Myocyte Nitroso-Redox Imbalance in Sepsis

NO Simple Answer

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Our initial understanding that the inhibition of inducible nitric oxide synthase (iNOS or NOS2) dependent NO production in cardiac myocytes would restore contractile function and improve outcome in septic shock failed. This was, in part, because of our then lack of understanding of the complexity of NO signaling in the cardiovascular system in health and disease. The initial paradigm that NO acts as a diffusible molecule to depress cardiovascular function uniformly and diffusely has been replaced by a more sophisticated yet incomplete model of NO signaling. It calls for NO to act in very defined signaling domains, through distinct signal transduction mechanism, sub-serving focused physiologic functions. It is now well established that NO and NO-related species are significant modulators of cardiac muscle excitation-contraction coupling and that these species affect a number of molecules and channels that coordinate this process. It has been further established that mammalian cardiac myocytes express all 3 of the isoforms of nitric oxide synthase: endothelial (eNOS or NOS3), neuronal (nNOS or NOS1), and iNOS or NOS2. Furthermore what determines the modulatory effects (sometimes directionally opposite) with regard to physiologic function is not only the signal transduction mechanism, but the spatial confinement of the enzyme within the cardiac myocyte. For example, NOS3 is targeted to the sarcolemmal and sarcoplasmic reticulum associated with the caveolar membrane protein caveolin 3. There, it is coupled through the β-3 adrenergic receptor, guanylyl cyclase and the second messenger cGMP to negatively regulate l-type Ca2+ channel-dependent Ca2+ influx. It thus acts as a negative modulator of adrenergic signalizing to balance and constrain positive inotropy. On the other hand nNOS is confined to the sarcoplasmic reticulum (SR) where it is associated with the ryanodine receptor (RYR 2), and positively modulates channel function, enhancing contractile response to β adrenergic stimuli and the contractile response to increasing pacing frequency. This signal transduction mechanism involves S-nitrosylation of cysteine thiols (an emerging critical redox-sensitive post translational protein regulatory mechanism) of the RYR which in turn regulates its gating function. Functional studies in NOS3−/− and NOS1−/− mice have confirmed these directionally opposite roles of NOS isoforms on contractile function which are dependent on their location within the cell. With regard to the iNOS however, it is present at very low concentrations in the cell and its expression is primarily induced in disease states.

Dysregulated NO signaling and NOS isoform function is implicated in a number of adaptive and disease processes such as heart failure, ischemia/reperfusion injury, aging and the contractile dysfunction associated with the septic shock. In the current issue, Ichinose and colleagues provide a tour de force of in vivo and in vitro physiologic, as well as biochemical data to support the protective role of cardiac myocyte-specific NOS3 overexpression (NOS3TG) in the myocardial depression mediated by sepsis. Using a multitude of techniques in vivo (pressure-volume loops), and in vitro (isolated myocytes studies), and using 2 models of sepsis (lipopolysacharide [LPS] administration and the cecal ligation and puncture technique), the investigators demonstrate that cardiac myocyte-specific overexpression of NOS3 markedly attenuates the myocardial contractile depression seen with sepsis. The investigators demonstrate that this preservation is in part a function of a significant but less depressed [Ca2+]i (compared with wild type [WT] LPS treated mice), as well an increase in myofilament Ca2+ sensitivity, which compensates in part for a depressed myocardial [Ca2+]i. This increased Ca2+ sensitivity in the NOS3TG is likely mediated by nitroso-redox modulation of the proteins involved in excitation-contraction coupling because the relatively depressed Ca2+ sensitivity in WT (compared with TG) is restored by the reducing agent dithiothreitol (DTT [DTT reduces thiols to the SH form]). Furthermore, the investigators demonstrate that NOS3TG animals have attenuated reactive oxygen species (ROS) production and that increased ROS production in LPS treated WT animals can be suppressed by the xanthine oxidase inhibitor allopurinol. This supports the enzyme as the primary source of ROS in this pathophysiologic process. Thus, overexpression of NOS clearly alters the nitroso-redox state of the myocyte promoting protection and maintenance of contractile function. Finally and most impressively is the demonstration that the protective effect of the NOS3TG on myocardial oxidant stress and function translates into a markedly enhanced survival.

Because the global “amount” of NO measured in both the TG (before) and WT mice following LPS administration are the same, we are left to speculate that it is the both the spatial and temporal distribution of the NO and the NOS from which it is derived, that modulates contractile response to the highly...
Subcellular location of myocyte NOS isoforms as well as potential site of the NOS3TG and proteins that may be altered by NOS3TG-mediated NO-dependent nitrosylation. They may thereby be “protected” from the profound oxidative and nitrosative stress induced by sepsis. Redox-dependent signaling represents a continuum from physiologic concentration of NO and ROS that can reversibly alter and modulate function of myocyte proteins (S-nitrosylation) to severe nitrosative and oxidative stress that may irreversibly alter protein function resulting in pathophysiologic disease processes. LPS stimulates cytokine production, cytokine receptor (CRs) activation resulting in a transcriptional upregulation of NOS2 and high levels of NO production. NOS3TG could potentially S-nitrosylate the ryanodine 2 receptor channel (RYR) at a single site preserving channel function and attenuating sepsis dependent reductions in [Ca\(^{2+}\)]i and contractile function. S-nitrosylation may also inhibit protein phosphatase 1 (PP1) activity maintaining phospholamban phosphorylation and thereby enhancing diastolic function and SR Ca\(^{2+}\) loading. In addition nitrosylation of contractile myofilaments may preserve Ca\(^{2+}\) sensitivity and diastolic function.

NOS3TG may also S-nitrosylate xanthine oxidase (XO) thereby inhibiting its activity and reducing oxidative stress. NOS3TG dependent-NO may also protect mitochondria and preserve mechano-energetics. LTCC (L-type Ca\(^{2+}\) channel): (+), positively modulate; (-), negatively modulate; Dashed arrow, inhibit; solid arrow, facilitate.

This idea is wholly consistent with the notion that NOS is colocalized with the proteins which it regulates (“stimulus-coupled regulation of S-nitrosylation within the confines of the signaling module”) a concept which is well summarized in a recent review by Drs Hare and Stamler. It is also consistent with the idea that the pathophysiologic alterations in NO signaling may not only be a function of “how much is produced” but “where it is produced”. This is exemplified, for example, in heart failure in which the translocation of NOS1 from its usual (SR) location contributes to myocardial dysfunction. This idea contrasts with an old paradigm which viewed NO as a freely diffusible molecule which has ubiquitous and uniform effects on the cell and its subcompartments. With regard to the temporal nature of NO signaling: The findings of the investigators suggests that the NO produced by myocardial over-expression NOS3 might form nitrosothiols before the oxidant stress and thus “protects” cysteine thiols that might otherwise be oxidized by ROS. Thus thiol modification by NO and ROS represents a continuum in which physiologic concentrations of NO and ROS within specific domains may “tune” physiologic function by altering specific cysteine residues reversibly in response to physiologic stress (eg, exercise). With increased (pathologic) concentrations of reactive oxygen and nitrogen species one gets irreversible modification of protein function and resultant pathophysiologic consequences.

The question then arises as to the subcellular location of the NOS3TG, so as to produce the observed effects. Given the known spatial location of NOS3 to the plasmalemma, its signaling through GC/cGMP, and its depressant effect on adrenergically mediated contraction (by cGMP and S-nitrosylation-dependent mechanisms), it seems unlikely that this is the site to which it is confined. On the other hand, the effects are more consistent with those of NOS1 (confined to the SR, colocalizes with the RYR, positively modulates RYR Ca\(^{2+}\) release, and enhances contractility). Is it possible that the NOS3TG is in fact confined to the SR? There is some evidence that the reductase domain of eNOS may in fact be a binding partner for the RYR and might thereby be responsible for the nitrosylation of the RYR. With regard to the RYR receptor, it is now well established that its function is sensitive to ROS and RNS. On the one hand ROS leads to irreversible activation of the RYR, Ca\(^{2+}\) SR leak and ultimately decreases SR Ca\(^{2+}\) stores (as seen in sepsis with WT mice). In addition polynitrosylation of the channel leads to channel dysfunction. On the other hand nitrosylation of a single thiol by physiologic concentrations of NO leads to channel activation and normally regulated function. The identification of the subcellular compartmentalization of NOS3TG by the investigators would go a long way to help explain the interesting pathophysiologic findings.

A further intriguing finding by the investigators relates to the observation that inhibition of XO by allopurinol before LPS stimulation significantly attenuates LPS dependent decreases in [Ca\(^{2+}\)]i and depressed Ca\(^{2+}\) sensitivity in LPS treated WT mice. This suggests that the source of the ROS is at least in part XO. This finding is interesting in light of the known interaction between NO and XO in general and in the cardiac myocyte specifically. For example, cardiac myocyte NOS-1 colocalizes with XO in the SR and NOS-1 dependent NO prevents XO-dependent ROS production. Indeed XO has been implicated in the pathophysiology of a number of cardiovascular diseases associated with oxidative stress. The mechanism by which NO may prevent XO-dependent ROS production remains unclear. XO, a molybdenum containing enzyme, expressed as a 150 kDa homodimer, produces superoxide in the process of purine metabolism. The enzyme has two forms: XO and xanthine dehydrogenase (XDH), the former, resulting from either irreversible proteolytic cleavage or reversible oxidation of sulfhydryl residues of XDH. It is interesting to speculate whether redox sensitive nitrosylation (by NO produced from the NOS3TG) of XDH sulfhydryl may prevent oxidation of these residues and thus prevent reversible activation of XO from XDH. This is even more intriguing given the idea that DTT, a sulfhydryl reducing reagent restores Ca\(^{2+}\) sensitivity in LPS treated mice. It should also be considered that irreversible proteolytic cleavage of XDH to XO rather than the reversible conversion may be the mechanism of activation. This is supported by the
experimental evidence in another pathophysiologic setting in which the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) inhibited radiation induced increases in XO activity and restored XDH activity in the liver, whereas the sulfhydryl reducing reagent DTT did not. These findings support the hypothesis that a major persistent and amplified effect of ionizing radiation (another profound cellular oxidative stress) is the irreversible conversion of XDH to XO. Thus interesting follow-up experiments would include whether DTT or the serine protease inhibitor would inhibit XO dependent ROS production in cardiac myocytes following LPS.

The investigators’ findings that the NOS3TG reduces ROS production in a sepsis model is at first glance inconsistent with the findings of other investigators, particularly as it relates to eNOS uncoupling and ROS generation in pathophysiologic situations such as atherosclerosis and myocardial hypertrophy and heart failure. NOS3 exists as a homodimer that generates NO from L-arginine. When exposed to an oxidant stress including peroxynitrite or when reducing co-factor tetrahydrobipterin (BH4) or substrate L-arginine are decreased, NOS3 uncouples to the monomeric form and produces O$_2^-$.

Thus, although apparently paradoxical, NOS3 TG mice demonstrate enhanced protection with regard to atherosclerosis because of a decrease in uncoupled eNOS derived O$_2^-$ whereas overexpression of NOS3 accelerates the development of atherosclerosis. Furthermore, NOS 3/− mice are protected from transverse aortic constriction-induced myocardial hypertrophy and ROS production. In addition BH4 treatment prevents NOS3 uncoupling and myocardial dysfunction in WT TAC mice. Interestingly, TAC mice also demonstrate an increase in XO activity consistent with the sepsis model findings. Given the enhanced peroxynitrite production in hearts from WT LPS mice, one might suspect that NOS may be the source of ROS. Because cytokine induced GTP cyclohydrolase (GTPCH) expression (the rate limiting enzyme in the production of BH4) occurs in coordination with NOS2 in endothelial cells and requires NFkB and stat activation, this could maintain BH4 levels for high level NOS2-dependent NO production.

In the models of sepsis described by the investigators, parameters of ventricular relaxation/diastolic function were also impaired. For example, Tau, the time constant of relaxation measured from P-V loops, and Tau cell relengthening, the diastolic parameter measured in isolated myocytes was prolonged in WT mice treated with LPS, but was not altered in NOS3TG mice. This is in turn associated with an increase in phospholamban (PLB) phosphorylation, which removes the constraint on the SR Ca$^{2+}$ uptake pump SERCA, and thus enhances or preserves diastolic relaxation. Again the question arises as to whether this phenomenon can be explained by a discrete NO/redox event that could be mediated by the spatially confined NOS3TG. PLB phosphorylation is not only dependent on the “on” kinase phosphorylation activity but on the “off” phosphatase activity and their dynamic balance. In fact targeted inhibition of protein phosphatase 1 (PP1) by increased activity of its inhibitor can enhance contractility and protect against the development of heart failure in a pressure loaded mouse model. There is emerging evidence that phosphatases may be regulated by S-nitrososthiols such that nitrosylation leads to loss of activity. This would tend to preserve and enhance PLB phosphorylation which would in turn enhance SERCA activity. This would support the findings of enhanced diastolic (and ultimately systolic) function. Furthermore SERCA can be regulated directly in a redox-sensitive fashion by S-nitrosylation in a manner analogous to RYR2.

One cannot fail to mention the possible role of the NOS3TG in the preservation of mitochondrial function and mecano-energetics in the septic heart. Suffice to say that the pathogenetic mechanism of mitochondrial dysfunction in sepsis is complex but central to the syndrome. Reactive nitrogen and oxygen species can directly inhibit mitochondrial respiration by competing with oxygen in binding to the cytochromes of the electron transport chain. It is emerging that nitrosylation of discrete proteins in the mitochondria might regulate function in addition to regulating/inhibiting mitochondrial driven cell death.

The cellular NO/redox balance is profoundly perturbed by sepsis. It should be remembered however that this balance is not only regulated by enzymes that produce reactive oxygen and nitrogen species but those endogenous antioxidant systems that are activated in response to the stress. Although those systems that are important in the inactivation of ROS have been well investigated, it has only recently been discovered that regulation of nitrosative stress is critical to the survival of the organism, because mice deficient in the enzyme GSNO reductase, an ancient enzyme which is conserved across most species have a markedly increased mortality when exposed to a profound nitrosative stress such as sepsis.

Thus, an emerging theme with regard to this work is that NO/redox disequilibrium contributes to specific alterations in the nitrosylation status of proteins that are involved in excitation-contraction coupling in the sepsis models. Although a candidate approach to identifying altered proteins will be informative, a proteomic/nitrosoproteomic approach might identify further candidates that are modified and could explain the effects of sepsis on these proteins and thereby the whole organism. Furthermore the identification of proteins that are “protected” by NOS3TG overexpression may lead to the development of other therapeutic strategies that could ultimately defend the heart against sepsis-induced myocardial depression.

Sources of Funding
This work was supported by grant R01 AG021523 from the NIH, grant CA00405 from the National Space Biomedical Research Institute through NASA, and grant NNH04ZUU005N from NASA.

Disclosures
None.

References
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Circ. Res. 2007;100:1-4
doi: 10.1161/01.RES.0000255898.65901.9d
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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