NO Way to Relax : The Complexities of Coupling Nitric Oxide Synthase Pathways in the Heart

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Editorial

NO Way to Relax

The Complexities of Coupling Nitric Oxide Synthase Pathways in the Heart

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The roles of nitric oxide (NO) in the heart have been studied intensely ever since the first report nearly 2 decades ago showing that endogenous NO modulates cardiac myocyte function.[1] All 3 NO synthase (NOS) isoforms have been found in mammalian heart tissues (see reviews in Massion et al[2] and Belge et al[3]). A complex array of myocyte and nonmyocyte cells in the heart express NOS isoforms, and the local generation of NO[2,3] and reactive oxygen species (ROS)[4] may exert both autocrine and paracrine effects on cellular function. Not only is the endothelial isoform of NOS (eNOS, or NOS3) robustly expressed in the endothelial cells of the cardiac vasculature, but eNOS is also present within cardiac myocytes, where the enzyme associates with the scaffolding/regulatory protein caveolin-3 in T tubules in plasmalemmal caveolae.[5] The neuronal NOS (nNOS, or NOS1) is also expressed in cardiac myocytes, where the enzyme appears to be localized in the sarcoplasmic reticulum and modulates phospholamban phosphorylation.[6] Although both eNOS and nNOS appear to be physiologically expressed in cardiac tissues, the inflammation-related NOS isoform (iNOS, or NOS2) only appears in the heart after immunoactivation; iNOS may modulate the decline in myocardial function seen in sepsis.[7] There have been innumerable studies of the cardiac effects of various NOS inhibitors, NO-donating drugs, and NO itself.[2] The cardiac phenotypes of NOS "knockout" mouse models have been characterized extensively, and mice lacking 1, 2, or all 3 NOS isoforms have been generated and characterized.[8] In addition, the roles of NOS substrates, cofactors, and allosteric modulators have been explored exhaustively in diverse cardiac model systems. Emerging from this broad range of experimental evidence is an increasingly clear view that NO and NOS are key determinants of cardiac function. Yet the molecular mechanisms and pathways whereby this ubiquitous, reactive, and pluripotent signaling system modulates cardiac contraction and relaxation remain incompletely defined.

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Into this complex cellular and molecular milieu, a recent study by Silberman et al[9] brings some new insights into NOS pathways in the heart. Using a series of carefully controlled invasive hemodynamic studies and noninvasive imaging methods, the authors studied a mouse model that develops diastolic dysfunction in the absence of systolic dysfunction or cardiac hypertrophy. Cardiac tissues from these mice showed increased oxidation of a key NOS cofactor, tetrahydrobiopterin (BH4), as well as increased superoxide formation. The heart preparations analyzed in this study contained both the eNOS and nNOS isoforms expressed within cardiac myocytes, in addition to the eNOS that is robustly expressed in cardiac endothelial cells, and this experimental system provides particular challenges to the dissection of the roles of cell- and isoform-specific NOS pathways at the molecular level. Analyses of cardiac preparations from $eNOS^{null}$ or $nNOS^{null}$ mice might have been informative in this experimental system; no doubt these mutants will be characterized in future studies and will help to further delineate the roles of specific NOS isoforms in diastolic function and dysfunction. Silberman et al[9] propose that cardiac NOS had been "uncoupled" in this model of diastolic dysfunction, and they show that BH4 feeding promoted the restoration of "coupled" eNOS and normalized diastolic function. To critically assess this interpretation, a clearer definition of what is meant by "NOS uncoupling" is in order. All 3 mammalian NOS isoforms catalyze the formation of NO by oxidizing the amino acid L-arginine to form NO plus L-citrulline (see review by Marletta[10]). NAPDH and molecular O_2 serve as obligate cosubstrates, and the ubiquitous calcium-binding signaling protein calmodulin is an essential allosteric activator that binds to and activates NOS. In addition, several redox-active cofactors are required for NOS catalysis, including FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide), heme, and BH4. Under different specific conditions, all 3 NOS isoforms also have the capacity to synthesize ROS such as superoxide (O_2^-) instead of their usual product, NO. NOS-dependent O_2^- formation was first described in studies of purified NOS proteins and was termed NOS "uncoupling" because the usual catalytic electron flow within the enzyme is uncoupled from NO synthesis and is instead diverted to molecular oxygen to yield O_2^-.[11,12] Purified NOS can produce O_2^- when the key NOS cofactor BH4 is oxidized to form dihydrobiopterin (BH2); importantly, O_2^- production from purified NOS is not substantively affected by the NOS substrate arginine or by most arginine-based NOS inhibitors.[11,12] Since the discovery of O_2^- production from purified NOS, the term "NOS uncoupling" has become broadly used to describe any of a variety of experimental systems in which an increase in ROS production is seen in cells or tissues that contain NOS. Yet is this extrapolation from the catalytic properties of purified NOS to the complex cellular milieu entirely justified? Sometimes yes, sometimes no: It is not clear that O_2^- formation in cells containing NOS perforce represents NOS uncoupling. Some studies have inferred a role for NOS uncoupling when observing effects of arginine or arginine

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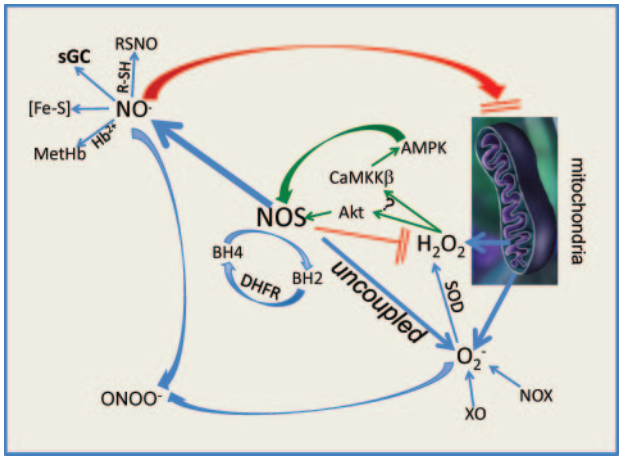

Figure. Principal reactions of NOS-derived products in cardiovascular tissues. NOS catalysis yields superoxide (O_2^-) when NOS is uncoupled by oxidation of BH4 to BH2. Reduction of BH2 to BH4 is catalyzed by dihydrofolate reductase (DHFR). NO-dependent activation of the soluble isoform of guanylate cyclase (sGC) leads to accumulation of cGMP. Formation of nitrosothiol (RSNO) adducts yields adducts that have distinct biochemical properties. NO can interact with [Fe-S] centers in mitochondria and elsewhere, and can lead to formation of methemoglobin (MetHb) through its interaction with ferrous (Fe^{2+}) heme. NOS-derived NO can inhibit mitochondrial respiration, as shown by the red arrow (the mitochondrion image is modified from http://www.nsf.gov/news/overviews/biology/interact08.jsp). Superoxide not only is formed by uncoupled NOS but also may arise from oxidative metabolism in mitochondria. NADPH oxidases (NOX), xanthine oxidoreductases (XO), and other oxidases also lead to the formation of ROS in cardiovascular tissues. Superoxide can undergo rapid dismutation to form hydrogen peroxide (H_2O_2), a stable ROS that plays key roles in cellular signaling. H_2O_2 promotes activation of eNOS through Akt-dependent phosphorylation, as noted by the green arrows. H_2O_2 leads to activation of the AMP-activated protein kinase (AMPK), a process that depends on specific AMPK kinases, including the calcium/calmodulin-dependent protein kinase-β (CaMKKβ). eNOS tonically inhibits endogenous H_2O_2 formation in endothelial cells. The complex interplay of reactive nitrogen and ROS modulates both physiological and pathophysiological responses in cardiovascular tissues. See the text for more details.

analogs on O_2^- formation from cells or tissues, yet studies of the purified NOS have documented that most of these compounds do not significantly affect O_2^- synthesis from the purified protein.[12] Moreover, there are many intracellular sources of O_2^-[4,13,14] and extracellular determinants of redox homeostasis.[15] The observation of increased O_2^- formation in cells or tissues in which NOS isoforms also happen to be present does not necessarily mean that the formation of superoxide is a direct consequence of NOS catalysis gone awry.

The major source for ROS in most mammalian tissues is not "uncoupled" NOS; rather, ROS principally derive from mitochondria (Figure) as a consequence of normal cellular oxidative metabolism.[4] Molecular oxygen is, of course, the ultimate acceptor of the electrons that flow through mitochondria, yielding H_2O as a final product. But along the way, a 1-electron reduction of O_2 can yield (sequentially) the ROS superoxide (O_2^-), hydrogen peroxide (H_2O_2), and the highly unstable hydroxyl radical ($HO^{\cdot}$) before H_2O is finally formed. It is estimated that up to 1% of the oxygen that is consumed in cellular respiration leads to the formation of such ROS,[4] a fact that has important implications for NOS and NO signaling in many cellular systems. The most abundant and stable of these ROS in most mammalian cells, H_2O_2, is a key signaling molecule[4,13] that promotes eNOS activation in endothelial cells.[16] O_2^- is rapidly reduced by superoxide dismutases to form H_2O_2, but O_2^- can also rapidly interact with NO to form peroxynitrite, which itself is a reactive oxidant molecule with important cellular effects. NO can also interact directly with mitochondrial electron transport proteins, and under some conditions, it may influence cellular respiration and ATP synthesis in the heart.[17] There are other physiological interactions between NOS pathways and ROS in cardiovascular tissues that may play key roles in oxidative metabolism. For example, eNOS appears to play a role in mitochondrial biogenesis,[18] and NO and NO adducts appear to modulate cardiac mitochondrial respiration.[17] Recently, eNOS was reported to exert a tonic inhibitory effect on H_2O_2 synthesis in cultured endothelial cells.[19] That study reported an increase in endothelial cell H_2O_2 synthesis after NOS enzyme inhibition, a situation sometimes ascribed to NOS "uncoupling." Yet small interfering RNA–mediated eNOS knockdown in endothelial cells also led to enhanced H_2O_2 synthesis, which argues strongly against eNOS serving as the H_2O_2 source in these cells. Indeed, the increase in H_2O_2 synthesis after eNOS knockdown was blocked by small interfering RNA–mediated knockdown of the AMP-activated protein kinase and related proteins in the AMP-activated protein kinase pathway, which implicates cellular phosphorylation pathways in at least some of the NOS-related changes in ROS formation (see Figure). Although the implications of these observations for organismal H_2O_2 homeostasis have not yet been established, such findings raise caveats about ascribing ROS production to NOS uncoupling without pursuing additional analyses. Moreover, NOS uncoupling can be influenced by factors independent of BH4. For example, posttranslational modifications may play a role in NOS-dependent O_2^- synthesis: Chen and colleagues[20] have shown that phosphorylation of eNOS at serine 1177 increases O_2^- synthesis by the enzyme, whereas eNOS phosphorylation at threonine 495 does not. These findings suggest that some of the effects of BH4 oxidation on NOS-dependent ROS synthesis may reflect changes in the NOS phosphorylation state.[21]

Several of the studies that have explored the pathways whereby BH4 ameliorates diastolic dysfunction[9] or endothelial dysfunction[22] have provided evidence that implicates the renin-angiotensin system in the pathophysiological response. Angiotensin II modulates ROS production in diverse cardiovascular tissues and has been shown to stimulate eNOS-derived O_2^- production, which in turn is suppressed by BH4.[22] The vascular dysfunction seen in models of diabetes, hypercholesterolemia, and hypertension is associated with enhanced oxidation of BH4 to BH2 and can be restored in vivo by BH4 supplementation.[14,23–25] Moens and colleagues[26] have elegantly documented that administration of BH4 can ameliorate the cardiac hypertrophy and myocardial fibrosis seen in a model of pressure overload, in association with an improvement in oxidative stress. These investigators provided evidence that indicated that the effects of BH4 were due to effects on NOS pathways in cardiac myocytes but did not involve activation of cGMP-dependent pathways in the heart. The study by Silberman et al[9] provides evidence implicating

the renin-angiotensin system in the diastolic dysfunction observed in this model system, accompanied by changes in phospholamban phosphorylation in cardiac myocytes that may reflect the involvement of oxidant-modulated phosphorylation pathways in the lusitropic response. Unraveling the complex relationships between BH4 metabolism and the numerous physiological and pathophysiological pathways in the vascular wall and in the heart, including the renin-angiotensin system, NOS catalysis, cardiac function, cellular redox balance, myocardial hypertrophy, and endothelial signaling, all remain active and important areas of investigation.

The central observation of the report by Silberman et al,[9] namely, that BH4 and related redox pathways play a key role in cardiac diastolic function, represents an important addition to our growing appreciation of the salutary biological effects of BH4 in cardiovascular homeostasis.[23,24,27] These observations have formed the basis for several clinical trials that have been exploring the therapeutic effects and safety of BH4 and related compounds in cardiovascular disease states. A clinical trial of the BH4 analog 6R-BH4 (sapropterin) for treatment of systemic hypertension failed to show efficacy for the drug, according to information released by the drug's manufacturer (see www.bmrn.com), but many other trials of BH4 and related compounds are ongoing. Current phase II clinical trials of BH4 and related compounds (listed at www.clinicaltrials.gov) include studies of endothelial dysfunction, peripheral vascular disease, sickle cell disease, coronary artery disease, systemic hypertension, endothelial dysfunction, pulmonary artery hypertension, chronic kidney disease, and cardiac diastolic function. These clinical trials are likely to provide critical information on the safety and efficacy of BH4 in cardiovascular therapeutics. At the same time, insights are emerging from ongoing basic research studies into the molecular mechanisms that control BH4-modulated cellular pathways, a confluence of research efforts that represents an exemplar of clinical science traveling from bench to bedside and back again.

Disclosures

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