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Nitric oxide and endotoxin-mediated sepsis: the role of osteopontin

Review Article

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Abbreviations: bactericidal/permeability-increasing, (BPI); basic fibroblast growth factor, (bFGF); cardiac index, (CI); cholesterol ester transfer protein, (CETP); chromatin immunoprecipitation, (ChIP); c-Jun N-terminal kinase, (JNK); cyclic monophosphate, (cGMP); endothelial NOS, (eNOS); Gly-Arg-Gly-Asp-Ser, (GRGDS); glycosylphosphatidyinositol, (GPI); heterogeneous ribonucleoprotein A/B, (hnRNP A/B); inducible NO synthase, (iNOS); interferon gamma, (IFN-γ); interleukin-1, (IL-1); lipopolysaccharide, (LPS); mean arterial pressure, (MAP); neuronal NOS, (nNOS); N^G-nitro-*L*-arginine methyl ester, (L-NAME); Nitric oxide, (NO); NO synthase, (NOS); osteopontin, (OPN); phorbol 12-myristate 13-acetate, (PMA); phospholipid transfer protein, (PLTP); platelet activating factor, (PAF); poly-ADP ribose synthase, (PARS); protein kinase RNA-regulated, (PKR); pulmonary vascular resistance, (PVR); reactive oxygen species, (ROS); suppression subtractive hybridization, (SSH); systemic inflammatory response syndrome, (SIRS); systemic vascular resistance, (SVR); TNFα receptor-associated factor-6, (TRAF6); Toll-like receptor 4, (TLR4); tumor necrosis factor-α, (TNF-α)

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Summary

Septic shock continues to be a life threatening complication of systemic infection despite advances in the clinical care of these patients. The incidence of severe sepsis in critically ill patients has increased annually by 8.7% and mortality rates are excessive, ranging from 30%-60%. Nitric oxide plays a central role in the molecular biology and biochemistry of septic shock. In endotoxin-mediated sepsis and septic shock, pro-inflammatory cytokines are elaborated and inducible nitric oxide synthase is systemically expressed in multiple cell types. The sustained production of nitric oxide in high concentration regulates multiple cellular and biochemical functions. Multiple studies have investigated the role of nitric oxide synthase antagonists in the treatment of septic shock in both animal models of endotoxemia and human clinical trials. However, cumulative data from these studies have not provided definitive evidence for a survival benefit in the use of these agents in humans. While the signalling pathways that activate iNOS expression or activity are well characterized, little is known about the endogenous molecular determinants that decrease NO. In this regard, osteopontin, recently identified as an intrinsic regulator of iNOS expression in endotoxin-stimulated macrophages, represents a novel target in the understanding of nitric oxide pathobiology in sepsis. The purpose of this review is to discuss the *S*-nitrosylation of heterogeneous ribonucleoprotein A/B in the transcriptional regulation of osteopontin in nitric oxide- mediated sepsis.

I. Introduction

Sepsis refers to a heterogeneous group of inflammatory syndromes that represent various stages involved in the host-response to infection. Septic shock has been previously defined as sepsis-induced hypotension that persists despite adequate fluid resuscitation with characteristic clinical manifestations such as lactic acidosis, oliguria or coagulopathy (Bone et al, 1992; Levy et al, 2003). There is a continuum and natural progression between the different stages of the inflammatory response from systemic inflammatory response syndrome (SIRS) to sepsis, severe sepsis, shock, and multiple organ dysfunction (Brun-Buisson, 2000). Risk factors identified as independently associated with severe sepsis include age, male sex, the presence of indwelling catheters or devices, chronic liver insufficiency, immunodepression, or severe underlying disease (Brun-Buisson et al, 1995, 2004; Balk, 2004). Septic shock continues to be a life threatening complication of systemic infection despite advances in the clinical care of these patients. The incidence of severe sepsis in critically ill patients has increased annually by

8.7% (Balk, 2004) with mortality rates ranging from 30%-60% (Brun-Buisson et al, 1995, 2004; Martin et al, 2003; Balk, 2004).

Nitric oxide (NO) plays a central role in the molecular biology and biochemistry of septic shock. In endotoxin-mediated sepsis and septic shock, pro-inflammatory cytokines are elaborated and inducible NO synthase (iNOS) is systemically expressed in multiple cell types. The sustained production of NO in high concentration regulates multiple cellular and biochemical functions, including inotropic and chronotropic cardiac responses, systemic vasomotor tone, intestinal epithelial permeability, endothelial activation, and microvascular permeability (Finkel et al, 1992; Kilbourn et al, 1997; Chavez et al, 1999).

In the decade since the discovery of NO as endothelium derived relaxing factor, multiple studies have investigated the role of NO synthase (NOS) antagonists in the treatment of septic shock in both animal models of endotoxemia and human clinical trials. The cumulative data from these studies do not reach consensus and conflict on whether NOS antagonists decrease sepsis-related mortality. Certainly, substantial evidence supports that NOS inhibition improves physiological endpoints during septic shock (Vincent et al, 2000; Cobb, 2001; Feihl et al, 2001). The non-selective and non-physiologic effects of these inhibitors used in model systems may account for some of the adverse effects observed in these studies and for the failure of these agents in increasing survival in clinical studies. Few studies have attempted to modulate iNOS expression by manipulating the intrinsic, homeostatic mechanisms that lead to iNOS down-regulation. Interestingly, while the signalling pathways that activate iNOS expression or activity are well characterized, little is known about the endogenous molecular determinants that decrease NO. In this regard, the recent discovery of osteopontin (OPN) as an intrinsic regulator of iNOS expression in endotoxin-stimulated macrophages represents an area of investigation that may yield novel targets for the therapeutic modulation of NO during sepsis.

In this discussion, we review the lipopolysaccharide (LPS) signalling pathways that lead to upregulation of iNOS expression and the biochemistry and physiology of NO in septic shock. In addition, we will describe the role of OPN in the regulation of NO and the identification of heterogeneous ribonucleoprotein A/B (hnRNP A/B) as an endogenous, NO-dependent, transcriptional regulator of OPN.

II. The LPS signalling pathway in sepsis

LPS endotoxin is the principal component of the outer membrane of Gram-negative bacteria. The structural components of LPS include an outer O-antigen polysaccharide region; outer, intermediate, and inner core polysaccharide regions; and the toxic lipid A moiety embedded deep within the outer membrane (Alexander and Rietschel, 2001; Lazaron and Dunn, 2002). Stimulation with LPS activates the cells of the innate immune system to produce a variety of inflammatory cytokines including interleukin-1 (IL-1), IL-6, IL-8, tumor necrosis factor-α (TNF-α) and NO. However, overstimulation of the monocytic signalling pathways with LPS can lead to systemic inflammation resulting in sepsis or shock.

The LPS signalling cascade involves the complex co-operation of a multitude of receptors, cofactors and messenger proteins (**Figure 1**).

The processing of LPS for signal transduction begins in the extracellular space with the ligation of LPS by LPS-binding protein (LBP). Derived from hepatic synthesis, LBP is secreted into the serum, and responds to LPS stimulation with a 5- to 20- fold increase in LBP concentration (Lazaron and Dunn, 2002). Sequence analysis and cloning of LBP cDNA has led to the identification of a family of related proteins that include bactericidal/permeability-increasing protein (BPI), cholesterol ester transfer protein (CETP), and phospholipid transfer protein (PLTP). The glycosylphosphatidyinositol (GPI)-linked membrane protein, CD14, is a myeloid surface antigen that lacks a transmembrane domain. A non-GPI-containing soluble form of CD14 is also secreted into the serum (Lazaron and Dunn, 2002). CD14 functions by recognizing the LPS-LBP complex (**Figure 1**). Loss-of-function studies have demonstrated that LBP and CD14 are necessary for the rapid and sensitive induction of the monocyte/macrophage inflammatory response to LPS (Diks et al, 2001). These cofactors appear to enhance the function of Toll-like receptor 4 (TLR4), the putative signalling receptor for LPS. Studies using murine macrophages with a targeted loss-of-function in TLR4 resulted in the ablation of the physiologic response to LPS (Guha and Mackman, 2001). TLR4 activity was found to be dependent on MD-2, a secreted protein that associates with TLR4 and enhances TLR4-dependent signalling pathways (**Figure 1**).

TLR4 regulates multiple intracellular, inflammatory signalling cascades including the NF-κB, ERK, JNK and p38 pathways. Cumulative data suggests that MyD88, IL-1 receptor-associated kinase (IRAK) and TNFα receptor-associated factor-6 (TRAF6) mediate TLR4 activation of NF-κB by enhancing phosphorylation of IKKβ, which in turn phosphorylates IκB and leads to the translocation of NF-κB p50 and p65 into the nucleus (Diks et al, 2001; Guha and Mackman, 2001). LPS also activates the extra-cellular signal-regulated kinase (ERK1/2) signalling pathway. LPS-mediated activation of MEK-ERK1/2 appears to occur via diverse mechanisms as both Ras/c-Raf -dependent and -independent pathways have been identified (Guha and Mackman, 2001). One downstream target of the MEK-ERK1/2 pathway is the transcription factor Elk-1, which co-operates with SRF to activate target genes. The c-Jun N-terminal kinase (JNK) signalling pathway can also be activated by LPS. Upstream activators of JNK include mPAK3, hPAK1, GCK, MEKK1 and MKK4/7 and the targeted transcription factors consist of c-Jun, ATF-2 and Elk-1 (Guha and Mackman, 2001). The p38 signalling pathway is yet another unique signalling pathway that is regulated by LPS. Cdc42, PAK , Rac1, protein kinase RNA-regulated (PKR) and MKK3/6 are some of the upstream signalling

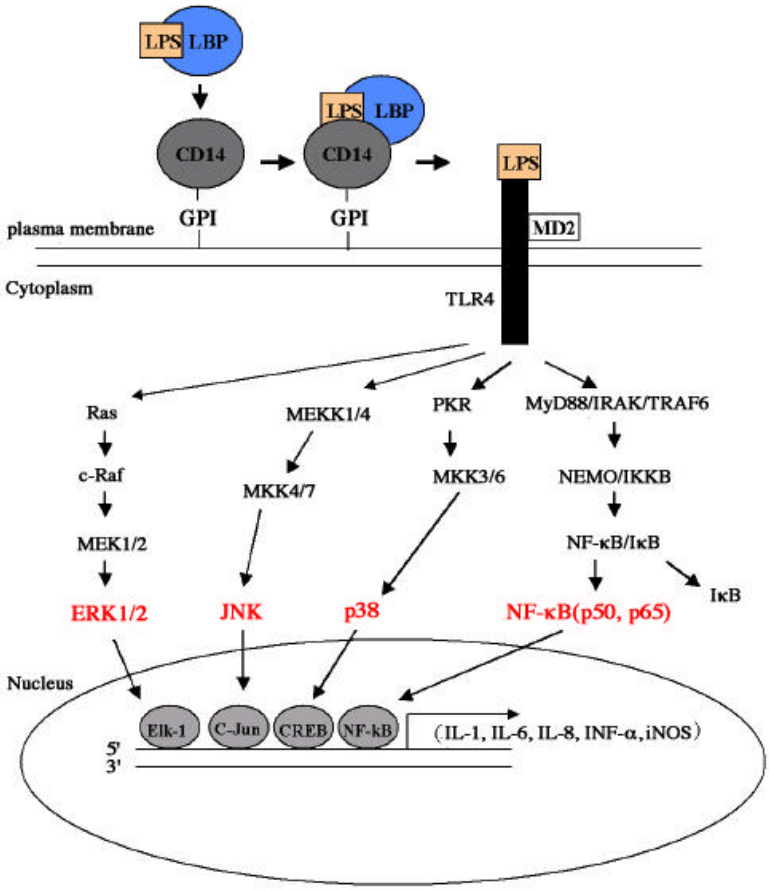

Figure 1. The LPS signalling pathway regulates transcription of the inflammatory mediator genes IL-1, IL-6, IL-8, TNF-α, and iNOS (Alexander and Rietschel, 2001; Diks et al, 2001; Guha and Mackman, 2001; Lazaron and Dunn, 2002). Please see text for details. Partialy reproduced from Guha and Mackman, 2001 with kind permission from Cellular Signalling.

molecules that activate p38. Target transcription factors activated by p38 include ATF-2, Elk-1, CHOP, MEF2C, Sap1a, CREB and ATF-1 (Guha and Mackman, 2001). Terminal signalling events from these different cascades regulate gene expression of TNF-α, IL-1, IL-6, IL-8, G-CSF, GM-CSF, MCP-1, IL-2 Rα and iNOS.

III. Increased nitric oxide production in sepsis

A. NO biosynthesis, mechanism of action, and pathophysiology

An important downstream effector of LPS signalling is iNOS, the primary regulator of NO production in sepsis. NO is a ubiquitous biological molecule produced by several cell types. The terminal guanidino group of the amino acid L-arginine gives rise to NO under redox-regulation by NOS in a calmodulin-dependent manner (**Figure 2**) (Nathan and Xie, 1994). The three known isoforms of NOS have been identified as neuronal NOS (nNOS/NOS-I), inducible NOS (iNOS/NOS-II), and endothelial NOS (eNOS/NOS-III). While the expression of nNOS and eNOS are constitutive, iNOS expression can be significantly upregulated in response to bacterial products and pro-inflammatory cytokines. NO production and iNOS expression play a central role in the pathobiology of sepsis. In the preceding section, we briefly reviewed some of the signalling pathways by which LPS signalling transcriptionally activates iNOS. However, a variety of stimuli, including microbes, IL-1, IL-6, IL-12, TNF, interferon-γ, α/β and platelet activating factor (PAF), can promote iNOS expression (Nathan and Xie, 1994; Nathan, 1997; Taylor and Geller, 2000). During sepsis, these agents act synergistically to induce iNOS gene transcription through complex signalling pathways that involve the NF-κb, cyclic AMP-CREB-C/EBP and Jak-Stat pathways (Nathan and Xie, 1994; Nathan, 1997; Taylor and Geller, 2000; Diks et al, 2001). Secondary auxiliary signalling pathways include AP-1, phospholipase C, protein kinase C, Ras-MAP kinase, and hypoxia inducible factor-1.

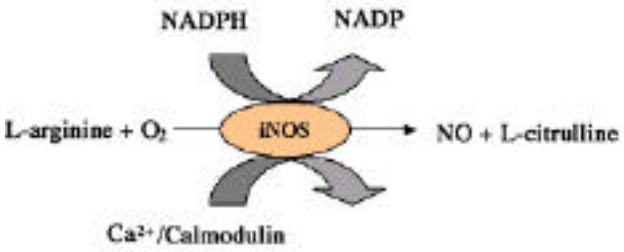

Figure 2. NO is produced by iNOS under redox conditions in a calmodulin-dependent manner. L-arginine and oxygen are catalytic substrates for iNOS during the production of NO and L-citrulline. The reaction occurs with the oxidation of NAPDH to $NADP^+$.

NO is a pleuripotent regulator of multiple cellular and biochemical functions, including allosteric receptor modification, enzymatic activity and transcriptional regulation (Crapo and Stamler, 1994; Morris and Billiar, 1994; Simon et al, 1996). NO, a highly-diffusable, gaseous free-radical, binds to heme-containing proteins such as guanylate cyclase which it activates to release guanosine 3'5'-cyclic monophosphate (cGMP), a potent intracellular second-messenger. NO also mediates *S*-nitrosylation of key target molecules in many biological processes (Feihl et al, 2001). Using these different mechanisms, NO can generate a variety of downstream activators. NO and its derivatives also possess innate biochemical properties as reactive oxygen species (ROS). ROS species include NO, its metabolic products (nitrite, nitrate and peroxynitrite) and other related-molecules such as superoxide anion, hydroxyl anion and hydrogen peroxide. Production of ROS is enhanced in sepsis and these products exert toxic effects on nucleic acids, lipids, and proteins (Symeonides and Balk, 1999). In particular, peroxynitrite impairs mitochondrial respiration, activates poly-ADP ribose synthase (PARS), reduces NAD pools, cellular glycolysis, electron transport, and limits ATP generation (Vincent et al, 2000). These free-radical species are thought to be responsible for significant cellular damage during severe sepsis.

In endotoxin-mediated sepsis and septic shock, the sustained production of NO in high concentration in multiple cell types modifies inotropic and chronotropic cardiac responses, systemic vasomotor tone, pulmonary vasomotor tone, intestinal epithelial permeability, endothelial activation, microvascular permeability, renal tubular-glomerular feedback, platelet adhesion and aggregation, and insulin metabolism (Finkel et al, 1992; Kilbourn et al, 1997; Chavez et al, 1999; Symeonides and Balk, 1999; Vincent et al, 2000). The natural history of septic shock stems from the combination of negative inotropic cardiac effects, pulmonary vasoconstriction and hypertension, decreased vasomotor tone and profound vasodilation with resultant hyperdynamic-cardiovascular collapse, leading to overwhelming tissue hypoxia and multiple organ dysfunction (Symeonides and Balk, 1999).

B. The negative feedback regulation of NO

Over the past decade, studies utilizing NOS antagonists to treat the deleterious effects of septic shock have produced conflicting results (Symeonides and Balk, 1999; Vincent et al, 2000; Cobb, 2001; Feihl et al, 2001). NOS antagonists can be categorized as amino-acid- or non-amino-acid-based competitive analogs whose members primarily exert either iNOS -selective or -non-selective effects (Vincent et al, 2000). In preclinical animal models of septic shock, the use of NOS inhibitors have shown that mean arterial pressure (MAP) and systemic vascular resistance (SVR) can be significantly increased (Symeonides and Balk, 1999; Vincent et al, 2000). Benefit on survival, however, has been less clear. Moreover, there are several potential detrimental effects to non-specific NOS inhibition including decreased organ perfusion, elevation of mean pulmonary artery pressure, pulmonary vascular resistance (PVR), renal vascular resistance and decreased renal blood flow (Vincent et al, 2000). The use of NOS inhibitors in animal models of endotoxemia has been associated with a decrease in cardiac index (CI) and tissue oxygen delivery and an increase in lactic acidosis and hepatic toxicity (Vincent et al, 2000; Cobb, 2001). In several studies, non-selective NOS inhibition was found to be associated with increased mortality (Symeonides and Balk, 1999; Vincent et al, 2000; Cobb, 2001). Clinical trials in human subjects have been performed and they revealed similar effects on SVRI, MAP, PVRI, CI, PCWP and CVP as those found in animal models of sepsis (Symeonides and Balk, 1999; Vincent et al, 2000; Cobb, 2001).

Many of these studies utilize compounds that are added exogenously to model systems. The investigation of *in situ,* homeostatic mechanisms that regulate iNOS expression and NO production represents a novel approach to understanding the complex biology of iNOS regulation and may yield new therapeutic targets. In contrast to iNOS activation pathways, the endogenous counter-regulatory pathways which inhibit iNOS expression and activity in a biologically relevant manner are largely unknown. Certainly, glucocorticoids, IL-4, IL-8, IL-10, transforming growth factor (TGF-β1, β2, β3), NAP110, kalirin and macrophage deactivating factor are among identified inhibitors of iNOS activation (Nathan and Xie, 1994; Nathan, 1997; Taylor and Geller, 2000). While TGF-β-exerts transcriptional and post-translational control of iNOS (Nathan and Xie, 1994), kalirin and NAP110 inhibit iNOS activity by preventing iNOS homodimer formation (Ratovitski et al, 1999a, b). Substrate and cofactor availability can also modulate iNOS activity (Nathan and Xie, 1994). Studies investigating these inhibitors have underscored the immense complexity and species-, signal- and cell-dependent nature of iNOS regulation. Moreover, the biological relevance of many of these inhibitors is unknown as their effects on iNOS activity have largely been determined in model systems in which they have been exogenously administered. In addition, the underlying signal transduction pathway for each inhibitory agent has not well characterized. An interesting and unique feature of iNOS counter-regulation is the negative feedback characteristic of NO (DelaTorre et al, 1997). NO, as the end-product of iNOS activity, can both directly and indirectly feedback inhibit iNOS expression. These endogenous inhibitory pathways by which NO feedback regulates iNOS expression remain poorly understood. NO may downregulate expression or activity of an iNOS inducing stimulus or conversely, upregulate expression or activity of an iNOS repressor. One example of how NO can biochemically trigger iNOS regulators is the *S*-nitrosylation of intermediary proteins. The biochemical kinetics of NO-mediated *S*-nitrosylation of NF-κB has been investigated and NO decreases the dissociation constant by four-fold. This suggests that NO modifies NF-κB active site-thiols and inhibits NF-κB DNA binding and subsequent iNOS gene transcription (DelaTorre et al, 1997). Critical thiol and non-heme iron groups which may serve as targets for NO are not limited to NF-κB. *S*-nitrosylation targets of NO include p53, caspase-8,

transglutaminase, glyceraldehyde-3-phosphate dehydrogenase, and glutiathione reductase (Calmels et al, 1997). The ubiquity of negative feedback regulation as a mechanism for modulation of protein activity suggests that inhibitory mechanisms for iNOS may be NO-dependent and that there exists a pool of NO-regulated genes and proteins, which potentially serve as mediators in NO-feedback regulation.

Using suppression subtractive hybridization (SSH), we have recently identified OPN as a regulator of iNOS that is itself NO-dependent (Guo et al, 2001). In ANA-1 murine macrophages, we hypothesized that endotoxin (LPS)-mediated NO production induces a specific set of genetic programs that may serve to alter cellular NO metabolism. To identify genes differentially expressed in LPS-stimulated cells producing NO, RNA from LPS-treated cells was used as a "tester" and RNA from LPS plus N^G-nitro-*L*-arginine methyl ester (L-NAME) was used as a "driver". Individual cDNA clones generated by SSH were used as probes in Northern blot analysis to identify differentially expressed genes. Using SSH, OPN was found to be specifically induced in the presence of LPS-induced NO synthesis.

IV. Osteopontin, nitric oxide synthase and hnRNP A/B

A. OPN structure, receptors and function

OPN is a hydrophilic and negatively charged sialoprotein of ~298 amino acids that contains a Gly-Arg-Gly-Asp-Ser (GRGDS) integrin-binding motif and additional domains for calcium-binding, phosphorylation and glycosylation (Wai and Kuo, 2004). Post-translational modifications account for cell-type and condition-specific OPN-isoforms, which can be measured between 41-75 kD (Wai and Kuo, 2004). This secreted phosphoprotein mediates diverse regulatory functions, including cell adhesion, migration, tumor growth and metastasis, atherosclerosis, aortic valve calcification, and repair of myocardial injury. Many of these functions appear to be regulated by signalling through the integrin and CD44 families of receptors (Wai and Kuo, 2004). OPN expression is tissue-specific and subject to regulation by many factors (Hijiya et al, 1994; Guo et al, 1995; Chellaiah et al, 1996; Wai and Kuo, 2004). Constituitive expression of OPN exists in several cell types but induced expression is found in T lymphocytes, epidermal cells, bone cells and macrophages in response to phorbol 12-myristate 13-acetate (PMA), 1,25-dihydroxyvitamin D, basic fibroblast growth factor (bFGF), TNF-α, IL-1, interferon gamma (IFN-γ) and endotoxin. Interestingly, OPN and iNOS are induced in response to many of the same agents such as TNF-α, IL-1β, IFN-γ, and LPS (Nathan and Xie, 1994).

B. OPN and inflammation

Recently the relationship between OPN, NO and inflammation has been examined by a number of investigators. Rollo et al, (1996) demonstrated that exogenous recombinant OPN protein was effective in blocking RAW264.7 murine macrophage NO production and cytotoxicity toward the NO-sensitive mastocytoma cells. Their work suggested that OPN in extracellular fluid protects certain tumor cells from the macrophage-mediated destruction by inhibiting the synthesis of NO. Singh et al, (1995, 1999) reported that a synthetic 20-amino acid OPN peptide analogue decreased iNOS mRNA and protein levels in ventricular myocytes and cardiac microvascular endothelial cells. Transfection of cardiac microvascular endothelial cells with an antisense OPN cDNA increased iNOS mRNA in response to IL-β and IFN-γ, suggesting that endogenous OPN inhibits NO production. Using an antibody directed against the OPN $\alpha_v\beta_3$ integrin receptor, Attur et al, (2001) demonstrated that ligand binding results in *trans*-dominant inhibition of NO production in human cartilage. Hwang et al, (1994a, b) found that OPN suppressed NO synthesis induced by interferon and LPS in primary mouse kidney proximal tubule epithelial cells. These studies clearly demonstrate that endogenous OPN can inhibit induction of iNOS and that OPN is an important regulator of the NO signalling pathway and NO-mediated cytoregulatory processes. However, the converse relationship, the role of NO in the induction of OPN synthesis, has not been well studied.

In our laboratory, we have recently demonstrated that LPS-induced NO synthesis up-regulates OPN promoter activity and protein expression (Guo et al, 2001). We have shown that LPS-treated ANA-1 and RAW 264.7 macrophages express high levels of OPN protein while untreated macrophages show no detectable level of immunoreactive OPN protein. The addition of L-NAME (competitive NOS inhibitor) to LPS-treated cells ablates OPN protein expression whereas the co-addition of the NO donor, *S*-nitroso-*N*-acetylpencillamine (SNAP), restores OPN expression in LPS + L-NAME treated cells. These data suggest that LPS-mediated NO production is associated with significantly increased OPN protein secretion in both ANA-1 and RAW 264.7 macrophages. Using nuclear run-on analysis, we showed that the NO-mediated increase in macrophage-OPN mRNA levels was the result of increased gene transcription. Transient transfection of plasmid constructs containing an 865-bp OPN promoter cloned upstream from a luciferase reporter gene, demonstrated that LPS-induced NO production increased OPN promoter activity by ~7-fold compared with controls (Guo et al, 2001). Together these data provide evidence to suggest that NO expression induced by LPS increases OPN promoter activity, and OPN mRNA and protein levels. We have also shown that blockade of the OPN-integrin cell surface receptor with GRGDSP increases macrophage NO production in response to LPS stimulation while the addition of exogenous OPN with LPS to ANA-1 cells maximally decreased nitrite levels by 50%. Together, these data suggest that OPN plays a functional role in regulating LPS-mediated NO production.

C. S-nitrosylation of hnRNP A/B regulates OPN transcription during endotoxin stimulation

Cloning of the human, porcine and murine OPN promoters has uncovered numerous consensus regulatory sequences (Wai and Kuo, 2004). Early investigations with the human OPN promoter revealed multiple candidate elements that contain consensus sequences for known transcription factors including TATA-like (-27 to -22 nt) and CCAAT-like (-73 to -68 nt) sequences, vitamin-D-responsive (VDR)-like motifs (-1892 to -1878 and -698 to -684 nt), GATA-1 (-851 to -847 nt), AP-1 (TGACACA, -78 to -72 nt), PEA3 (-1695 to -1690 and -1418 to -1413 nt) and Ets-1 (-47 to -39 nt) binding sequences and multiple TCF-1 sites (31). Craig and Denhardt identified similar sequences in the murine OPN promoter: a characteristic TATA box (-27 to -22 nt), an inverted CCAAT box (-53 to -49 nt), a positive transcription element (-543 to -253 nt) and a negative transcription element (-777 and -543 nt) (Craig and Denhardt, 1991). Several investigators have since shown that transcriptional regulation of OPN is complex and involves multiple pathways. Several inter-related signalling pathways and transcription factors regulate the OPN promoter including AP-1, Myc, Oct-1, USF, v-Src, Runx/CBF, TGF-B/BMPs/Smad/Hox, Wnt/ß -catenin/APC/GSK-3ß/Tcf-4, Ras/RRF and TP53 (Wai and Kuo, 2004).

Recently, we have identified heterogeneous nuclear ribonucleoprotein A/B (hnRNP A/B) as a constitutive transcriptional repressor of OPN whose DNA binding activity is decreased by LPS-mediated *S*-nitrosylation of a key cysteine thiol. hnRNPs were originally described as a group of chromatin-associated RNA-binding proteins that form complexes with RNA polymerase II transcripts. The hnRNP family is a collection of more than 20 proteins that contribute to the complex around nascent pre-mRNA and are thus able to modulate RNA processing (Krecic and Swanson, 1999). Members of the group are characterized by their ability to bind to RNA with limited specificity, and they are among the most abundant of all of the nuclear proteins. Despite its function in RNA handling, the precise physiological role of hnRNPs has yet to be fully defined and may include *trans*-regulatory effects. Recent studies have shown that the hnRNPs D0B, E2BP, and K are able to bind to double-stranded DNA motifs within the complement receptor 2, hepatitis B virus, and c-myc promoters, respectively (Tay et al, 1992; Tomonaga and Levens, 1995; Tolnay et al, 1999). hnRNP K possesses both transcriptional activator and repressor functions (Michelotti et al, 1996).

hnRNP A/B is a unique member of the hnRNP family in that it possesses a DNA-binding sequence domain that is separate from the repression domain. The p40 isoform contains 331 amino acid residues, whereas p37 contains 284. The amino acid sequences are identical with the exception of an additional 47 amino acids at the C-terminal region of p40. In this regard, Yabuki et al, (2001) found that hnRNP A/B p40 binds to the rat aldolase B promoter to inhibit activity, whereas hnRNP A/B p37 had no effect. Further studies by this group found that the DNA-binding region for both isoforms reside with amino acids 67-159, 67-75, and 147-159 are absolute requirements for binding activity (Saitoh et al, 2002). This 67-159-amino acid region contains the *S*-nitrosylation target Cys 104, which was found to be responsible for NO-mediated inhibition of DNA binding in our experiments.

Using OPN promoter deletion constructs cloned upstream from a luciferase reporter gene we localized a NO-sensitive *cis*-acting element in the OPN promoter (-174 to -209 nt). Deletion of this segment resulted in > 4-fold increase in OPN promoter activity (Gao et al, 2004). Electromobility shift assay demonstrated that nuclear protein is bound to the OPN promoter in the region of nt -183 to nt -196 in unstimulated control cells. In the presence of LPS and NO, binding is ablated, and OPN promoter activity is increased. Utilizing the biotin-streptavidin DNA affinity technique with the identified DNA-binding sequence, the candidate repressor-transcription factor was then purified and isolated from nuclear extract isolated from unstimulated control RAW 264.7 macrophages. The purified proteins were separated by SDS-PAGE and analyzed after tryptic digestion and yielded results that matched with hnRNP A/B (GenBankTM accession number NM 010448). Supershift assays confirmed the identity of the gel-shift band and chromatin immunoprecipitation (ChIP) -assay analysis demonstrated *in vivo* binding of hnRNP A/B to the OPN promoter (Gao et al, 2004). This binding was inhibited in the presence of NO that was either endogenously induced by LPS or exogenously delivered. Finally, we demonstrated that *S*-nitrosylation of hnRNP A/B p37 is significantly enhanced in the presence of LPS-mediated NO synthesis and that *S*-nitrosylation of the p37 cysteine residue at position 104 is associated with diminished DNA binding in gel shift assays. Together these data suggest that LPS-induced *S*-nitrosylation of hnRNP A/B inhibits its activity as a constitutive repressor of the OPN promoter (**Figure 3**).

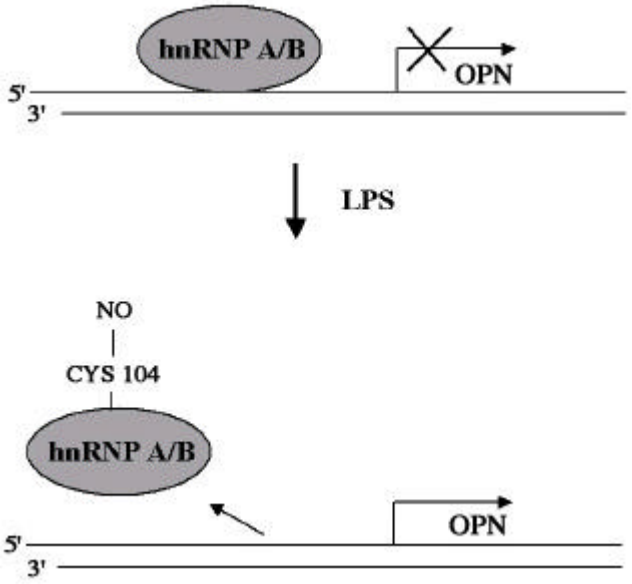

Figure 3. *S*-nitrosylation of hnRNP A/B relieves transcriptional repression of OPN during LPS-mediated production of NO and serves as a negative feedback mechanism for iNOS regulation.

V. Conclusion

In sepsis, endotoxin-mediated production of NO involves complex signalling pathways that regulate iNOS expression. NO has wide-ranging biochemical and physiologic effects in multiple organ systems and mediates some of the processes that lead to cardiovascular collapse, tissue hypoxia and organ failure in the septic patient. While many studies have focused on the modulation of NO production as a means of reducing the mortality associated with septic shock, little is known about the endogenous, homeostatic pathways that lead to down-regulation of NO synthesis. Our current findings suggest that LPS-induced *S*-nitrosylation of hnRNP inhibits its activity as a constitutive repressor of the OPN promoter. This represents a novel target for *S*-nitrosylation regulatory functions as hnRNP proteins are better characterized as participants in telomere biogenesis, splicing, and mRNA transport. Further study to determine the potential role of *S*-nitrosylation in these other hnRNP-dependent functions may expand the known regulatory roles for NO and *S*-nitrosylation.

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