac myocytes and also the constitutive NOS in the coronary endothelium and the endocardium (reviewed in [5]). In addition to the expression of iNOS, impairment of eNOS in the coronary microvasculature may also play a role and lead to coronary vasoconstriction and myocardial ischaemia and decreased cardiac performance. Similarly it is proposed that the reduced production of NO from the endothelium would also enhance adhesion and aggregation of platelets and neutrophil granulocytes (via a reduction in cGMP) to the intima, possibly leading to further development of endothelial tissue injury (via oxygen radical and peroxynitrite-dependent mechanism) [5].

7. NO and liver function in septic shock

It is clearly established that iNOS is induced in the liver during endotoxic shock [19,30,83,84,92,93]. During sepsis, bacterial products interact with the Kupffer cells within the liver to cause the release of cytokines (IL-1β, IL-6, TNF-α) which interact with the adjacent hepatocytes to alter parenchymal cell function, ultimately leading to liver cell damage and liver failure. The studies of Billiar et al. have demonstrated that co-cultures of hepatocytes and Kupffer cells in the presence of LPS or cultures of hepatocytes in medium obtained from LPS+IFN-γ-stimulated Kupffer cells result in an L-arginine-dependent acute phase reactant synthesis and inhibition of protein synthesis (reviewed in [93]). This effect is coincident with the production of $NO_2^- + NO_3^-$, is inhibited by L-NMMA and reproduced using synthetic NO donors, suggesting the involvement of NO. Subsequent studies using both rat and human parenchymal cells have indicated that a mixture of cytokines, including IL-1β, TNF-α, IFN-γ plus LPS, act synergistically to induce iNOS mRNA and protein, while LPS alone has no effect [24,93]. A similar inhibition of albumin synthesis by Kupffer cell-conditioned medium has also been observed in cultured hepatocytes, which can be mimicked by a combination of IL-1β, IL-6 and TNF-α [95]. The mechanism underlying the effects of NO on protein synthesis remains to be established; however, it appears to reside at the level of translation [93].

Hepatic glucose production and hyperglycaemia are markedly increased in patients with sepsis, insulin resistance contributing to this process in the hypermetabolic phase of sepsis [96]. With overwhelming sepsis or animals treated with bacterial endotoxin (hypometabolic phase), an inhibition of hepatic gluconeogenesis in the face of elevated peripheral glucose utilisation occurs and hypoglycaemia develops (reviewed in [6,97]). NO has been implicated as a possible mediator in the inhibition of gluconeogenesis from in vitro [98] and in vivo [99] studies using artificial NO donors. This is supported by other studies indicating that the induction of NO synthase in cultured hepatocytes with a combination of LPS, IFN-γ, IL-1β and TNF-α coincides with a decreased rat of glucose formation [24,100–102]. The work of Stadler et al. [100] indicated that the inhibition of glucose output (glycogenolysis plus gluconeogenesis) by this combination of cytokines can be inhibited by L-NMMA suggesting an involvement of NO; however, attenuation of the response by NOS inhibitors has not been found in other studies, either in cultured hepatocytes [24] or hepatocytes freshly prepared from endotoxic rats treated with either L-

NMMA or aminoguanidine [101]. Similarly the inhibition of gluconeogenesis by endotoxin is still evident in the iNOS deficient mouse [101]. A more recent study has indicated that this cytokine combination results in an inhibition of both glycogenolysis and gluconeogenesis in cultured hepatocytes and that chronic treatment of the cells with glucagon attenuates iNOS induction and the inhibition of glucose synthesis [102]. The use of NO synthase inhibitors in this study reveals that NO plays an important role in the inhibition of glucose output when cells are stimulated with insulin; however, it makes a very minor contribution to the inhibition of glucose synthesis observed in steroid-treated cells. Under the latter conditions, the formation of reactive oxygen intermediates is proposed to make the major contribution to the inhibition of glucose output [102], although a ceramide-dependent inhibition of glucose output has also been reported which is independent of NO synthesis [6]. It is postulated that the extent to which NO is involved in this process is dependent upon the hormonal milieu and the site(s) at which metabolic control is exerted within the pathway at any given time. Recent evidence in favour of the involvement of NO in the development of hypoglycaemic phase of sepsis has come from the study of Ruetten and Thiemermann [38] which demonstrates that administration of the calpain 1 inhibitor to rats to prevent IκB degradation prevents both iNOS induction in both lung and liver and also the development of the hypoglycaemia characteristic of endotoxin treatment.

A significant feature of septic or endotoxic shock is hepatocellular failure and liver damage, with the release of aspartate aminotransferase, ornithine carbamoyl transferase and lactate dehydrogenase into the bloodstream as seen in the onset of MODS. A number of studies have implicated NO as a contributing factor to liver damage by employing in vivo treatment of animals with NOS inhibitors; however, the precise role for NO has been difficult to determine because of the use of non-specific NO synthase inhibitors. Billiar et al. have demonstrated that in the *C. parvum* plus LPS murine model of endotoxic shock, a bolus injection of L-NMMA together with the LPS prevents NO formation; however, it substantially exacerbates the endotoxin-induced liver injury [93]. The liver damage is associated with increased numbers of microinfarcts and platelet thrombi within the small vessels and it has been suggested that inhibition of NO synthesis causes disturbances of blood flow resulting from platelet adhesion and aggregation, vasoconstriction, clot formation and subsequent ischaemia with O_2^- production. In these studies, the effect of L-NMMA was exaggerated by the presence of aspirin, suggesting that NO and prostacyclins act together to maintain hepatic blood flow during sepsis, whereas it is abrogated by co-administration of superoxide dismutase or deferroxamine, suggesting that NO may also function by preventing damage by reactive oxygen radicals ([93] and references therein). A more recent study employing infusion of a combination of either non-specific or more iNOS-selective inhibitors together with LPS has confirmed that the non-selective inhibitors lead to an exacerbation of liver damage and an increase in LPS-induced necrosis [94]. The increased necrosis is associated with, but independent of increased ICAM-1 expression and neutrophil migration into the liver. Co-infusion of O^2-vinyl 1-(pyrrolidin-1-yl)diazen-1-ium-1,2-diolate (V-PYRRO/NO) to deliver exogenous NO at the level of the liver reversed the effects of the non-selective NOS inhibitors, confirming a protective effect of NO. V-PYRRO/NO infusion did not inhibit the basal LPS-induced damage indicative of the involvement of non-NO mediated mechanisms [94], which is supported by the finding that liver injury is also a feature of the iNOS-deficient mouse following treatment with LPS [29]. In the study of Ou et al. specific iNOS inhibition did not affect liver necrosis, ICAM-1 expression or neutrophil infiltration suggesting that NO produced by eNOS has the major protective role in limiting inflammation-induced tissue damage. In contrast to the above study, a model using a bolus injection of selective iNOS inhibitors has suggested that overproduction of NO by iNOS may also play a significant role in inducing hepatic damage and MODS in rodent models of endotoxic shock (reviewed in [4]). These inhibitors, based upon substituted guanidines or isothiurea derivatives, have been shown to attenuate liver dysfunction, under conditions where L-NMMA exacerbated hepatic damage. In support of this, prevention of IκB degradation by the calpain 1 inhibitor or blocking NF-κB activation with dexamethasone to prevent iNOS induction attenuates the

onset of MODS and liver damage in the rat model of endotoxic shock further strengthening the role of enhanced NO formation by iNOS being detrimental [38]. However, other mechanisms in addition to iNOS induction cannot be completely eliminated using this approach as the induction of other proteins such as COX-2 which are also induced via an NF-κB-dependent mechanism would also be inhibited. Although the extent of the involvement of iNOS in causing liver necrosis is unclear, it is evident that it is the inhibition of the constitutive eNOS and not iNOS which exacerbates the LPS-induced liver damage in studies employing non-specific NOS inhibitors. It is likely that in reality liver damage and MODS are actually a combination of excessive NO, alterations in liver perfusion as a result of disturbances in the hepatic microcirculation and the generation and interaction of other factors with NO, e.g. reactive oxygen intermediates.

A similar situation to that in liver has also been observed in the rat small intestine. Induction of iNOS following in vivo endotoxin challenge is associated with a reduction in cell viability, mitochondrial dysfunction, vasocongestion and plasma exudation into the intestinal lumen [103–105]. Pretreatment with L-NMMA prevents NO formation; however, it enhances the endotoxin-induced intestinal damage and plasma leak [104], while NO donors attenuate this damage [106]. Inhibition of iNOS induction by pretreatment with dexamethasone or specific inhibition of NO formation with selective iNOS inhibitors also results in attenuation of cell damage, mitochondrial dysfunction and intestinal hyperpermeability [103,105], suggesting that NO formed by the constitutive enzyme aids in the maintenance of microvascular flow and epithelial cell integrity, while excessive NO production due to iNOS contributes to cell damage and accumulation of fluid within the intestinal lumen [103,105].

8. NO and apoptosis in septic shock

In addition to death by necrosis, cells may also undergo programmed cell death or apoptosis. Apoptosis and DNA fragmentation have been demonstrated in a variety of tissues following endotoxin treatment of the rat, including liver, lung, kidneys, thymocytes and intestine [107,108], although in the liver of non-sensitised animals this occurs in a very small proportion of cells [94,107]. The importance of NO in apoptosis is complex and outside the scope of this review; however, NO has been shown to suppress apoptosis in a number of cell types including endothelial cells, lymphocytes, eosinophils and hepatocytes, whereas it has been shown to induce apoptosis in macrophages, β-cells and thymocytes (reviewed in [109]). The ultimate effect of NO is likely to depend upon the tissue type, source and concentration of NO and duration of treatment [109]. Recent studies in liver have demonstrated that significant apoptosis only occurs when LPS is infused together with a NOS inhibitor [94]. This can be prevented by simultaneous administration of the liver-specific NO donor, V-PYRRO/NO, indicative of a protective role of NO. In contrast to necrosis, iNOS-specific inhibitors are equally potent with non-specific inhibitors in inducing apoptosis, suggesting that the inhibition of apoptosis is the result of iNOS expression in the liver or a combination of both iNOS and eNOS activity [94]. The mechanisms underlying the effects of NO on apoptosis remain to be fully elucidated. NO appears to be able to protect cells from apoptosis by several mechanisms. Large quantities of NO generated by iNOS protect from TNF-α-induced apoptosis by induction of heat shock protein 70, whereas small quantities block via mechanisms involving inhibition of caspase-3-like protease activation, possibly via *S*-nitrosylation of the protein ([94,109] and references therein). In contrast, the induction of apoptosis by high concentrations of NO may reflect direct DNA damage in the susceptible tissues [109].

9. Conclusion

In conclusion, it is evident that excessive production of NO following the induction of iNOS as a result of an increased circulating cytokine concentration makes a major contribution to the development of the characteristic symptoms of septic or endotoxic shock. However, it is evident that in many cases an increase in NO by itself may be insufficient, and the generation of other mediators, e.g. reactive oxygen intermediates, may also play a crucial role in the

tissue dysfunction and damage observed in septic shock and MODS. Indeed NO produced by both eNOS and in some cases iNOS may be beneficial and ameliorate the tissue damage and apoptosis associated with septic shock. Much of our knowledge concerning the involvement of NO has been derived from isolated cell or tissue systems (frequently using rodent tissue and rodent models of shock), and the use of non-specific inhibitors of NO synthases. Although these produce useful insights into the physiological situation in man, extrapolation of the data needs to be approached with some caution. For example, in vitro experiments rarely take into account the interplay with the effects of counter-regulatory factors which are also elevated during septic shock, e.g. increased circulating concentrations of cytokines (IL-10, TGF-β), steroid hormones and cAMP mediated hormones (glucagon and catecholamines), while rodent tissue is relatively insensitive to endotoxin and cytokines, relative to that of humans [5]. Similarly the use of non-specific NO synthase inhibitors has led to contradictory interpretations because of the inhibition of both the constitutive and inducible forms of NOS. With the development and increased availability of iNOS specific inhibitors, this problem should be resolved and the results from clinical trials employing these agents should allow us to better understand the role of excessive NO production in shock in man.

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