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Complexity of Inducible Nitric Oxide Synthase
Cellular Source Determines Benefit Versus Toxicity

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Background—Inducible nitric oxide synthase (iNOS) has been shown to have both beneficial and detrimental effects in sepsis. We focused on a single organ, the heart, and used 2 distinct cell types that express iNOS—the cardiac myocyte and the infiltrating neutrophil—to study the distinct functional effects of iNOS derived from heterogeneous cellular sources.

Methods and Results—In the first series of experiments, extravascular neutrophils were exposed to isolated single endotoxemic cardiac myocytes. Adhesion of wild-type neutrophils caused a rapid decrease in myocyte shortening and a concomitant increase in neutrophil-derived intracellular oxidative stress within the myocytes that was not observed with neutrophils from iNOS-deficient animals. We previously demonstrated that neutrophil-derived superoxide was essential for myocyte dysfunction; however, superoxide production was not compromised in the iNOS-deficient neutrophils. Because both superoxide and NO were essential for the neutrophil dysfunction, we probed for but could not detect any peroxynitrite assessed by detection of nitrotyrosine. There was a significant increase in length shortening in response to β-adrenergic stimulation of wild-type myocytes. Surprisingly, myocyte iNOS activity was essential rather than detrimental for the development of β-adrenergic receptor–mediated increases in shortening in endotoxemic iNOS-deficient myocytes.

Conclusions—These results demonstrate that iNOS, when expressed in isolated cardiac myocytes, can regulate the response to β-adrenergic stimulation during sepsis. However, as the neutrophils migrate in proximity to myocytes, iNOS now becomes essential for the ability of neutrophils to damage myocytes. These findings demonstrate that cellular source strongly modulates the beneficial and detrimental effect of iNOS. (Circulation. 2003;108:1107-1112.)

Key Words: nitric oxide ● myocytes ● inflammation ● leukocytes

The cellular and molecular mechanisms that contribute to the initiation and progression of inflammation in pathological conditions such as endotoxia are not completely understood. The available data regarding the role of nitric oxide (NO) in endotoxia are controversial and have been interpreted in terms of NO or its byproducts being detrimental and contributing to the pathogenesis of endotoxia or NO being beneficial and having antiinflammatory properties that turn off the proinflammatory processes during endotoxia. At present, these seemingly diametrically opposing views were rationalized by a working hypothesis that states that the constitutive NO isoforms (endothelial NO synthase and neuronal NO synthase) (which produce small amounts of NO) are beneficial whereas activation of the inducible isoform of NO (inducible NO synthase [iNOS]) (which produces much more NO) causes injury. One possible explanation for the variable results after iNOS inhibition during endotoxemia is that systemic inhibition of iNOS may affect different organs in distinct ways. Therefore, one alternative approach to gain understanding of how injury or benefit of target tissue can occur after activation of a single isoform such as iNOS is to focus on a single organ, for example the myocardium, and to gain insight into the mechanisms that underlie lipopolysaccharide (LPS) responses. Although the available data consistently show that LPS can induce iNOS in mammalian myocardium, discrepant data have been reported for the role of iNOS in this organ. Some investigators have reported that treatment of myocytes or animals with LPS induces iNOS-dependent myocardial depression. In contrast are reports that when iNOS is inhibited in endotoxemia, vascular dysfunction increases, cardiac function is depressed, and mortality increases. Clearly, iNOS can have beneficial or detrimental effects even in a single organ. However, it needs to be emphasized that the experimental design of these studies is based on the assumption that iNOS functions in a homogenous fashion, without consideration of the cellular source. In the case of sepsis, both myocytes and inappropriately activated immune cells (neutrophils and macrophages) are significant sources of iNOS. Although the quantity of iNOS may differ between neutrophils and myocytes, more important are the microenvironments provided by each of these cellular sources, neutrophils...
producing many reactive oxidants in addition to NO, whereas, relatively, myocytes produce far fewer oxidants.

In this study, we have used iNOS knockout animals and developed experimental paradigms to allow us to study the functional significance of iNOS in defined populations of single cells, namely ventricular myocytes and emigrated neutrophils. Our results demonstrate that iNOS seems to be essential for the ability of emigrated neutrophils to cause myocyte injury. By contrast, the role of iNOS in the isolated ventricular myocyte is essential for the myocyte to maintain responsiveness to β-adrenergic stimulation during sepsis.

Methods

All experimental protocols were reviewed and approved by the University of Calgary Animal Resource Center and conformed to Canadian Guidelines for Animal Research. All wild-type and iNOS-deficient mice used were on a C57Bl6 background. Unloaded cell shortening was measured using isolated murine ventricular myocytes from wild-type or iNOS-deficient mice and emigrated neutrophils from wild-type mice as previously described.11 A cytochrome c reduction assay was used to measure the production of superoxide from emigrated neutrophil suspensions, and single-cell imaging was used to measure oxidant production in myocytes exposed to emigrated neutrophils.13

To determine if peroxynitrite is generated by the neutrophil-myocyte interaction, a nitrotyrosine antibody was used as a marker of peroxynitrite production. For positive or negative controls, neutrophils and myocytes were treated with stock peroxynitrite or degraded peroxynitrite (Upstate Biotechnology). Cells were labeled with 6 μg/mL rabbit anti-mouse nitrotyrosine antibody (Upstate Biotechnology) or isotype-matched control antibody and then labeled with 1.4 μg/mL Cy3-conjugated goat anti-rabbit IgG (Jackson Immunoresearch). Finally, cells were labeled with 0.25 μg/mL bisbenzimide (DAPI, Hoechst No. 33342; Sigma) and mounted onto glass slides for deconvolution fluorescence imaging (DeltaVision, Applied Precision). All images were acquired using a 40×1.35 NA objective and n=1.518 immersion oil.

Analysis of iNOS mRNA Expression by Reverse Transcription and Polymerase Chain Reaction

iNOS mRNA was measured as previously described.14 Total RNA was extracted from the cells, and reverse transcription and polymerase chain reaction (RT-PCR) was performed using OneStep RT-PCR kit (Qiagen Inc). The primer pairs were as follows: iNOS (sense 5′/3′) TCACTGGGACAGCAGAGAAAT and (antisense 5′/3′) TGAAGCCTAGCCTTTGCGATAGATG, with PCR product size of 1423 bp. GAPDH cDNA was coamplified as an internal control using the following primer sequences: (sense 5′/3′) CGGAGTCACACGTTCGTCGATG and (antisense 5′/3′) AGCCTTCTCCATGTTGGTGAAAGAC, with a final PCR product size of 302 bp. The RT-PCR condition was optimized so that both iNOS and GAPDH mRNA were expanding linearly, as follows: 50 ng total RNA, 0.5 μmol/L of each iNOS primers, and 0.2 μmol/L of each GAPDH primer in 35 cycles. PCR products were electrophoresed through 2% agarose gel containing 0.5 μg/mL ethidium bromide. Bands were visualized and analyzed using a Fluor-S MAX Multi-Imager and Quantity One software (Bio-Rad Laboratories).

Determination of Myeloperoxidase Activity

Myeloperoxidase (MPO) activity was measured in emigrated neutrophils from wild-type and iNOS-deficient mice.4-15 The assay was modified for emigrated neutrophil suspensions (2.5×10⁶ cells in 500 μL H-Tab), with the reagent volumes adjusted for use in 96-well plates, and absorbance changes at 450 nm were determined over a 60-second period.

Results

Emigrated Neutrophil-Derived NO Contributes to Ventricular Myocyte Dysfunction

Figure 1A shows the pattern of shortening of an electrically stimulated wild-type myocyte. This cell shortened approximately 12 μm, or 9% of its resting cell length. The rate of contraction and the rate of relaxation were approximately 250 and 160 μm/s, respectively. The responses over a 10-minute period of field stimulation remained constant (multiple overlapping traces), demonstrating no alteration in function with time. Addition of isoproterenol showed the characteristic positive inotropic responses to β-adrenergic agonist (isoproterenol 0.1 μmol/L).

Statistical Analysis

All data are presented as mean±SEM. The data within groups were compared using a paired Student’s t test. Unpaired t tests were used to compare between groups. A Bonferroni correction for multiple comparisons was used as required. Statistical significance was set at P<0.05.

Figure 2A illustrates the cumulative myocyte shortening data after the addition of emigrated neutrophils. A 34±7%
decrease in cell shortening was noted after 5 minutes of adhesion of neutrophils, and this decrease persisted throughout the experiment. In addition, 10 minutes after neutrophil adhesion, the maximal rate of contraction and relaxation was reduced by 40% (Figures 2B and 2C). Interestingly, the temporal dissociation between the impaired rate of contraction/relaxation (generally seen at 10 minutes) and the depressed unloaded cell shortening (seen as early as 5 minutes) was consistently observed. Although the number of neutrophils adherent to myocytes varied from as few as a single neutrophil to as many as 10 cells, only 1 adherent neutrophil was required to induce the same amount of myocyte dysfunction as multiple neutrophils.

In contrast, neutrophils from iNOS-deficient mice did not alter cell-shortening patterns (94±7% and 96±3% of baseline) at either 5-minute (data not shown) or 10-minute measurements, respectively (Figure 2A). Interestingly, the temporal dissociation between the impaired rate of contraction/relaxation (generally seen at 10 minutes) and the depressed unloaded cell shortening (seen as early as 5 minutes) was consistently observed. Although the number of neutrophils adherent to myocytes varied from as few as a single neutrophil to as many as 10 cells, only 1 adherent neutrophil was required to induce the same amount of myocyte dysfunction as multiple neutrophils.

Figure 2. A, Cumulative myocyte shortening data from endotoxemic (LPS 10 mg/kg IP, 4 hours) wild-type mice recorded after 10 minutes for control (no neutrophils, N=4), wild-type neutrophils (WT PMN, N=6), and iNOS-deficient neutrophils (iNOS KO PMN, N=5). B and C, Rates of contraction and relaxation, respectively, before (baseline) and 10 minutes after the addition of no neutrophils (control), wild-type neutrophils, or iNOS-deficient neutrophils. *P<0.05 between indicated groups.

Neutrophil preparations (Figure 3A). Superoxide production has been reported previously to be an essential neutrophil-derived mediator of myocyte dysfunction. Accordingly, superoxide production was measured from the wild-type and iNOS-deficient neutrophils. Again, no difference in superoxide production could be detected between neutrophils of iNOS-deficient or wild-type mice (Figure 3B). Finally, MPO activity was measured from wild-type and iNOS-deficient neutrophil suspensions. MPO activity was low in both samples; however, there was no significant difference in activity between samples (Figure 3C).

Figure 3. There is no difference in the average number of neutrophils that adhered to myocytes (A), the amount of superoxide produced (B, N=2), or myeloperoxidase activity in emigrated neutrophils (C, N=4) isolated from wild-type and iNOS-deficient (KO) mice.

Figure 4 demonstrates that isolated emigrated neutrophils from wild-type donors do synthesize measurable amounts of iNOS. There was no iNOS expression in controls or in neutrophils from iNOS-deficient donors.

Figure 4. iNOS RNA expression in emigrated neutrophils (5×10⁶ cells) from wild-type (WT) and iNOS-deficient (KO) mice.
Absence of iNOS Reduces Myocyte Intracellular Oxidative Stress

Figure 5A consists of 4 images of wild-type neutrophils adhering to a myocyte isolated from an endotoxemic mouse. Both cells were loaded with 6-carboxy-2',7'-dichlorodihydrofluorescein diacetate acetoxymethyl ester to provide a visual indicator of overall oxidative stress within these cells. Fluorescence in the ventricular myocyte is first detected in the area of neutrophil attachment (Figure 5A, II). With time, it spreads throughout the myocyte (Figure 5A, III and IV).

Figure 5B demonstrates that control myocytes showed no significant increase in fluorescence above baseline within the 10-minute monitoring period. In contrast, the addition of wild-type neutrophils to ventricular myocytes from endotoxemic donors caused a 5-fold increase in fluorescence intensity over myocytes alone (10-minute value). Surprisingly, neutrophils deficient in iNOS did not cause any significant increase in fluorescence levels within myocytes (Figure 5B). It has been suggested that superoxide and NO can form the highly toxic peroxynitrite. Therefore, in additional experiments, we probed for nitrotyrosine as an indicator of peroxynitrite production (Figure 6). We were not able to detect nitrotyrosine on the adherent neutrophil surface, the intracellular space of the myocyte, nor within the subjacent space (between the neutrophil and the myocyte) (Figure 6E, compared with IgG control, Figure 6F). Application of peroxynitrite did cause positive nitrotyrosine staining (Figure 6A), suggesting that our preparation did not produce peroxynitrite or that it was at such a low level that it was not detected by our assay.

All of the above experiments were performed using myocytes isolated from wild-type endotoxemic mice, which allowed us to delineate the importance of NO derived exclusively from iNOS in neutrophils. Figure 7 demonstrates that using RT-PCR, no message was detectable in isolated cardiac myocytes from untreated mice, but significant levels of iNOS mRNA was detectable in the myocytes isolated from endotoxin-treated wild-type donors.

iNOS-Deficient Myocytes From Septic Mice Have Altered Function

When isolated myocytes from iNOS-deficient endotoxemic mice were used, very surprising responses were obtained. iNOS-deficient myocytes (from endotoxemic mice) responded similarly to myocytes from wild-type mice (first contraction, Figure 8). However, addition of isoproterenol did not induce the characteristic responses to β-adrenergic stimulation seen in Figure 8A. Some of the myocytes initially responded with the characteristic 2-fold increase in cell shortening. However, these myocytes were unable to recover...
to baseline levels of cell shortening after 10 minutes of washout. More often, a depressed response was noted, and frequently, these myocytes exhibited dysrhythmic contractions (Figure 8B).

In additional experiments, L-N\textsuperscript{6}-(l-iminoethyl) lysine (L-NIL), an iNOS inhibitor, was given with endotoxin to the animals 4 hours before myocyte isolation. Although positive inotropic responses to isoproterenol were noted in 80% of cells, the recovery was impaired in 60% of the myocytes (data not shown). A normal positive inotropic response to isoproterenol was rarely seen in the iNOS-deficient cells, but the impaired recovery was very similar in iNOS-deficient myocytes or myocytes treated with L-NIL. The subtle difference may be related to incomplete inhibition of iNOS with L-NIL or attributable to some potential defect in iNOS-deficient myocytes that occurs over time and manifests in endotoxemia.

**Discussion**

The role of iNOS in inflammatory conditions is controversial, because several proinflammatory and antiinflammatory results have been reported. We propose that the cellular source of NO and the microenvironment in which it is produced will dictate its biological or pathological activity. In this study, we have demonstrated at least 2 separate functions of iNOS that depend on the cellular source (neutrophils versus myocytes).

Important initial work on the function of iNOS demonstrated an inhibitory (antiadhesive) effect on neutrophil-endothelial cell interactions. Previous findings based on intravital microscopy work, which permitted direct visualization of leukocyte behavior in microvessels, demonstrated that inhibition of NO production increased P-selectin/PSGL-1–dependent leukocyte rolling\textsuperscript{17} and CD18/ICAM-1–dependent leukocyte adhesion.\textsuperscript{3,18} An interesting extension herein is that once outside the vasculature, the neutrophils adhered with equal intensity to cardiac myocytes regardless of the presence or absence of iNOS. The explanation is likely related to the fact that very different adhesive mechanisms are invoked in neutrophils once they migrate outside the vasculature and bind to cardiac myocytes.\textsuperscript{12} Indeed, the CD18/ICAM-1 interaction that dominates in the vasculature becomes less important as the emigrated neutrophils begin to express \( \alpha_\text{IIb} \)-integrin and adhere to myocytes.\textsuperscript{12} Clearly, the antiadhesive properties of NO are restricted to the vasculature and not to extravascular events.

Neutrophils that migrate outside the vasculature and interact with myocytes cause a depression in myocyte contractile activity. When wild-type neutrophils bound to wild-type myocytes, an increase in oxidative stress in the myocytes was consistently observed. Interestingly, with iNOS-deficient neutrophils, the resultant depression in myocyte function was not observed, nor was the associated oxidative stress within the myocytes. Clearly, the neutrophil-derived oxidative stress was NO-dependent. Indeed, neutrophils have been shown to produce NO from iNOS, particularly after their exodus out of the vasculature and into tissues.\textsuperscript{19} Our data show that this source of NO can indeed cause depression in myocyte function. We have previously reported that NADPH oxidase–deficient neutrophils (which do not produce superoxide) were also unable to depress myocyte function.\textsuperscript{11} Thus, both superoxide-generating\textsuperscript{11} and NO-generating (this study) systems are required for the neutrophil-induced myocyte dysfunction observed in our model. Indeed, superoxide and NO can form peroxynitrite, which may be more toxic than superoxide.\textsuperscript{16} In the present study, we could not detect any increase in nitrotyrosine despite the enhanced nitrotyrosine signal seen with exogenously applied peroxynitrite. This is not entirely surprising, because peroxynitrite formation requires a 1 to 1 ratio of superoxide and NO.\textsuperscript{20} and this stoichiometry may not occur in our experimental model.

It is well-known that engagement of integrins causes enhanced production of superoxide from neutrophils and monocytes.\textsuperscript{21,22} However, to date an association between engagement of an integrin and NO production has not been demonstrated. Our previous work demonstrated that emigrated neutrophils express \( \alpha_\text{IIb} \)-integrin and bind to myocytes to induce intracellular oxidative stress and myocyte dysfunction.\textsuperscript{11} It is possible that the NO-producing capabilities of the neutrophil may be related to engagement of \( \alpha_\text{IIb} \)-integrin. Although we have not determined the \( \beta_\text{subunit involved in the murine model, previous data from the rat model suggest that it is the \( \beta_\text{I} \)-integrin.\textsuperscript{19} It has been suggested that integrin-linked kinase (an ankyrin-repeat containing serine/threonine protein kinase that interacts with the cytoplasmic domain of the \( \beta_\text{I} \)-integrin) can regulate iNOS expression in macrophages.\textsuperscript{23} Furthermore, coexpression of iNOS and apical \( \beta_\text{I} \)-integrins has been demonstrated.\textsuperscript{24} It seems likely in our model that the engagement of the \( \alpha_\text{IIb} \)-integrin and the induction of iNOS are both essential for neutrophil-induced myocyte dysfunction. In principle, this could explain the fact that some investigators have been unable to detect NO production from circulating (nonadherent) neutrophils, even in the setting of inflammation.\textsuperscript{19,25}
Many studies have reported that septic myocytes have the capacity to produce iNOS; however, whether iNOS is protective or detrimental in the myocardium is unclear. In this study, when myocytes from iNOS-deficient endotoxicemic mice were exposed to a β-agonist, the inotropic response was dramatically altered in every cell examined. In some myocytes, a characteristic doubling of cell shortening was noted, however, the myocytes were unable to subsequently return to normal baseline levels. In other cells, there was no response to isoproterenol or a depressed response or even dysrhythmic response was noted. These in vitro data could potentially translate into detrimental effects if β-adrenergic stimulation is increased during endotoxemia.

In summary, endotoxemia in the setting of sepsis is a multifactorial pathology that involves inappropriate activation of the immune system. A key feature is the inappropriate recruitment of neutrophils into tissues wherein release of toxic molecules may cause parenchymal cell damage. Our data would suggest that NO, perhaps in combination with superoxide generated from infiltrating neutrophils, certainly has the capacity to injure parenchymal cells. Our data also suggest, however, that parenchymal cells use iNOS-derived NO during endotoxemia as an important mediator of β-adrenergic responses. Because the absolute absence of iNOS in the myocyte caused inappropriate responses to this stimulus, it is clear that cell-selective iNOS inhibition could provide some partial benefit but global iNOS inhibition will likely cause significant deleterious effects.

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