Published in final edited form as:
Nitric Oxide. 2008 May ; 18(3): 153–156.

The fork in the nitric oxide road: Cyclic GMP or nitrosylation?

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Cardiac contractility is regulated by a process termed excitation–contraction coupling (ECC) [1]. An increase in the body's metabolic demands results in an increase in contractility. However, because every myocyte contracts with each beat, the heart cannot recruit additional myocytes to achieve this phenomenon. Hence, the myocyte has evolved numerous cellular mechanisms to regulate ECC. The β-adrenergic signaling pathway is a primary regulator of ECC [2].

Nitric oxide and cardiac function

Another key signaling pathway that regulates ECC is nitric oxide (NO) [3]. The heart is able to generate NO on a beat-per-beat basis via nitric oxide synthase (NOS) [4]. Cardiac myocytes constitutively express two NOS isoforms: endothelial NOS (NOS3) and neuronal NOS (NOS1). Both of these enzymes produce low levels of NO from the precursor L-arginine in a Ca^{2+}/calmodulin-dependent manner. NOS1 and NOS3 differentially regulate contractility due to their differences in cellular localization [5,6]. Another isoform (inducible NOS, NOS2) is not normally expressed in healthy myocytes. However, its expression is induced during the inflammation that occurs in many cardiomyopathies (e.g., heart failure [7]). Once expressed, NOS2 produces much higher levels of NO (and related congeners) in a Ca^{2+}-independent manner, leading to cardiac dysfunction [8–10].

Several studies, using a multitude of tools (various NO donors, specific and non-specific NOS inhibitors, cGMP analogs, transgenic and knockout mice, etc.) have examined the functional impact of NO and its signaling pathways and end targets. The results to date have yielded exciting information. However, the data generated have often been complex, leading to much controversy. Accordingly, NO has been found to be both a negative and positive inotropic agent. This dual effect, in part, has been found to be due to using diverse NO species and different NO concentrations, leading to S-nitrosylation or cGMP- or cAMP-dependent signaling [11–14]. These factors work in concert to exert positive or negative inotropic effects. For example, activation of the cGMP-dependent protein kinase through high [$NO^{\bullet}$] stimulation of guanylate cyclase will decrease the L-type Ca^{2+} current (I_{Ca}) [8,15]. Conversely, conditions that favor NO^{+} generation lead to nitrosylation of the L-type Ca^{2+} channel and an increase in I_{Ca} [15]. Nonetheless, inconsistencies have been observed for each effect. For example, studies have shown that cGMP is able to increase I_{Ca} [16], and nitrosylation can decrease I_{Ca}.[17]. These differences have been presumed to be due to different experimental protocols, NO donors, and/or animal species. In this issue of *Nitric Oxide*, Gonzalez et al. [18] have further addressed these factors by performing identical experimental protocols using the same preparation (isolated rat heart) and NO donor (SNAP, at different concentrations). It should be

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noted that additional factors, not studied by Gonzalez et al., also influence the functional response to NO, such as adrenergic state [19], site of NO production [20], and NOS isoforms [5].

Biphashic effect of the NO donor SNAP

In these well-designed experiments, Gonzalez et al. [18] have verified the biphasic effects of NO in an isolated rat heart preparation. That is, low concentrations of SNAP (0.1, 1, and 10 μM) exhibited a positive inotropic and lusitropic effect (increased left ventricular pressure development (LVP_{max}), contractility (dP/dt_{max}), and ventricular relaxation (dP/dt_{min})). However, a high concentration of SNAP (100 μM) induced a negative inotropic and lusitropic effect in LVP_{max}, $(dP/dt)_{max}$, and $(dP/dt)_{min}$. These results demonstrate that the contractile effect is dependent on the concentration of NO generated.

These authors further investigated the signaling pathways activated by the different concentrations of SNAP/NO. The data suggest that whole-heart cGMP levels were increased only in the presence of the highest concentration of SNAP (100 μM). cGMP was confirmed as the signaling molecule for the functional effects of the high concentration of SNAP (100 μM) by using ODQ, an inhibitor of NO stimulation of guanylate cyclase. ODQ abolished the negative functional effects of high SNAP. However, ODQ did not abolish the positive inotropic effect of the low doses of SNAP, demonstrating that the effects of low SNAP are via the cGMP-independent pathway. Further experiments revealed that the cGMP-dependent protein kinase (PKG) was responsible for the cGMP-induced negative inotropic effects.

The superoxide radical is needed to react with NO to form nitrosylating agents (i.e., NO^+, peroxynitrite, etc. [21]). The authors used tempol, a superoxide scavenger, to examine if the functional effects of low-dose SNAP occur via redox-mediated modifications such as S-nitrosylation or S-glutathiolation. Tempol did not have any effect on the functional response to high-dose SNAP, which is consistent with high SNAP being cGMP-dependent. However, tempol did inhibit the functional effects of low-dose SNAP (1 μM). Although tempol had no effect on the high -dose SNAP, it should be noted that ODQ induced a further increase in $(dP/dt)_{max}$ in the presence of low-dose SNAP (1 μM). Intriguingly, high-dose SNAP in the presence of ODQ elicited a positive inotropic effect. These data suggest an intricate balance between the cGMP and nitrosylation signaling pathways that warrants further investigation. An interesting experiment would be to repeat the ODQ/tempol experiments with 10 μM SNAP. This concentration of SNAP seemed to yield a variable increase in total cGMP (although not significant), with a corresponding lower increase in $(dP/dt)_{max}$. These experiments may better reveal the balance between the cGMP and nitrosylation signaling pathways. For example, would tempol induce a negative inotropic effect with 10 μM SNAP? Because cGMP signaling is compartmentalized [22], a change in whole cell cGMP may not have been observed, but there could have been a sufficient increase in local cGMP levels to elicit a negative inotropic effect. Because ODQ + 1 μM SNAP induced a larger positive inotropic effect than 1 μM SNAP alone, this suggests that there is compartmentalized cGMP signaling that is superseded by nitrosylation. Hence, ODQ should elicit a much larger positive inotropic effect with 10 μM SNAP than with 1 μM SNAP.

A possible determinant of which pathway (cGMP or nitrosylation) dominates could be the ECC proteins that are modified. The authors did not specifically examine which protein(s) was phosphorylated by PKG, but speculated that troponin I (TnI) is involved (Fig. 1, left panel). PKG phosphorylation of TnI will desensitize the myofilaments to Ca^{2+}, which could indeed explain the negative inotropic effect[23]. Also speculated was PKG phosphorylation of $Ca_{v1.2}$ which would limit Ca^{2+} influx [24] and also lead to a decrease in contractility (Fig. 1, left panel). However, the authors also observed a slower rate of relaxation, $(dP/dt)_{min}$, which

cannot be explained by phosphorylation of TnI or $Ca_{v1.2}$. The authors did not report the effect of PKG inhibition on relaxation; therefore, we do not know if this effect is also via PKG or some other cGMP-mediated pathway and further work needs to be done to determine the ECC protein(s) involved. Previous studies have also shown that ECC proteins can be nitrosylated (or at least modified by the cGMP-independent pathway) such as RyR (Fig. 1, right panel). S-nitrosylation of RyR will increase its open probability [25], allowing more release of Ca^{2+} from the SR to increase contractility. However, this increase in RyR open probability alone will not be sufficient to maintain the increase in contractility [26]. In addition, modification in RyR cannot be responsible for the increased lusitropy. Thus, there must be another primary target of nitrosylation, which could be the SERCA/phospholamban complex (Fig. 1, right panel). An increase in SERCA activity would increase the rate of relaxation [1], as well as increase the SR Ca^{2+} load, and, along with increased RyR open probability, would increase contractility. It has been demonstrated that SERCA1 (not the cardiac isoform) can undergo S-glutathiolation to increase its activity[27].

Relevance to endogenous NO production

The divergent contractile effects of NOS1 and NOS3 were hypothesized to be due to their different localization [5]. Differential effects of NO on contractility may be more complicated than merely different NOS isoforms. As suggested by Gonzalez et al. [18], these effects could be due to which specific signaling pathway is activated by each NOS isoform.

There may be discrete local redox environments enveloping each NOS isoform. Studies have shown that within cardiac myocytes NOS1 colocalizes with xanthine oxidoreductase [28], a producer of superoxide radicals and important for the generation of nitrosylating agents. Hence, the signaling pathway activated via NOS1 may be through the formation of peroxynitrite leading to S-nitrosylation. Previous studies have shown that NOS1 signaling leads to positive inotropic and lusitropic effects [5,6], which is consistent with Gonzalez et al. [18] (i.e., nitrosylation leads to positive inotropic and lusitropic effects). Thus, NOS1 may predominately signal via nitrosylating agents leading to increased contractility (Fig. 1, right panel).

The local redox environment for NOS3 may be different from that for NOS1. NOS3 colocalizes with superoxide dismutase [29], a scavenger of superoxide radicals, which will lead to enhanced activation of guanylate cyclase and cGMP production. Further evidence supporting the activation of the cGMP pathway primarily by NOS3 is the observation that the cGMP-specific phosphodiesterase (PDE5) is associated with NOS3 [30]. We have demonstrated that NOS3 signaling leads to negative inotropic effects via inhibition of I_{Ca} [31], which is consistent with Gonzalez et al. [18] (i.e., cGMP leads to negative inotropic effects). Thus, NOS3 may predominately signal via cGMP, leading to decreased contractility (Fig. 1, left panel).

A potentially important factor in determining which signaling pathway (cGMP or nitrosylating) will be dominant is the balance between NO and reactive oxygen species (ROS) known as the nitroso-redox balance (Fig. 1, middle circle). This critical balance is altered during heart failure [32] and may have profound effects on NOS1/NOS3 signaling. Much higher levels of NO (via NOS2) and superoxide (via NADPH, xanthine oxidoreductase, mitochondria) are generated during many cardiomyopathies. Altered reactive nitrogen species (RNS) and ROS production could disrupt the spatially localized redox environment encircling NOS1/NOS3. This, in turn, could change which signaling pathway is activated by each NOS isoform. For example, after myocardial infarction NOS1 is translocated from the SR to the caveolae, which depresses the response to β-adrenergic stimulation[33]. This NOS1-mediated decrease in the β-AR response has also been observed in normal myocytes [34]. Also, in patients with right ventricular hypertrophy, cGMP levels increased by PDE5 inhibition increased (not decreased) contractility [35]. In addition, pathophysiological levels of peroxynitrite have been shown to lead to

decreased (not increased) cardiac inotropy by modulation of RyR and phospholamban [9,36]. Thus, the isoform-specific production of NO may not be as important as which signaling pathway is activated and/or dominant.

In summary, NO is an important modulator of cardiac contractility, resulting in either positive or negative inotropy. Many factors have been found to be responsible for this NO-induced biphasic effect, including NOS isoforms and the concentration of NO. In their study, Gonzalez et al. have nicely demonstrated that the contractile effects of NO may be more complex and dependent on an intricate balance between the cGMP and nitrosylating signaling pathways.

Acknowledgements

M.T.Z. is supported by the National Institutes of Health (R01 HL079283).

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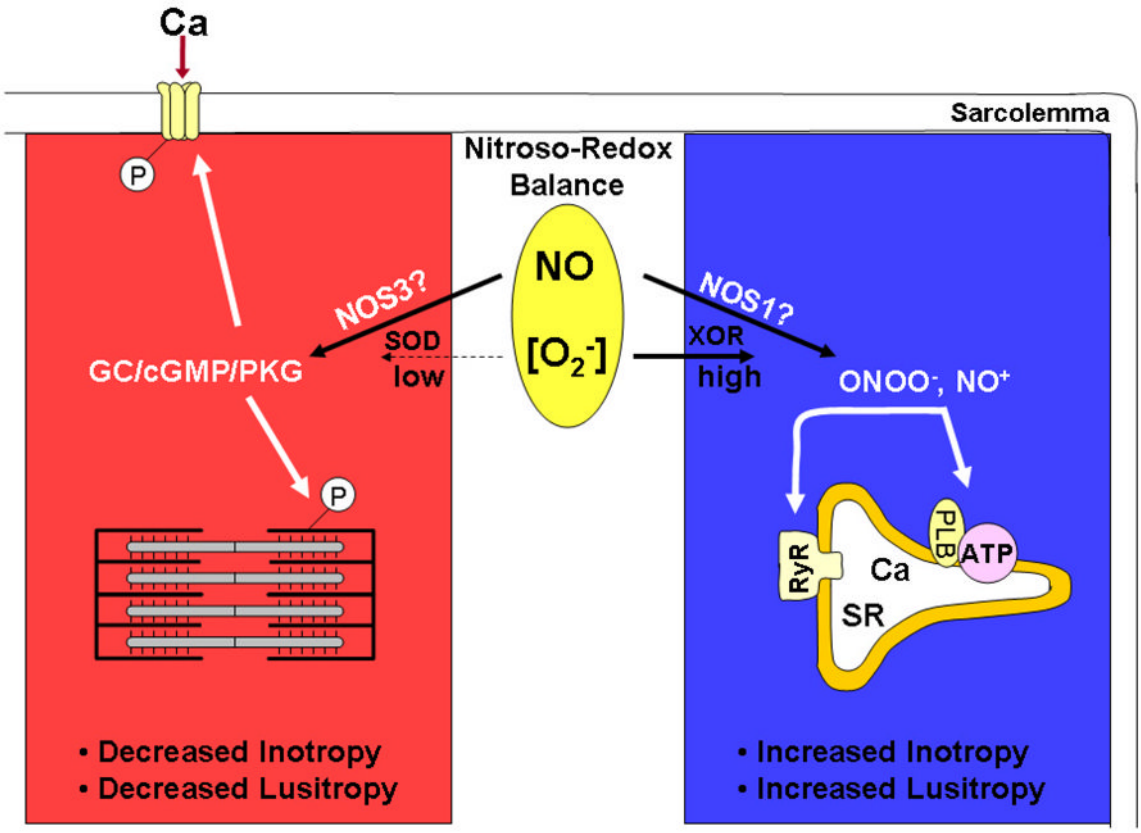

Fig. 1.

NO signaling pathways in cardiac myocytes. O_2^-,superoxide radical; SOD; superoxide dismutase; GC, guanylate cyclase; P, phosphorylation; XOR, xanthine oxidoreductase, $ONOO^-$, peroxynitrite; NO^+, nitrosonium ion; SR, sarcoplasmic reticulum; ATP, SR Ca-ATPase; PLB, phospholamban; RyR, SR Ca release channel (see text for other abbreviations).