Modulation of In Vivo Cardiac Function by Myocyte-Specific Nitric Oxide Synthase-3

Hunter C. Champion, Dimitrios Georgakopoulos, Eiki Takimoto, Takayoshi Isoda, Yibin Wang, David A. Kass

Abstract—Nitric oxide (NO) functions principally as a diffusible paracrine effector. The exception is in cardiomyocytes where both NO synthases (NOS) and target proteins coexist, allowing NO to work in an autocrine/intracrine fashion. However, the abundant myocyte isoform (NOS3) is far more expressed in vascular endothelium; thus, the in vivo contribution of myocyte-NOS3 remains less clear. The present study tested this role by transfecting whole hearts of NOS3-null (NOS3−/−) mice with adenovirus-expressing NOS3 coupled to a α-MHC promoter (AdV NOS3), comparing results to hearts transfected with marker-gene β-galactosidase (AdV β gal). Total myocardial NOS3 protein and activity were restored to near wild-type (WT) levels in NOS3−/−+ AdV NOS3 hearts, and NOS3 relocalized normally with caveolin-3. Ejection function by pressure-volume analysis was enhanced in NOS3−/−+ AdV β gal over WT or NOS3−/−+ AdV NOS3. More prominently, isoproterenol (ISO)-stimulated systolic and diastolic function in WT was amplified in NOS3−/−+ AdV β gal, whereas NOS3−/−+ AdV NOS3 returned the response to control. ISO-activated systolic function was inhibited 85% by concomitant muscarinic stimulation (carbachol) in NOS3−/−+ AdV NOS3 but not NOS3−/−+ AdV β gal hearts. Lastly, NOS3−/−+ AdV β gal mice displayed enhanced inotropy and lusitropy over WT at slower heart rates but a blunted rate augmentation versus controls. A more positive rate response was restored in NOS3−/−+ AdV NOS3 (P<0.001). Thus, myocyte autocrine/intracrine NOS3 regulation in vivo can underlie key roles in β-adrenergic, muscarinic, and frequency-dependent cardiac regulation. (Circ Res. 2004;94:657-663.)

Key Words: nitric oxide synthase ■ myocyte ■ adenovirus ■ β-adrenergic ■ hemodynamics

Nitric oxide (NO) is principally a paracrine effector, being synthesized by one cell type to diffuse and modify the biology of neighboring cells. Cardiomyocytes are the unique counterexample in that they contain both NOS synthases and the key protein targets for NO modulation. Early evidence supporting a role of NO for affecting heart function was obtained using nonselective NOS antagonists1,2 and appeared to more prominently affect heart function was obtained using nonselective NOS antagonists1,2 and appeared to more prominently affect β-stimulated3 over basal function.4 More recent insights regarding the role for specific NOS isoforms followed generation of mouse null-mutant models, and both isoforms now appear to contribute to various aspects of excitation/contraction regulation.5–9

Both neuronal NOS1 and endothelial NOS3 are not solely expressed in myocytes, yet knockout models delete expression from all cells. In the case of the more abundant NOS3 isoform, endothelial expression exceeds that in myocytes by more than 4:1,10 and this has raised speculation that a substantial degree of physiological NOS3 signaling in vivo is paracrine in nature. Fueling this controversy are data obtained from isolated myocytes of NOS3-null hearts that failed to show differences in the calcium current (I Ca) response to β-stimulation or modulation of such stimulation by muscarinic agonists.10–13 This led some to propose that endothelial sources are indeed central to intact heart effects.10 Other studies have raised doubts regarding the role of myocyte NOS3 for modulating the functional responses to heart rate (force-frequency relation, FFR). Although FFR is enhanced by nonselective NOS inhibitors,14 more recent data found this to be principally mediated by the neuronal (NOS1) isoform.15

To more specifically test the functional role of myocyte-NOS3 in vivo, we developed a method for adenovirus gene transfer in the intact murine heart16 and linked the NOS3 gene to an α-MHC promoter to achieve myocyte selectivity. Reintroduction of myocyte-targeted NOS3 into NOS3−/− hearts resulted in hearts with only myocyte-NOS3 expression. Functional studies were then performed to test modulation of basal, β-stimulated, and frequency-dependent function, and muscarinic modulation. The re-
Materials and Methods

Animals

C57Bl/6 mice with or without the null mutation for NOS3 (6 to 8 weeks, Jackson Laboratories, Bar Harbor, Maine) were studied. The study was approved by the Institutional Animal Care and Use Committee and complies with the guidelines of the American Physiological Society.

Viral Vectors

Replication-deficient recombinant adenovirus serotype 5-encoding nuclear-targeted β-galactosidase (AdVβgal) or NOS3 (AdVNOS3) driven by a mouse α-MHC promoter possessing an HA epitope tag were prepared.17 Adenovirus was plaque purified and titer determined by plaque assay on cultured myocytes. Titers for AdVβgal and AdVNOS3 were 1.3×10^12 and 1.8×10^12 pt/mL, respectively. After purification, virus was suspended in PBS (pH 7.4) with 3% sucrose and stored at −80°C until use.

In Vivo Myocardial Gene Transfer

Details of the transfection procedure have been reported.16 Mice were anesthetized with isoflurane (2%), endotracheally intubated, and ventilated in a supine position on a thermoregulated surgical table at 37°C. The left external jugular vein was exposed by blunt dissection and cannulated. Topical 2% lidocaine gel was applied to the chest, and the thorax entered to reveal the mid-descending thoracic aorta. Mice were cooled with a water jacket to a core temperature of 19 to 21°C (heart rate ~100 bpm), the distal aorta clamped, and AdVβgal or AdVNOS3 injected IV (30 μL total volume). The aortic clamp was released after 9 minutes, inotropic and/or pacing support provided as needed, the mouse was warmed to 37°C over 30 to 40 minutes, and the chest closed.

As recently reported, this method of gene transfer yields broadly distributed transfection throughout the myocardium, with approximately 60% of individual myocytes being transfected (based on analysis of β-gal expression).16 AdV-based gene expression peaks at 3 to 4 days and gradually declines thereafter returning to baseline over the ensuing 1 to 2 weeks. Therefore, we performed all functional analyses 3 to 4 days after gene transfer.

Isolation of Cardiac Endothelial Cells and Myocytes

Isolation of murine coronary endothelial cells was performed using anti-endoglin (CD-106) antibodies (Transduction Labs) with a mini-MACS separation unit (Miltenyi Biotec, Bisley, catalog No. 421-01).

Further confirmation of a selective cell population was performed by flow cytometry. Labeled cells were incubated with MACS magnetic goat anti-rat IgG (HIL) (Miltenyi Biotec, catalog No. 481-01) MicroBeads and streptavidin (Miltenyi Biotec, catalog No. 481-01) MicroBeads, and then separated using a high-gradient magnetic separation column 1 (Miltenyi Biotec catalog No. 422-01). Cardiomyocytes were obtained from freshly isolated hearts using collagenase digestion as described.7

Expression of β-Galactosidase

β-Galactosidase expression was assessed by enzyme activity using a commercial kit, and by histochemistry as recently described in detail.15

Expression of NOS3 and Colocalization With Caveolin

Immunoprecipitation of NOS3 and colocalization with caveolin-1 was performed as described.18 Mid-LV myocardium from NOS3+/− with or without AdV NOS3 (n = 5) was cryopreserved, sectioned, and fixed in 4% paraformaldehyde/0.5% Triton X-100, and incubated with CAV1 (secondary incubation with anti-rabbit Alexa 488 and anti-mouse Alexa 536, Molecular Probes). Tissue was imaged on a Nikon Diaphot 300 inverted epifluorescence microscope attached to a PCM-2000 laser confocal scanning system (Nikon, Inc). NOS3 enzyme activity was assessed by l-arginine to l-citrulline conversion.17

Pressure-Volume Loop Studies

Intact heart hemodynamic analysis was performed using miniaturized pressure-volume catheterization.19 Studies used a 4-electrode pressure-volume catheter (model SPR-719, Millar Instruments) placed through the LV apex in the open chest anesthetized animal and advanced along the long axis. Pressure-volume data were obtained at baseline and 5 minutes after infusion of isoproterenol. Muscarinic regulation was assessed by infusion of carbachol (CCh; 30 mg/kg per minute IV) with heart rate fixed constant by atrial pacing via an intraesophageal lead (NuMed). Studies were performed in the presence of the I channel blocker ULFS-49 to blunt sinus activity,19 and atrial pacing achieved over a range of 450 to 750 minutes. 

Statistical Analysis

Data are expressed as mean±SEM. ISO and rate-dependent changes were analyzed based using two-way ANOVA, with an interaction term defining differences due to genotype or transfection effects.

Results

Selectivity of NOS3 Gene Transfer to Myocytes

Figure 1A shows levels of myocardial NOS3 protein expres-
Influence of Myocyte-Derived NO on Baseline Cardiac Function

The Table summarizes baseline data for the three principal experimental groups. For WT and NOS3−/− controls, data were obtained with and without AdVβgal transfection and results combined as there was no significant independent influence of reporter gene transfection itself. Hearts lacking NOS3 had slightly greater basal systolic function versus WT based on ejection-phase parameters such as maximal power index21 and end-systolic pressure/volume ratio (Ees). However, isovolumic contraction (dP/dtmax) was unchanged, and dP/dtmax divided by instantaneous pressure (dP/dtmax/IP) was actually lower in null mutants. The latter has been previously reported,6 but this may reflect higher pressure loads in these hearts rather than contractile depression per se. Relaxation rates were slightly longer consistent with pressure load and a small positive effect of basal NO on diastolic relaxation. Most importantly, transfection with myocyte-targeted NOS3 restored basal contractile function toward control in both systolic and diastolic parameters.

<table>
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<tr>
<th>Comparison of Baseline Cardiac Function in WT Controls [With or Without Transfection With β-Galactosidase (β-Gal) and NOS3−/− Transfected With Either β-Gal or Myocyte-Targeted (α-MHC Promoter) NOS3]</th>
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<td>Heart rate, bpm</td>
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<td>dp/dtmax/IP, mm Hg/s</td>
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<td>Tau, ms</td>
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PMXEDV indicates maximal power divided by end-diastolic volume (EDV); Ees, end-systolic elastance; tau, time constant or pressure relaxation; and NS, not significant.

*P<0.02 vs Control; †P<0.02 vs NOS3−/− + AdVβgal; ‡P=0.07 vs NOS3−/− + AdVβgal.
Influence of Myocyte-Derived NO on β-Adrenergic Stimulation

We next tested the role of myocyte-NOS3 on in vivo β-adrenergic stimulation. Isoproterenol (ISO; 0 to 75 μg/kg per minute IV) increased HR in each group similarly (Figure 3A). However, NOS3/+/Adv gal displayed a greater increase in systolic pressure and contractility with ISO versus controls (Figure 3B), whereas hearts from NOS3/+-AdvNOS3 displayed a response similar to controls. The rate of pressure decay was less responsive to ISO in NOS3/+/Adv gal, but this too was restored with myocyte-NOS3 reexpression (Figures 3C and 3D). Thus, myocyte-NOS3 could largely recapitulate functional modulation of β-adrenergic stimulation by NO in WT in vivo hearts.

Myocyte-Derived NO and Muscarinic Modulation of β-Adrenergic Stimulation

To test the role of myocyte-NOS3 to muscarinic modulation of in vivo β-stimulated function, ISO was administered before and after carbachol (CCh, 30 mg/kg per minute), with HR maintained constant by atrial pacing. Pressure-volume loops and systolic relations (Figure 4A) revealed a persistent positive contractile response to ISO despite CCh infusion in NOS3/+/Adv gal (left), very similar to the ISO response alone (Figure 4B). This contrasted to a −35% decline in this response in WT controls. However, AdvNOS3-transfected hearts (Figure 3B, right) showed minimal ISO-response in the presence of CCh. Isovolumic contractile function (Figure 3C) mirrored other systolic parameters (eg, ejection fraction, end-systolic volume). Diastolic effects (eg, relaxation rate) of β-adrenergic stimulation were not influenced by CCh.

Myocyte-Derived NO and the Force-Frequency Relationship

Figure 5 displays the results of FFR analysis using a preload-independent ejection-phase index (maximal power/ED volume) to assess systole. There was greater absolute enhancement of frequency-dependent function in WT versus NOS3/−+/Adv gal (P=0.007), despite slightly elevated function in the latter at slower rates. Similar results were obtained with the isovolumetric index: dP/dtmax/EDV (not shown). This was essentially restored by NOS3-myocyte transfection. Relaxation rates also displayed a blunted frequency response over the broad HR range in the

Figure 3. Influence of myocyte-selective NOS3 expression on in vivo modulation of β-adrenergic stimulation with isoproterenol (ISO). Whereas heart rate increase was similar among groups, NOS3/− transfected with β-gal had an enhanced contractile response (maximal rate of pressure rise normalized to end-diastolic volume; dP/dtmax/EDV) and systolic pressure rise. Relaxation response was blunted, however. Restoration of myocyte-NOS3 restored the ISO response to that observed in WT controls.

Figure 4. A, Pressure-volume loops showing ISO response after carbachol (CCh) injection. In NOS3/−/+Adv gal, a positive contractile response to ISO was still observed, with the loop and systolic pressure-volume relation (dashed line) shifting leftward. CCh blocked the ISO response in NOS3/− with myocyte-NOS3 restored. B, Summary contractility data showing ISO response before and after CCh in NOS3/− hearts with or without myocyte-NOS3 restoration.
NOS3-null animals, and this too was enhanced by myocyte-NOS3 transfer.

Discussion

Despite its description as an important contributor to myocardial regulation more than a decade ago, the functional role of myocyte-NOS3 has remained somewhat controversial. Nonselective pharmacological inhibition was first used to establish NO modulation of β-adrenergic stimulation, frequency-dependent function, and adrenergic inhibition by cholinergic agonists.1,3,14 However, such studies could not identify the cellular source or NO isoform involved for each modulation. The latter was more recently addressed by the use of genetically engineered mice harboring null mutations specific to NOS3 or NOS1,7,8,10,11,15 or with myocyte-targeted NOS3 overexpression.13 However, knockout models delete isoforms from myocytes as well as other tissues (ie, vascular or neural), and in vivo biology is always the composite from all changes. Furthermore, embryological models can contain many concealed adaptations that may influence the net behavior. Use of isolated myocytes might seem to circumvent some of these problems, but such preparations themselves alter ambient neurohormonal stimulation and redox state that are important to NO biochemistry.22,23 The present approach of viral-gene transfer provided a novel means to address this question in the in vivo heart. It provided targeted protein reexpression over a relatively short time frame (3 to 4 days, as compared with embryological and postnatal development), increasing the likelihood that responses would be more specific to the reexpressed NOS3 protein.

One important caveat to the present study is that protein expression and calcium-dependent NOS activity in NOS−/− + AdVΝΟ3 were higher than normal given that myocyte-NOS3 accounts for ≈20% of total NOS3 in the heart, and myocytes were not uniformly transfected. This suggests that the observed modulation of function may have been somewhat enhanced over that normally present. Similar limitations apply to previously used transgenic approaches that enhanced myocyte-NOS3.13 It should be noted that myocyte-NOS3 transfection restored basal (WT) function and reserve responses rather than generate an overshoot as might be anticipated if functional overexpression had been achieved.

Myocyte-NOS3 and Basal Function

Although the influence of NOS3-derived NO on basal contractility and lusitropy is accepted as modest, the direction itself has remained somewhat controversial. Prior studies have reported positive influences of NO on the L-type calcium channel24 and ryanodine receptor,25 although these investigations were not performed with NOS3−/− and the impact of basal NOS3 on nitrosylation-based channel modulation remains unclear. Petroff et al26 found NOS3-derived NO contributed to the positive effect of sarcomere stretch on myocyte shortening. However, the majority of cell/organ studies using null mutants have reported unaltered basal function (as reviewed by Massion and Balligand27) or slightly diminished function,6 in part depending on the index used. In the present study, ejection function was slightly enhanced in NOS3−/−, but as reported by Hare,6 the isovolumic index dP/dtmax/IP was reduced in these hearts. The latter may belie the effects of higher chronic pressure loading in the null mutants. The decline in contractility observed with myocyte-AdVΝΟ33 restoration agrees with results from myocyte-targeted NOS3-overexpressing transgenics.13 Diastolic function with or without restoration of myocyte-NOS3 was unaltered. This suggests that NO derived from alternative NOS3 (ie, cardiac or vascular endothelium), NOS-1, or exogenous sources more likely underlies reported effects of NO on diastolic properties.28

Myocyte NOS3 and Adrenergic/Muscarinic Modulation

Myocyte-derived NOS3 was far more potent in blunting β-adrenergic stimulation. Intact NOS3−/− hearts display augmented systolic responses to dobutamine or ISO stimulation,7,29 as observed in our study. However, ISO-stimulated Icα8,10 is similar in WT and NOS3−/− myocytes, and this has been cited as evidence that paracrine (vascular) NOS3 is central to this effect.10 Alternative signaling to L-type currents would appear to be active, as both myocyte sarcomere shortening and whole cell peak calcium transients have been recently shown to be more enhanced by ISO in NOS3−/− compared with control cells.7 Other NO targets such as cGMP phosphorylation of phospholamban and troponin I likely contribute to the altered systolic force generation and relaxation independent of Icα.30

The involvement of NO in muscarinic-receptor suppression of β-adrenergic stimulation1 has been more controversial. Han et al8 found the CCh-mediated inhibition of ISO-stimulated Icα in WT mice was absent in NOS3−/−, whereas others have not confirmed these effects.10–12 In the present study, we observed minimal CCh antagonism of ISO-stimulated function in NOS3−/− transfected with the reporter gene, whereas hearts with myocyte-NOS3 revealed inhibition. While supporting a role, we cannot fully rule out a concentration effect (ie, overexpression) that may have am-
plified the importance of NOS3 to this signaling. In a previous report, acetylcholine inhibition of norepinephrine stimulation was similar between isolated control hearts and those with myocyte-specific NOS3 overexpression. One possibility for prior negative results for muscarinic modulation is that myocyte (or muscle) isolation removes normal neural inputs, and this maybe particularly important to NO-mediated effects. It is intriguing in this regard the gene transfer of NOS1 to guinea pig atria results in increased protein expression and localization of NOS1 in cholinergic ganglia.31

**Myocyte NOS3 and Force-Frequency Modulation**

Augmentation of cardiac function at faster frequencies is ascribed to enhanced sarcolemmal calcium entry and uptake by the sarcoplasmic reticulum (SR).32 Early studies showing negative modulation of the force-frequency relation by NO were based on broad NOS inhibitors.14,34 Since then, the NOS1 isoform has been localized to the SR,33 and recent analysis using NOS1 and NOS3 null mutant models suggests the latter to be more involved with frequency modulation.15 Although the present data does not refute prior findings regarding NOS1, it does suggest a role for NOS3 as well. Differences in HR range (ie, we included data at rates below 650 minutes−1), analysis of absolute rather than percent change, and indexes used may have contributed. Our data do seem consistent with a reduced phospholamban/SERCA2a ratio in NOS3−/− hearts,13 which would be expected to enhance basal contractility but limit frequency reserve,34,35 as observed in the PLB−/− mice.35 Additional factors such as the impact of NOS3 on PLB phosphorylation remain unknown. Admittedly, of the three control mechanisms studied, myocyte-NOS3 effects on frequency modulation were the most modest.

**Limitations**

The present study, although the first to demonstrate a role specifically for myocyte-derived NO in vivo, does have limitations. First, embryologic deletion of NOS3 has been shown to trigger alterations in gene expression and neurohumoral signaling such as increased ANP secretion5 and enhanced cytochrome P450 activity.36 One potential way to circumvent this limitation would be use of a conditional rather than embryological-derived NOS3-null mutant. Such models do not currently exist. An additional limitation is the that in vivo gene transfer in adult murine hearts results in about 60% transfection efficiency, raising the question as to how this could lead to sufficiently global restoration of functional effects in the intact heart. However, as with most transgenic models, the correspondence between expression level and phenotype is highly nonlinear, and 60% cell transfection that is diffusely distributed around the myocardium is sufficient. Alternatively, they may be some paracrine myocyte-myocyte signaling, although to our knowledge, this has never been directly confirmed.

**Conclusions**

The present study demonstrates that myocyte-derived NO itself plays a substantial and important role in modulating cardiac function in vivo. The impact on β-adrenergic stimulation matched that observed in WT hearts suggesting the former could recapitulate normally combined endothelial/myocyte NOS3 signaling modulation. The present data also support in vivo muscarinic modulation by myocyte-NOS3, whereas frequency modulation was indeed less prominent but still present. Numerous disease conditions affect NOS3 signaling in the vasculature but the impact on cardiomyocytes is not necessarily concordant. Although the present data do not negate the importance of endothelium-derived NO, they are important by demonstrating that endothelium-dependent NO signaling is not required to provide key components of NOS3 regulation of cardiac function.

**Acknowledgments**

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