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Review article

Nitric oxide signaling and the regulation of myocardial function

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ABSTRACT

Nitric oxide, which is produced endogenously within cardiac myocytes by three distinct isoforms of nitric oxide synthase, is a key regulator of myocardial function. This review will focus on the regulation of myocardial function by each nitric oxide synthase isoform during health and disease, with a specific emphasis on the proposed end-targets and signaling pathways.

Contents

1. Nitric oxide and the myocardium

The role of nitric oxide (NO) signaling has been well defined in such processes as neural transmission and the dilation of blood vessels. Although the function of NO remains less well defined in the heart, NO has been shown to be a key regulator of excitation-contraction coupling (ECC) [1]. The process of ECC underlies myocardial contraction [2]. The β-adrenergic receptor (β-AR) signaling pathway is also a critical modulator of ECC and produces positive inotropic and lusitropic effects upon activation [3]. Balligand et al. first demonstrated that endogenous NO plays a role in the mediation of β-AR signaling as well [4].

NO is synthesized upon the cleavage of L-arginine into L-citrulline by three distinct isoforms of NO synthase (NOS) within the myocardium [5,6]. Neuronal NOS (nNOS, NOS1) and endothelial NOS (eNOS, NOS3) are constitutively expressed in cardiac myocytes. These two isoforms are considered to be low output enzymes and produce NO in phase with myocyte contraction due to Ca-calmodulin regulation. In early studies, the use of NO donors or nonspecific NOS inhibitors made it difficult to distinguish between NOS1 and NOS3 signaling. However, recent studies have found that although NO is a highly diffusible signaling molecule, signaling via NOS1 and NOS3 is compartmentalized, and NOS1 and NOS3 differentially modulate

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cardiac function [5,7,8]. Inducible NOS (iNOS, NOS2), on the other hand, is only expressed during inflammatory responses and has been shown to be present during many pathophysiological conditions of the myocardium (e.g. ischemia–reperfusion injury, septicemia, aging, heart failure, etc.). When expressed, NOS2 produces much higher levels of NO independent of $[Ca^{2+}]_i$, compared to the constitutive NOS isoforms [6,9].

2. Nitric oxide signaling

NO has been shown to signal through at least two distinct pathways: cGMP-dependent and cGMP-independent [10]. The cGMP-dependent effects of NO result from the NO-induced activation of guanylate cyclase, leading to increased cGMP levels, which modulate the activity of protein kinase G (PKG), as well as cGMP-regulated phosphodiesterases (PDE; cGMP-stimulated: PDE2; cGMP-inhibited: PDE3). cGMP-independent effects occur mainly via *S*-nitrosylation, an important protein modification related to cell signaling [11]. NO can also directly activate adenylate cyclase, thus increasing cAMP levels and myocardial contractility [12]. Additionally, NO may couple with other reactive oxygen and nitrogen species, leading to the formation of related congeners, such as peroxynitrite ($ONOO^-$). These related species may also influence cardiac contractility, and in some cases produce markedly differing effects from those observed with NO alone. Therefore, it is not surprising that paradoxical results have been reported in the literature, as both positive and negative effects of NO and related congeners have been observed. However, recent studies are resolving these apparent contradictions by determining that the contractile effects of NO are greatly influenced by NOS isoform localization [5,6], and the activation of distinct cGMP-dependent and cGMP-independent signaling pathways which target individual ECC proteins in the cardiac myocyte. Additional studies have determined that these contractile effects are further confounded by such factors as gender [13], site of production [14,15], species produced [16–18], concentration [19,20], and cardiac myocyte contractile state [17,20]. These factors are relevant to the contractile effects of NO and related congeners during both health and disease and are sensitive to cellular redox state.

3. NOS1 expression in the myocardium

The neuronal isoform of NOS (NOS1) was originally characterized in the forebrain [21], but has also been found to be constitutively expressed in cardiac myocytes. NOS1 has been shown to be localized to the sarcoplasmic reticulum (SR), and co-immunoprecipitates with the SR Ca^{2+} release channel or ryanodine receptor (RyR) under physiological conditions [5,7]. Although sex hormones such as estradiol have been demonstrated to increase NOS1 mRNA levels [22], the effect of gender on the expression of NOS1 remains less well defined. One study demonstrated higher levels of NOS1 expression in female hearts compared to male [23], while another study found no difference between male and female hearts [24]. However, this discrepancy may result from species differences (rat vs. mouse) and/or female estrus cycle variance.

4. Contractile effects of NOS1-derived NO

The force frequency response (FFR) is an important mediator of contractility [25], and is partly modulated by NOS1 signaling. For instance, *in vivo* measurements have demonstrated that NOS1 knockout ($NOS1^{-/-}$) mice exhibit a blunted FFR (contraction and relaxation), which was also apparent in isolated $NOS1^{-/-}$ trabeculae and myocytes [5,26,27].

Several studies have shown that NOS1 is also capable of regulating the β-AR pathway. Specifically, *in vivo* and whole heart experiments demonstrated that the knockout of NOS1 leads to a reduced contractile response to β-AR stimulation [5,28,29]. We have recently demonstrated that myocytes isolated from $NOS1^{-/-}$ hearts also had a blunted response to β-AR stimulation, observed as a decrease in $[Ca^{2+}]_i$ transient and cell shortening amplitudes compared to WT [27].

NOS1 expression and activity may also be upregulated in certain disease states. For example, one study noted gender-dependent changes in NOS1 activity following pressure overload [30]. NOS1 has also been shown to translocate to the sarcolemma and localize with caveolin-3 during disease states [31,32], or with conditional overexpression [33]. Conditional cardiac-specific overexpression of NOS1 resulted in decreased contractile function, while $NOS1^{-/-}$ mice exhibited increased mortality, hypertrophy, and left-ventricular dilation after myocardial infarction [28,34]. Although NOS1 appears to be cardioprotective, the mechanism(s) for these effects are unknown.

5. End-targets and signaling pathways of NOS1

Phospholamban (PLB) is a key ECC protein which modulates SR Ca^{2+}-ATPase activity (SERCA). As such, PLB is a participant in the FFR and is also the major phosphoprotein in the β-AR pathway [35]. We and others have demonstrated that PLB is a key target of NOS1 signaling [27,36]. In WT myocytes, acute NOS1 inhibition resulted in decreased basal and β-AR-stimulated contraction, and slowed $[Ca^{2+}]_i$ decline (Fig. 1B; similar to $NOS1^{-/-}$). However, with acute NOS1 inhibition in $PLB^{-/-}$ myocytes, we noted no effect on contraction or $[Ca^{2+}]_i$ decline. We further examined the effects of NOS1 signaling on PLB, and observed that NOS1 inhibition decreased PLB phosphorylation [27], which was shown to be due to enhanced protein phosphatase activity [36]. We also observed a decreased SR Ca^{2+} load, an important determinant of myocyte contraction [37], with NOS1 knockout or inhibition. Interestingly, $NOS1^{-/-}$ hearts have decreased expression of PLB and increased expression of RyR and calsequestrin [26,38]. These changes appear to be compensatory in an attempt to increase SR Ca^{2+} uptake, SR Ca^{2+} load, and SR Ca^{2+} release. During β-AR stimulation, PLB phosphorylation levels are similar between $NOS1^{-/-}$ and WT myocytes [36]. This normalized PLB phosphorylation leads to similar $[Ca^{2+}]_i$ decline and myocyte re-lengthening rates between $NOS1^{-/-}$ and WT myocytes [27,36]. However, we have shown that there is still a reduced contractile response to β-AR stimulation in $NOS1^{-/-}$ myocytes [27], suggesting additional protein targets.

NOS1 has also been shown to target RyR [39], as one study demonstrated that $NOS1^{-/-}$ myocytes had an enhanced diastolic leak via RyR. This enhanced leak could also contribute to the reduction in SR Ca^{2+} load. NOS1 signaling has also been found to target the L-type Ca^{2+} current (I_{Ca}). Interestingly, this NOS1-mediated decrease in I_{Ca} led to decreased basal and β-AR-stimulated contraction [38]. NOS1 also interacts with non-ECC proteins, as NOS1 has been shown to bind with sarcolemmal Ca^{2+} pump 4b (PMCA) [40], which regulates NOS1 activity by modulating $[Ca^{2+}]_i$ levels. Overexpression of PMCA was shown reduce NOS1 activity, and decrease the response to β-AR stimulation, with a trend toward decreased basal contraction (similar to $NOS1^{-/-}$). Notably, several of these studies showed that these effects were through cGMP-independent signaling pathways (Fig. 1A, solid lines) [27,36,39].

Since NOS1 co-immunoprecipitates with xanthine oxidoreductase [41], a superoxide (O_2^-) producing enzyme, low levels of peroxynitrite may be formed. Additionally, it is possible for NOS1 to produce both NO and superoxide [42], although this is more likely to occur with uncoupling in disease states [43]. Thus peroxynitrite, produced by the reaction of NO and superoxide, may be a potential signaling molecule for NOS1. We investigated the role of peroxynitrite in NOS1 signaling and upon perfusion with FeTPPS, a peroxynitrite decomposition catalyst, we were able to mimic the effects of NOS1 knockout or acute inhibition (decreased contraction, slowed $[Ca^{2+}]_i$ decline, decreased PLB phosphorylation) [27]. In another study, we demonstrated that a