Original Article

Differential Expression of Inducible Nitric Oxide Synthase in Septic Shock

D. Bradford Sanders, BS; Douglas F. Larson, PhD, CCP; Curt Jablonowski, MS, CCP; Laura Olsen, BS

Circulatory Sciences Graduate Perfusion Program, Sarver Heart Center, University Medical Center, University of Arizona, Tucson, Arizona

Keywords: nitric oxide, septic shock, interleukin-1β, tumor necrosis factor-α, lipopolysaccharide, interferon-γ, hypotension

Presented at the 37th International Conference of the American Society of Extra-Corporeal Technology, New Orleans, Louisiana, April 8–11, 1999

ABSTRACT

During cardiopulmonary bypass (CPB), the septic patient has markedly decreased peripheral vascular resistance as a consequence of endotoxin release from microorganisms. This decrease in peripheral vascular resistance is the result of endotoxin-induced nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS). iNOS and eNOS are responsible for the synthesis of NO because of various stimuli, including the bacterial endotoxin, lipopolysaccharide (LPS). We tested the hypothesis that a differential expression of iNOS among human endothelial cells and murine macrophage is dependent upon exposure to endotoxin and various pro-inflammatory cytokines. Using a human endothelial cell line, ECV-304 and murine macrophage cell line, RAW 264.7, we quantified the expression of iNOS with specific FITC-conjugated antibodies using fluorescence activated cell sorter (FACS) and NO production with a Bioxytech nitric oxide spectrophotometric assay. This in vitro septic model utilized LPS supported with species-specific interferon-γ, interleukin-1β, and tumor necrosis factor-α. The cell types were stimulated for 8 hours with combinations of the cytokines mentioned. The FACS data demonstrated a significant stimulus-dependent increase in iNOS expression among the macrophage groups; however, the stimulated endothelial cells showed no significant change in iNOS expression. The nitric oxide production data demonstrated significant increases in NO production among macrophage stimulated groups; whereas, endothelial stimulated groups exhibit no significant change. We conclude that NO secreted during septic shock is the result of activated macrophage, not the endothelium. The clinical relevance is that the more severe the infectious process, the lower the PVR may be during CPB because of increased NO production from activated macrophage.

Address correspondence to:
D. Bradford Sanders
Circulatory Sciences Graduate Perfusion Program
Sarver Heart Center
University of Arizona
Tucson, AZ 85724
INTRODUCTION

During the coming year approximately 250,000 people will die from complications of sepsis (1). Sepsis can be categorized into four progressive stages, systemic inflammatory response syndrome, sepsis, septic shock, and multiple organ dysfunction (2). Septic shock during cardiopulmonary bypass (CPB) is usually characterized by refractory hypotension that is unresponsive to treatment with phenylephrine, norepinephrine, and epinephrine. During CPB, hypotension is especially dangerous, because perfusion of most major organ systems is pressure dependent because of the loss of vascular tone associated with septic shock (3). Nitric oxide (NO) is the principal mediator of profound vasorelaxation during septic shock (3).

The production of NO during septic shock is primarily the result of iNOS protein induction (3). iNOS can be expressed by numerous cell types including: endothelial cells, macrophage, neutrophils, and vascular smooth muscle cells (4). When the iNOS protein is induced during sepsis, the NO generation can increase to as much as fourfold (5).

NO binds to soluble guanylate cyclase (sGC) to activate the conversion of guanosine-5’-triphosphate (GTP) to cyclic guanosine-5’-monophosphate (cGMP) (6). Accumulations of cGMP interrupt the Ca²⁺ homeostasis, driving intracellular Ca²⁺ into the sarcoplasmic reticulum. In the absence of sufficient Ca²⁺ levels in the vascular smooth muscle cell, no contraction occurs; hence, vasorelaxation predominates. This interference of the calcium equilibrium can likely explain the refractoriness of vasoconstriction following administration of 2-adrenergic agonists, such as phenylephrine, norepinephrine, and epinephrine. As a result of the Ca²⁺ disturbance, agents dependent upon normal intracellular concentrations of Ca²⁺ lose their efficacy.

The vascular endothelium has been established as the primary regulator of vascular tone during physiologic conditions (5). However, during pathophysiologic conditions, macrophage, neutrophils, and vascular smooth muscle cells may overwhelm the endothelial regulation of the vasculature (6, 7). LPS is a principal molecule leading to dysregulation of vascular tone (8). Exposure to LPS initiates a cascade of cytokines that induce the inducible nitric oxide synthase isoform (3). Interleukin-1β (IL-1β), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) are the primary cytokines in the inflammatory response. These factors along with LPS amplify the vasoplegia during septic shock through the aforementioned NO pathway. Numerous studies have demonstrated that various cytokines, in the presence of LPS, display a potent effect on the expression of iNOS and NO production in macrophage. However, this finding is not transferred to the endothelial cell (1, 2, 9, 10). CPB has also been shown to increase the production of proinflammatory cytokines (11–13).

This immunological correlation has prompted our group to investigate the origin of septic shock-induced iNOS and NO production. We quantified the iNOS protein and NO production utilizing an in vitro model, endothelial cells, macrophage and tested the hypothesis that iNOS expression and NO production are similar between the two cell types. We found that macrophage express significantly more iNOS and produce more NO than endothelial cells.

METHODS AND MATERIALS

CELL CULTURE

A human endothelial cell line, ECV-304 and murine macrophage, RAW 264.7, were grown in a humidified incubator in room air and 5% carbon dioxide at 37°C. Endothelial cells were placed in Dulbecco’s modified Eagle’s medium DME/F12 (7.8 g, 1 g NaHCO₃, 1.19 g HEPES into 475 mL of ddH₂O) enriched with 20% fetal calf serum, first endothelial growth factor (15 mg/500 mL or 0.03 mg/mL), gentamicin (10 mg/mL), amphotericin (10 mg/mL), heparin (10,000 units/500 mL) and grown to confluency under sterile conditions. Macrophage were established in DME/F12 (7.8 g, 1 g NaHCO₃, 1.19 g HEPES into 475 mL of ddH₂O) supplemented with 10% fetal calf serum, penicillin/streptomycin (1 mL/500 mL), gentamicin (10 mg/mL), amphotericin (10 mg/mL). Macrophage were grown to confluency under sterile conditions.

STIMULATION TREATMENT

Upon confluent growth, endothelial cells were stimulated in the following schemes: (1) LPS (10 μg/mL); (2) LPS (10 μg/mL) and hIFN-γ (200 units/mL); (3) LPS (10 μg/mL), hIFN-γ (200 units/mL), hIL-1β (5 units/mL), and hTNF-α (10 units/mL); and (4) a control group receiving no stimulation. All concentrations are per milliliter of culture medium. Macrophage were stimulated in the following schemes (1) LPS (10 μg/mL); (2) LPS (10 μg/mL) and mIFN-γ (125 ng/mL); (3) LPS (10 μg/mL), mIFN-γ (125 ng/mL), mIL-1β (10 ng/mL), and mTNF-α (10 ng/mL); and (4) a control group receiving no stimulation. All concentrations are per milliliter of culture medium.

FLUORESCENCE STAINING PROTOCOL

Cells were collected separately from each flask and rinsed with PBS solution and centrifuged at 500 g. Cells were re-suspended and frozen at −70°C in DMSO (10% solution). At the time of staining, cells were thawed and rinsed again with PBS solution and centrifuged at 500 g. All cells were lysed

a American Type Cell Culture, Rockville, MD  
b Sheldon Laboratories, Cornelius, OR  
c Sigma, St. Louis, MO  
d Hyclone Laboratories, Logan, UT  
e R & D Systems, Minneapolis, MN  
f Calbiochem, San Diego, CA  
g Beckman, Palo Alto, CA
for 25 min with Fix and Perm fixation\textsuperscript{h}. Cells were then rinsed with PBS solution and centrifuged. The cells were suspended in the primary antibody\textsuperscript{i} (rabbit antimouse, 1:200 dilution) for iNOS and incubated in darkness for 90 min. The cells were then rinsed, centrifuged, and the secondary antibody\textsuperscript{j} (swine antirabbit, 1:20 dilution) was applied in darkness for a period of 60 min. The cells were then rinsed of the secondary, centrifuged, and resuspended in PBS solution. The samples were thoroughly covered for protection from light and delivered the FACS device\textsuperscript{k} for analysis.

**SPECTROPHOTOMETRIC NO ANALYSIS**

Spectrophotometric NO analysis kit\textsuperscript{l} was utilized to conduct this examination. This kit is utilized to reduce nitric oxide radicals, such as NO\textsuperscript{-}, NOO\textsuperscript{-}, and NO\textsuperscript{3-} to nitrite, which is detected by differences in absorbance of light. These radicals are collectively referred to as NO\textsubscript{X}. The treated medium of each flask was removed immediately before cell collection and stored in \textdegree C until the assay was performed. The cadmium beads included in the kit are used for the conversion of nitrate to nitrite. All cadmium beads are rinsed three times with H\textsubscript{2}O, 0.1M HCl, and 0.1M NH\textsubscript{4}OH before use. Each sample of 75 \textmu L is adjusted to 190 \textmu L with ddH\textsubscript{2}O. 10 \textmu L of ZnSO\textsubscript{4} is added for the precipitation of protein present in the medium. These samples are vortexed, incubated for 15 min at room temperature, and centrifuged 3000–4000 rpm for 5 min. The resulting supernatants are incubated with cadmium beads and agitated overnight at room temperature. Duplicate 100 \textmu L samples are combined with 50 \textmu L of color reagent #1 and shaken. 50 \textmu L of color reagent #2 is added, and the sample is agitated. The microtiter plate reader\textsuperscript{m} is set to an absorbance of 540 nm, and the sample is analyzed.

**STATISTICAL METHODS**

The sample data were compared utilizing analysis of variance (ANOVA) statistical analysis. In consideration of sample variation, the low cytometry data was standardized as fluorescence ratio. The fluorescence ratio is derived as the quotient of variation, the low cytometry data was standardized as fluorescence (ANOVA) statistical analysis. In consideration of sample variation, the low cytometry data was standardized as fluorescence (ANOVA) statistical analysis. In consideration of sample variation, the low cytometry data was standardized as fluorescence (ANOVA) statistical analysis. In consideration of sample variation, the low cytometry data was standardized as fluorescence (ANOVA) statistical analysis. In consideration of sample variation, the low cytometry data was standardized as fluorescence (ANOVA) statistical analysis. In consideration of sample variation, the low cytometry data was standardized as fluorescence (ANOVA) statistical analysis.

**RESULTS**

**INDUCTION OF iNOS EXPRESSION IN ENDOTHELIAL AND MACROPHAGE CULTURES**

Endothelial cells and macrophage were separated into groups and exposed to either LPS, LPS and IFN-\gamma, or LPS, IFN-\gamma, IL-1\beta, and TNF-\alpha along with a control group that received no stimulation. Cells were incubated with a primary antibody for iNOS, subsequently conjugated with a FITC-secondary antibody, and analyzed with FACS. The fluorescence ratio iNOS expression was ascertained. Table 1 demonstrates the mean iNOS expression as fluorescence ratio (N = 6). The mean and standard deviation data represented in Table 1 reflect iNOS protein expression as a fluorescence ratio in the three treatment groups.

The endothelial cells demonstrated no significant increase in iNOS protein expression, irrespective of treatment. The mean expression of iNOS in the endothelial cells treated with LPS was 1.151; LPS and IFN-\gamma was 1.185; LPS, IFN-\gamma, IL-1\beta, and TNF-\alpha was 1.125. These data represent a 15% increase over control with LPS exposure; a 19% increase over control with LPS and IFN-\gamma exposure; and a 13% increase over control with exposure to LPS, IFN-\gamma, IL-1\beta, and TNF-\alpha. ANOVA of the three treatment groups showed no significant difference among the groups at 0.05 level of significance.

The macrophage cells demonstrated a significant, stimulus-dependent increase in iNOS protein expression. The mean expression of iNOS in the macrophage treated with LPS was 1.47; LPS and IFN-\gamma was 3.74; LPS, IFN-\gamma, IL-1\beta, and TNF-\alpha was 3.21. These data represent a 47% increase over control with LPS exposure, a 274% increase over control with LPS and IFN-\gamma exposure; and a 221% increase over control with exposure to LPS, IFN-\gamma, IL-1\beta, and TNF-\alpha. ANOVA of the three treatment groups showed a statistically significant difference among the two treatment groups that received LPS in combination with cytokines (p < .001). However, LPS alone did not produce a statistically significant effect on iNOS expression at 0.05 level of significance.

Regression and correlation analysis was performed on the macrophage to determine the relationship between the expres-

<table>
<thead>
<tr>
<th>Cell type</th>
<th>LPS</th>
<th>LPS and IFN-\gamma</th>
<th>LPS, IFN-\gamma, IL-1\beta, and TNF-\alpha</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECV-304, endothelial cells</td>
<td>1.15 ± 0.14</td>
<td>1.18 ± 0.29</td>
<td>1.12 ± 0.11</td>
</tr>
<tr>
<td>RAW 264.7, macrophage</td>
<td>1.47 ± 0.57</td>
<td>3.74\textdagger ± 0.88</td>
<td>3.21\textdagger ± 1.66</td>
</tr>
</tbody>
</table>

\textdagger Denotes p < .001

The mean intensity of the treated group was divided by the intensity of the control for each group to ascertain each value. These data represent the mean intensity plus or minus the standard deviation at a 0.05 level of significance.
sion of iNOS and the production of NO; that is, the controls and treatment groups were examined for dependence of NO production to the iNOS fluorescence ratios. The correlation value of 0.817 (p < .001) indicates a relationship between fluorescence ratio and NOX production. The regression analysis confirmed a relationship between iNOS protein expression and NO production with an R² = 0.727 (Figure 1 demonstrates the nonlinear regression analysis of iNOS protein expression and NOX generation).

**SPECTROPHOTOMETRIC NITRIC OXIDE PRODUCTION ASSAY**

The media culture supernatants from each treatment were collected and analyzed for the presence of nitrite. Nitrite is the reductive end product of nitric oxide and nitrate degradation. Table 2 illustrates the means of each treatment data collected.

The endothelial cells demonstrated no significant increase in the presence of nitrite following stimulation. The mean detection of nitrite in the supernatant of the endothelial cells treated with LPS was 0.178 μM; LPS and IFN-γ was 0.155 μM; IFN-γ, IL-1β, and TNF-α was .182 μM. The control group was 0.142 μM. ANOVA statistical analysis of the three treatment groups and control showed no significant difference among the groups at .05 level of significance.

The macrophage cells demonstrated a statistically significant difference in the presence of NOX. The mean detection of nitrite in the supernatant of the macrophage treated with LPS was 0.276 μM; LPS and IFN-γ was 0.421 μM; LPS, IFN-γ, IL-1β, and TNF-α was 0.404 μM. The control group was 0.173 μM. ANOVA of the three treatment groups showed significant difference among the groups (p < .001).

**DISCUSSION**

The results of this research indicate that a majority of nitric oxide generated during septic shock could be the product of macrophage localized to the inflammatory region. The concept of an infiltrating cell modulating the vasomotor regulation is relatively new. The endothelium may not play as much a role in sepsis-mediated systemic vasodilation as previously reported (14). The nitrite assay demonstrates significant increases in production among the macrophage treatment groups; whereas, the endothelial groups demonstrate no significant change to the various stimuli. We have demonstrated a significant increase in iNOS expression and nitric oxide production when LPS was added to macrophage, and further increases resulting following supplementation with IFN-γ, TNF-α, and IL-1β. Conversely, the endothelial cells did not exhibit a similar effect. The iNOS expression and nitric oxide production remained relatively constant for the endothelial cells and showed no significant increase in iNOS protein. The nitric oxide assay shows similar results.

The family of NOS proteins have enjoyed the notoriety of scholarly attention, and NOS research has been awarded the most recent Nobel Prize for science. All nitric oxide synthase isoforms catalyze a five-electron oxidation of a guanidine nitrogenous group of the amino acid L-arginine evolving NO and L-citrulline (4). Elemental oxygen and reduced nicotinamide adenine dinucleotide phosphate (NADPH) serve as cosubstrates, and flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), tetrahydrobiopterin (BH₄), and heme are co-factors of the NOS isoforms (4). Figure 2 presents all of the substrates, co-factors, and co-substrates necessary for the synthesis of NO via iNOS. NO, a freely diffusible molecule, can traverse easily through cells to mediate its effect. However, because of its very short half life of approximately 6 sec (4), the outcome is localized to the immediate area of synthesis. The downstream effects of the increased NO production include vasorelaxation, platelet inhibition, decreased leukocyte adhesion, and augmented bactericidal effects. Several studies have shown that employing selective inhibitors of iNOS, such as aminoguanidine or such suicide substrates as L-N⁶-monomethyl-arginine, reduce the production of nitric oxide from iNOS, mitigating the vasoplegia (10, 15, 16). The focus of this discussion is the modulation of the vasculature. The most prevalent binding site for NO is iron, which is complexed as a heme group or an iron-sulfur compound, similar to that of soluble guanylate cyclase (17).

Soluble guanylate cyclase (sGC) in the vascular smooth muscle is the target of NO in an overwhelming majority of interactions. As a result, sGC has been labeled the “NO receptor” (17, 18). sGC is found in virtually all mammalian cells. NO binds to sGC to activate the conversion of guanosine-5’-triphosphate (GTP) to cyclic guanosine-5’-monophosphate (cGMP). Catalytic conversion of GTP to cGMP is dependent on the presence of divalent cations, such as Mn²⁺ and Mg²⁺ (5). cGMP is a common signal transduction pathway employed by a number of messengers. In the instance of vasorelaxation, cGMP serves to evacuate Ca²⁺ from the cytoplasm.

The governing mechanism of smooth muscle reactivity remains unclear. However, there is consensus the process revolves around interaction and modulation of the Ca²⁺ homeo-

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Table 2: Spectrophotometrically derived concentration of nitrate

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Control</th>
<th>LPS</th>
<th>LPS and IL-1β</th>
<th>LPS, IFN-γ</th>
<th>LPS, IFN-γ, IL-1β, and TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECV-304,</td>
<td>142 ± 0.2</td>
<td>.178 ± 0.5</td>
<td>.155 ± 0.3</td>
<td>.182 ± 0.6</td>
<td></td>
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<tr>
<td>endothelial</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RAW 264.7,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>macrophage</td>
<td>.173† ± .06</td>
<td>.276† ± .08</td>
<td>.421† ± .06</td>
<td>.404† ± .03</td>
<td></td>
</tr>
</tbody>
</table>

†denotes p < .001

The presence of nitrite was tested. The data is expressed in μM. These data represent the mean concentration plus or minus the standard deviation at a 0.05 level of significance.
stasis. An increase in the concentration of Ca\textsuperscript{2+} levels is required for the activation of the myosin light-chain kinase. Myosin light-chain kinase phosphorylates the myosin light-chain, inducing vascular smooth muscle contractions or vasoconstriction (6, 7, 18, 19). When NO concentration is high, GTP is degraded to cGMP concomitantly. This action results in a shift of the Ca\textsuperscript{2+} homeostasis to a lower concentration; hence, no phosphorylation of the myosin light-chains occur, and vasorelaxation predominates. Conversely, if cGMP levels are low, vasoconstriction ensues. Figure 3 illustrates a simplified version of how NO affects the vasorelaxation of vascular smooth muscle cells.

Sepsis-induced cytokine regulation is a key determinant in the degree of vasorelaxation observed in septic shock.

The cytokines, IFN-γ, TNF-α, and IL-1β, mediate their activity through the potentiation of LPS. Figure 4 illustrates how the iNOS promoter is activated by each of the cytokines.

The TATA box is the site on the iNOS promoter region that RNA polymerase recognizes and begins to synthesize mRNA. LPS is bound extracellularly by lipopolysaccharide binding protein (LBP) and CD-14, which transduces an intracellular signal via cascade of cytoplasmic mediators (19). As a result, nuclear factor-κB (NF-κB) is dephosphorylated, traverses the nuclear membrane, and binds the κB response element on the promoter of iNOS to activate gene transcription and subsequent protein translation (20). IL-1β binds to its extracellular receptor and initiates a similar cascade to that of LPS, resulting in NF-κB B-mediated gene transcription augmentation (21). IFN-γ, on the other hand, binds its extracellular receptor, to initiate an intracellular cascade that activates the gamma-activating sequence (GAS) on the iNOS promoter (22). The
result is, again, iNOS gene transcription and protein translation. Finally, TNF-α demonstrates a dual pathway of iNOS activation. TNF-α can operate via NF-κB activation (23), similar to that of LPS and IL-1β, or it can initiate an intracellular cascade causing the dimerization and translocation of c-fos and c-jun to the nucleus. The dimer then binds activating protein-1 (AP-1) on the iNOS promoter generating an increase in gene transcription and subsequent protein translation (24). Alone, IFN-γ, TNF-α, and IL-1β have little or no effect in cell culture. However, when these cytokines are co-incubated with LPS, the activation of iNOS protein expression is appreciated.

In contrast to the endothelial cells, LPS, alone in macrophage culture, has the capacity to augment the expression of iNOS protein. When IFN-γ is added to the culture media, iNOS protein increases significantly, by as much as fourfold. The treatment of macrophage with LPS, IFN-γ, TNF-α, and IL-1β generated significant increases in iNOS expression. These effects were not appreciated in the endothelial cell, suggesting either a negative feedback loop (10), lack of extracellular receptors, too few binding sites on the iNOS promoter, or some other deficiency in the pathway. The statement of other researchers that iNOS expression can be blocked and the clinical hypotension corrected confirms that iNOS is the origin of NO in septic shock.

**CONCLUSION**

Sepsis is a catastrophic complication of infectious disease that accounts for many deaths. Septic shock is characterized by profound hypotension, increased cardiac output, and low preload. During CPB, the vasodilation associated with sepsis must be dealt with pharmacologically and with perfusion pressure in an effort to maintain adequate tissue perfusion. Sepsis-induced vasorelaxation is mediated by NO in the vascular smooth muscle cell by a high output nitric oxide synthase isoform, iNOS. Endotoxin and proinflammatory cytokines can potentiate iNOS expression by affecting gene transcription and subsequent protein translation.

**REFERENCES**

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