Nitric Oxide Regulation of Myocardial Contractility and Calcium Cycling

Independent Impact of Neuronal and Endothelial Nitric Oxide Synthases

Shakil A. Khan,* Michel W. Skaf,* Robert W. Harrison,* Kwangho Lee, Khalid M. Minhas, Anil Kumar, Mike Fradley, Artin A. Shoukas, Dan E. Berkowitz, Joshua M. Hare

Abstract—The mechanisms by which nitric oxide (NO) influences myocardial Ca²⁺ cycling remain controversial. Because NO synthases (NOS) have specific spatial localization in cardiac myocytes, we hypothesized that neuronal NOS (NOS1) found in cardiac sarcoplasmic reticulum (SR) preferentially regulates SR Ca²⁺ release and reuptake resulting in potentiation of the cardiac force-frequency response (FFR). Transesophageal pacing (660 to 840 bpm) in intact C57Bl/6 mice (WT) stimulated both contractility (dP/dt_max normalized to end-diastolic volume; dP/dt-EDV) by 51±5% (P<0.001) and lusitropy (tau; τ) by 20.3±2.0% (P<0.05). These responses were markedly attenuated in mice lacking NOS1 (NOS1⁻/⁻) (15±2% increase in dP/dt-EDV; P<0.001 versus WT; and no change in τ; P>0.01 versus WT). Isolated myocytes from NOS1⁻/⁻ (~2 months of age) also exhibited suppressed frequency-dependent sarcomere shortening and Ca²⁺ transients ([Ca²⁺]) compared with WT. SR Ca²⁺ stores, a primary determinant of the FFR, increased at higher frequencies in WT (caffeine-induced [Ca²⁺], at 4 Hz increased 107±23% above 1 Hz response) but not in NOS1⁻/⁻ (13±26%; P<0.01 versus WT). In contrast, mice lacking NOS3 (NOS3⁻/⁻) had preserved FFR in vivo, as well as in isolated myocytes with parallel increases in sarcomere shortening, [Ca²⁺], and SR Ca²⁺ stores. NOS1⁻/⁻ had increased SR Ca²⁺ ATPase and decreased phosphohamilb protein abundance, suggesting compensatory increases in SR reuptake mechanisms. Together these data demonstrate that NOS1 selectively regulates the cardiac FFR via influences over SR Ca²⁺ cycling. Thus, there is NOS isoform-specific regulation of different facets of rate-dependent excitation-contraction coupling; inactivation of NOS1 has the potential to contribute to the pathophysiology of states characterized by diminished frequency-dependent inotropic responses. (Circ Res. 2003;92:1322-1329.)

Key Words: nitric oxide ■ force-frequency response ■ SERCA2a ■ sarcoplasmic reticulum ■ excitation-contraction coupling

Nitric oxide (NO) modulates cardiac function, in large part, by influencing calcium channels critical to excitation-contraction coupling.¹ In this regard, NO influences on β-adrenergic signaling are exerted at the level of the L-type Ca²⁺ channel² and the sarcoplasmic reticulum (SR) ryanodine receptor (RyR).³,⁴ We have recently shown that different NO synthases are compartmentalized in proximity to effector Ca²⁺ channels, allowing specific physiological control of events controlled by the protein in question.⁴ For example, neuronalonal NOS (NOS1) is localized to the SR,⁵ where it enhances β-adrenergic–stimulated contractility by increasing SR Ca²⁺ release, and endothelial NOS (NOS3) is localized to the sarcolemma where it inhibits β-adrenergic inotropic responses via an effect on Iₑ.⁴ Although a paradigm of spatial confinement of NO synthase with effector proteins offers insight into the mechanism(s) by which NO affects β-adrenergic contractile reserve,⁶ the role that various NOS isoforms play in regulating myocardial contractility in general remains highly controversial.⁶,⁷ In this regard, Petroff and colleagues have implicated NOS3, not NOS1, in stretch induced myocyte Ca²⁺ transients,⁶ and Ashley et al⁷ have suggested that NOS1 may, under certain experimental conditions, suppress rather than stimulate SR Ca²⁺ release.

To address these controversies, we tested the hypothesis that NOS1 plays a primary role in stimulating SR Ca²⁺ cycling. In order to focus on physiological conditions, we studied both in vivo and isolated myocyte force-frequency

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responses (FFRs), a contractile reserve mechanism mediated largely by SR Ca\(^{2+}\) cycling,\(^8,9\) in intact mice lacking NOS1 (NOS1\(^{-/-}\)),\(^10\) NOS3 (NOS3\(^{-/-}\)),\(^11\) and their respective wild-type (WT) controls (C57Bl/6). Given its localization to the SR, we predicted that NOS1 would modulate the FFR as opposed to NOS3. We performed complementary in vitro studies to measure SR Ca\(^{2+}\) stores in isolated myocytes and to assess abundance of major proteins involved in diastolic Ca\(^{2+}\) removal.

Materials and Methods

Integrated Hemodynamic Analysis

The animal protocol conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996) and was approved by the Johns Hopkins Animal Care and Use Committee. We studied mice deficient in NOS1 (NOS1\(^{-/-}\))\(^9,10\) and NOS3 (NOS3\(^{-/-}\))\(^11\) both due to homozygous deletion mutations, and C57Bl/6 wild-type mice (Jackson Laboratories, Bar Harbor, Maine) at 2 to 5 months of age. Both knockout strains were backcrossed with C57Bl/6 mice for greater than 10 generations. In preliminary studies, we showed that NOS1\(^{-/-}\) mice on a mixed C57Bl/6 SV129 background had identical force-frequency characteristics to backcrossed mice (data not shown). Mice were anesthetized with etomidate (1.3 mg/g), urethane (0.9 mg/g), and morphine (0.5 mg/g) and were instrumented with a micromanometer-conductance catheter (SPR-719, Millar Instruments Inc).\(^12\) Volume measurements were calibrated by correlation with stroke volume measured with an ultrasonic flow probe and by saline calibration of end-diastolic volume.\(^12\) Cardiac pacing (from 660 to 840 bpm in 60 bpm increments) was accomplished with a pacing wire advanced to the distal esophagus.\(^13\)

Myocardial systolic and diastolic performance was assessed from pressure-volume data.\(^12\) Transient occlusion of the inferior vena cava was used to generate the end-systolic pressure–volume relationship (ESPVR) from which Ees and the slope of the relationship between the peak rate of rise in LV pressure (dP/dt\(_{max}\)) and end-diastolic volume (EDV) are determined. Cardiac preload was indexed as EDV and left ventricular end-diastolic pressure (EDP); cardiac afterload was evaluated as effective arterial elastance (Ea, ratio of LV end-systolic pressure to stroke volume), and myocardial contractility was indexed by the slope of the ESPVR (Ees) and by the dP/dt\(_{max}\)/EDV relationship (dP/dt\(_{EDV}\)).

Isolated Myocyte Preparation

Myocytes from 2-month-old mice were studied, an age at which cardiac architecture was unchanged from wild type.\(^4\) Cardiac myocytes were isolated as described in detail in the expanded Materials and Methods section (available in the online data supplement at http://www.circresaha.org). Myocytes were incubated with 5 \(\mu\)m/L Fura-2/AM (Molecular Probes) then transferred to a Lucite chamber superfused with Tyrode’s containing 1.8 \(\mu\)m/L Ca\(^{2+}\) and 0.5 mmol/L probenecid. Sarcomere length (SL) and Ca\(^{2+}\) transient ([Ca\(^{2+}\)]\(_i\)) were measured in myocytes stimulated at 1, 2, and 4 Hz. The time-constant of relaxation for SL and [Ca\(^{2+}\)]\(_i\), (\(\tau\)) were used to estimate diastolic relaxation and Ca\(^{2+}\) removal. Caffeine (10 mmol/L) was rapidly infused after a 10-second pause following steady-state stimulation at increasing rates. The decay of Ca\(^{2+}\)\(_i\) (\(\tau\)) after caffeine infusion was used to approximate NCX activity.\(^14\) All experiments were conducted at 37°C and in room air (Po\(_2\) \(>\) 20%) as increased O\(_2\) concentrations are known to disrupt NO influences over the RyR.\(^15\)

SL was recorded with an IonOptix iCCD camera. Change in average SL was determined by fast Fourier transform of the Z-line density trace to the frequency domain, and SL Shortening was calculated as follows: (diastolic SL – systolic SL)/diastolic SL. [Ca\(^{2+}\)]\(_i\) was measured using the Ca\(^{2+}\)-sensitive dye Fura-2 and a dual-excitation spectrofluorometer (IonOptix), alternately excited with a xenon lamp at wavelengths of 365 and 380 nm. The emission fluorescence was reflected through a barrier filter (510±15 nm) to a photomultiplier tube. The Fura-2 fluorescence ratio, the ratio of the photon live count detected by the excitation at 365 nm compared with 380 nm, represents [Ca\(^{2+}\)]\(_i\).

Response to Pharmacological NOS1 Inhibition

To assess the impact of acute NOS1 inhibition, wild-type myocytes were incubated with S-methyl-L-thiocitrulline (SMTC, 10\(^{-5}\) mol/L for 20 minutes), a potent isoform selective inhibitor of NOS1.\(^16\) FFR was assessed in aliquots of myocytes incubated with and without SMTC.

Western Blots

To compare the levels of the major proteins involved in Ca\(^{2+}\) reuptake, Western blot analysis was performed on total protein from WT (n=7), NOS3\(^{-/-}\) (n=4) and NOS1\(^{-/-}\) (n=7) mice (2 to 3 months old). Western blots were performed as described in detail in the expanded Materials and Methods (see online data supplement).

Statistical Analysis

Data are reported as mean±SEM. Statistical significance was determined by one-way or two-way ANOVA and Student-Newman-Keuls post hoc test (GraphPad Instat and SAS statistical software). Values of P<0.05 were considered significant.

Results

Baseline Hemodynamics

Baseline hemodynamic parameters are illustrated in Table 1. NOS3\(^{-/-}\) but not NOS1\(^{-/-}\) mice had elevated systolic blood pressure (P<0.01 versus WT), yet the coupling of ventricular to arterial elastance (Ees/Ea) was similar in all strains of mice as previously described.\(^3\)

Force-Frequency Response in Intact Mice

To determine the effect of NO synthases on the force-frequency response, we recorded pressure-volume data in intact NOS1\(^{-/-}\), NOS3\(^{-/-}\), and WT controls (n=5 for each group) during pacing at rates from 660 to 840 bpm in 60 bpm increments. Example steady-state pressure-volume data recorded at 660 and 780 bpm are shown in Figure 1A. A positive force-frequency response as evidenced by an increased slope of the end systolic pressure volume relationship (Ees), was observed in both WT and NOS3\(^{-/-}\) but was attenuated in NOS1\(^{-/-}\) mice. Ees at 720 bpm increased by 9±4% in NOS1\(^{-/-}\) mice (P<0.05 versus WT) compared with 38±11% and 33±12% in WT and NOS3\(^{-/-}\), respectively (Figure 1B).

Increasing pacing frequency also increased dP/dt\(_{EDV}\) in WT and NOS3\(^{-/-}\) mice (P<0.05 versus BL), whereas this response was markedly blunted in NOS1\(^{-/-}\) (P<0.001 versus WT; Figure 1C).

We also examined frequency related lusitropic responses. The time-constant of LV relaxation, \(\tau\), shortened in both WT and NOS3\(^{-/-}\) mice, but remained unchanged in NOS1\(^{-/-}\) mice. Ees at 720 bpm increased by 9±4% in NOS1\(^{-/-}\) mice (P<0.05 versus WT) compared with 38±11% and 33±12% in WT and NOS3\(^{-/-}\), respectively (Figure 1B). Increasing pacing frequency also increased dP/dt\(_{EDV}\) in WT and NOS3\(^{-/-}\) mice (P<0.05 versus BL), whereas this response was markedly blunted in NOS1\(^{-/-}\) (P<0.001 versus WT; Figure 1C).

Cardiac Myocyte Frequency Response

To test whether these responses were due to intramyocyte signaling and independent of autonomic influences, we mea-
sured frequency-dependent responses in isolated myocytes from mice at 2 months of age. Baseline characteristics were similar among the three groups of myocytes at 1 Hz (Table 2). Increasing stimulation rate augmented WT myocyte contraction and Ca\(^{2+}\) transients. Sample transients of sarcomere shortening and [Ca\(^{2+}\)] at 1 and 4 Hz are shown in Figure 2A. Peak SL shortening and [Ca\(^{2+}\)] (Figures 2B through 2E) increased in parallel with higher pacing frequencies in the WT myocytes (mice, n=11; 3 to 4 cells per heart) demonstrating a positive FFR. In contrast, the FFR was blunted in NOS1\(^{-/-}\) mice, n=9; 3 to 4 cells per heart), as evidenced in SL shortening and [Ca\(^{2+}\)], at higher stimulation frequencies (P<0.01 versus WT).

To further confirm that the FFR was attenuated due to NOS1 deficiency and not diminished NO pathway activity, we studied NOS3\(^{-/-}\) mice, n=12, 2 to 3 cells per heart). Like WT myocytes, both SL shortening and [Ca\(^{2+}\)],

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<th>TABLE 1. Baseline Hemodynamics</th>
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<td>Mice, n</td>
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<td>WT</td>
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<td>NOS3(^{-/-})</td>
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<td>NOS1(^{-/-})</td>
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<td>HR, bpm</td>
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<td>SBP, mm Hg</td>
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<td>ESV, µL</td>
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<tr>
<td>dP/dt-Pid, mm Hg/sec per µL</td>
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<tr>
<td>dP/dt-Pi, 1/sec</td>
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<tr>
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<tr>
<td>Ea, mm Hg/mL</td>
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<tr>
<td>Ees/Ea</td>
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<td>r, msec</td>
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HR indicates heart rate; SBP, systolic blood pressure; ESV, end-systolic volume; dP/dt-Pd, dP/dt-Pi, normalized to instantaneous developed pressure; and r, time constant of LV relaxation.

*P<0.05 vs both WT and NOS3\(^{-/-}\); †P<0.01 vs WT; and ‡P<0.05 vs NOS1\(^{-/-}\).

**Figure 1.** Left ventricular pressure-volume data obtained during increasing stimulation frequency in WT, NOS3\(^{-/-}\), and NOS1\(^{-/-}\) mice. Depicted are representative steady-state left ventricular pressure-volume loops and the ESPVR obtained by transient IVC occlusion at baseline (solid lines) and in response to pacing at 780 bpm (dashed lines) (A). NOS1\(^{-/-}\) mice have an attenuated contractile response to increased pacing frequency (contractility indexed by the slope position of Ees; upward deflection signifies positive inotropy). In contrast, NOS3\(^{-/-}\) have a similar response compared with WT at 780 bpm. Also shown are pooled data of the rate-stimulated inotropy (B). At 720 bpm, Ees increase was significantly attenuated in NOS1\(^{-/-}\) mice compared with WT (*P<0.05). C, NOS1\(^{-/-}\) mice have an attenuated contractile response (% change in dP/dt-EDV) compared with WT and NOS3\(^{-/-}\) (*P<0.05 vs BL; †P<0.001 vs WT; ‡P<0.01 vs NOS3\(^{-/-}\); §P<0.05 vs WT). D, Time-constant of LV relaxation, r, shortened in both WT and NOS3\(^{-/-}\) mice at increased pacing rates, but remained unchanged in NOS1\(^{-/-}\) (*P<0.05 vs BL; †P<0.05 vs both WT and NOS3\(^{-/-}\)).
Table 2. Baseline Myocyte Characteristics at 1 Hz

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>NOS3&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>NOS1&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Mice, n&lt;sup&gt;+&lt;/sup&gt;</td>
<td>15</td>
<td>12</td>
<td>10</td>
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<tr>
<td>Diastolic SL, μm</td>
<td>1.79±0.02</td>
<td>1.80±0.01</td>
<td>1.77±0.01</td>
</tr>
<tr>
<td>SL shortening, %</td>
<td>2.12±0.2</td>
<td>1.89±0.2</td>
<td>2.22±0.2</td>
</tr>
<tr>
<td>[Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sup&gt;i&lt;/sup&gt;, %</td>
<td>23.8±1.1</td>
<td>24.3±1.4</td>
<td>25.9±1.4</td>
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*3 to 4 cells were studied per heart.

SL shortening indicates (diastolic SL−systolic SL)/diastolic SL; [Ca<sup>2+</sup>]<sup>i</sup>, change in the ratio of the photon live count detected by excitation at 365 nm compared with 380 nm during contraction.

Increased in parallel at higher pacing frequencies, and the magnitude of the FFR was nearly identical to that observed in WT (Figure 2).

As an added control, we tested the effects of pharmacological NOS1 inhibition with SMTC in WT myocytes. As illustrated in Figure 3, the SMTC-treated myocytes (mice, n=3; 2 to 3 cells per heart) at 4 Hz had blunted increases in sarcomere shortening and [Ca<sup>2+</sup>]<sup>i</sup>, compared with untreated cells (both P<0.01). The effects of acute NOS1 inhibition were similar in magnitude to that observed in NOS1<sup>−/−</sup>.

Myocyte relaxation (Table 3) was similar with regards to both SL shortening and [Ca<sup>2+</sup>]<sup>i</sup>, among all three strains stimulated at 1 Hz. Frequency-dependent relaxation was suppressed in NOS1<sup>−/−</sup> compared with WT; however, cytosolic removal of Ca<sup>2+</sup> (as indexed by τ [Ca<sup>2+</sup>]<sup>i</sup>) was similar, consistent with the findings presented below of reduced PLB/SERCA ratio in NOS1<sup>−/−</sup> mice.

Sarcoplasmic Reticulum Ca<sup>2+</sup> Stores

As SR stores are a primary determinant of frequency-dependent responses, we estimated SR Ca<sup>2+</sup> stores by rapidly infusing caffeine (10 mmol/L) at rising pacing frequencies (Figure 4). At 1 Hz, calcium stores were similar in WT (111±10, n=9), NOS3<sup>−/−</sup> (121±17, n=7), and NOS1<sup>−/−</sup> (122±9, n=5, 2 to 3 cells per heart in each strain). Increasing stimulation frequency to 4 Hz augmented SR Ca<sup>2+</sup> stores to a similar extent in WT (227±25%, n=3, 1 to 2 cells per heart) and NOS3<sup>−/−</sup> (234±39%, n=4, P<0.05 compared with 1 Hz caffeine responses). In contrast, caffeine-induced Ca<sup>2+</sup> release did not increase in NOS1<sup>−/−</sup> at 4 Hz relative to 1 Hz (138±32, n=4, P<0.01 versus WT).

Protein Abundance

In order to investigate potential mechanisms for reduced Ca<sup>2+</sup> stores, we performed Western blot analysis of the major proteins involved in diastolic Ca<sup>2+</sup> removal (Figure 5). Interestingly, SERCA2a was increased in NOS1<sup>−/−</sup> hearts.

Table 3. Myocyte Relaxation Values

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>[Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sup&gt;i&lt;/sup&gt; at 1 Hz, ms</th>
<th>[Ca&lt;sup&gt;2+&lt;/sup&gt;]&lt;sup&gt;i&lt;/sup&gt; at 4 Hz, %</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>146±15</td>
<td>−58±4*</td>
<td>166±10</td>
</tr>
<tr>
<td>NOS3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>141±16</td>
<td>−58±6*</td>
<td>200±17</td>
</tr>
<tr>
<td>NOS1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>173±25</td>
<td>−54±4†</td>
<td>175±20</td>
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</table>

*P<0.001 vs 1 Hz; †P<0.05 vs WT; and §P<0.05 vs NOS3<sup>−/−</sup>.
forms. These data support the contention that NOS isoforms regulate cardiac contractile function and clarify divergent results regarding the roles of the various NOS isoforms in regulating cardiac contractile function and clarify divergent results regarding the roles of the various NOS isoforms. These data support the growing body of evidence that spatial confinement of NOS isoforms plays a central role in regulating cardiac contractile function and clarify divergent results regarding the roles of the various NOS isoforms.

**Discussion**

The major new finding of this study is that NOS1 influences the frequency-mediated rise in cardiac contractility and Ca\(^{2+}\) cycling in a manner unique from NOS3. NOS1\(^{-/-}\) mice have a depressed FFR, both in vivo and in isolated myocytes, accompanied by reduced Ca\(^{2+}\) release and SR Ca\(^{2+}\) stores at higher pacing frequencies. On the other hand, NOS3\(^{-/-}\) mice have a normal FFR in both intact animals and isolated myocytes. These data support the growing body of evidence that spatial confinement of NOS isoforms plays a central role in regulating cardiac contractile function and clarify divergent results regarding the roles of the various NOS isoforms.

Excitation-contraction coupling, and that NOS1 is the primary isoform regulating SR calcium cycling. NO influences many major facets of cardiac myocyte function, including signal transduction, \(^{1,17}\) Ca\(^{2+}\) cycling, \(^{3,18}\) and mitochondrial respiration. \(^{19,20}\) The precise role for NO in each of these functions continues to be vigorously debated. \(^{1,21,22}\) We have previously shown that NOS1 and NOS3, which localize to different subcellular compartments, can exert opposite effects on cardiac \(\beta\)-adrenergic signal transduction. \(^{4}\) Our present data demonstrate how the paradigms of spatial localization and isoform specificity help explain NO regulation of frequency-related inotropic reserve and SR calcium cycling. As predicted by its localization to the SR, NOS1 preferentially influences the FFR.

**Nitric Oxide and the Force-Frequency Response**

The manner in which NO influences myocardial contractility and the FFR remains extremely controversial. \(^{1}\) Studies using NO donors and NOS inhibitors in both experimental systems and in humans have failed to detect NO influences on the FFR, \(^{23,24}\) whereas others have shown an inhibitory influence with similar interventions. \(^{25,26}\) Neither positive nor negative studies explored isoform specificity. Our data suggest that nonspecific inhibition of NOS isoforms or generalized activation of their effector molecules may obscure the differences in the effects of NOS1 and NOS3 on the FFR.

Recently, Ashley et al\(^7\) reported that mouse myocytes FFR responses were absent in both WT and NOS1\(^{-/-}\), and that NOS1 contractility was augmented over the range of stimulation frequencies studied (1 to 6 Hz). Although an attenuated

![Figure 4](image-url.png)

**Figure 4.** Sarcoplasmic reticulum Ca\(^{2+}\) stores at increasing frequencies. Isolated cardiac myocytes were rapidly exposed to caffeine (10 mmol/L) at increasing frequencies after a 10-second pause. SR Ca\(^{2+}\) reserves were measured as the percent change from baseline [Ca\(^{2+}\)]. A, Sample transients depict the SR Ca\(^{2+}\) reserves at 1 and 4 Hz. B, WT and NOS3\(^{-/-}\) had increasing Ca\(^{2+}\) stores at higher frequencies, whereas NOS1\(^{-/-}\) myocytes did not. \(*P<0.05\) vs 1 Hz response; \(†P<0.01\) compared with WT at 4 Hz; \(‡P<0.05\) compared with NOS3\(^{-/-}\) at 4 Hz.

![Figure 5](image-url.png)

**Figure 5.** Western blot analysis of major proteins involved in diastolic Ca\(^{2+}\) removal. A through D, SERCA2a protein abundance was increased in NOS1\(^{-/-}\) \((P<0.05)\), compared with WT, whereas PLB was decreased in both NOS1\(^{-/-}\) and NOS3\(^{-/-}\) \((P<0.001\) both vs WT). NCX levels were similar among all 3 strains. E, The ratio of PLB/SERCA2a was markedly reduced in NOS1\(^{-/-}\) \((P<0.01\) vs WT) and also diminished in NOS3\(^{-/-}\) \((†P<0.05\) vs WT).
FFR in NOS1−/− myocytes is concordant with the present results, we have shown that the FFR is positive in WT, consistent with many other recent studies demonstrating positive FFRs in murine cardiomyocytes,27,28 trabeculae,29,30 and intact hearts.13 Sears and coworkers31 attributed increased resting contraction in NOS1−/− to elevated \( I_{\text{Ca}} \). Interestingly, although we observed a nonsignificant elevation in SL contraction in NOS1−/− relative to WT, our present and previously published4 intact heart data support an elevated resting contractile state (measured as peak \( +dP/dt \) normalized to LV pressure) in NOS1−/−. Thus, as previously described, NO influences on myocardial contractility appear to be biphasic.32,33

It is important to note that the NOS1−/− myocytes exhibited a positive FFR, despite modest changes in \([Ca^{2+}]\), and a minimal increase in SR Ca\(^{2+}\) stores. This may be attributed to other mechanisms regulating the FFR, such as CAMKII activation,4 myofibrillar calcium responsiveness,34 or PLB phosphorylation,9 and is consistent with the role of NO as a modulator of some of these signaling processes.

**Diastolic Calcium Removal**

There are two potential explanations for reduced SR Ca\(^{2+}\) stores in NOS1−/−: decreased Ca\(^{2+}\) uptake or increased Ca\(^{2+}\) leak (or a combination thereof). We cannot exclude ineffective SERCA2a activity and a role for NOS1 modulating SR Ca\(^{2+}\) reuptake.35 Despite explicit study by several other groups, whether or not NO directly affects SR reuptake continues to be controversial.5,15,35 Nevertheless, our observation of reduced PLB/SERCA2a ratios, which would be expected to enhance SR Ca\(^{2+}\) reuptake, in combination with evidence of reduced SR stores strongly suggests a compensatory mechanism, and, is consistent with RyR leak36 in NOS1−/− myocytes.

This conclusion is supported by abundant in vitro data demonstrating that NO increases the open probability of purified cardiac and skeletal muscle ryanodine receptors (RyRs) via thiol nitrosylation of cysteine 3635,3,15,37. Importantly, this reaction occurs in a reversible manner allowing for both activation and deactivation of RyR stimulation,3 permitting this mechanism to be physiologically relevant. The absence of NOS1, which is closely associated with the RyR,4,5 may lead to diastolic Ca\(^{2+}\) leak, a phenomenon that worsens at higher frequencies,38 thereby attenuating the FFR. Furthermore, NO activation of the RyR contrasts to that by superoxide.3,39,40 Superoxide irreversibly activates the RyR and enhances SR leak, whereas NO does so reversibly and is thus potentially modulated during the cardiac cycle.4 Interestingly, an analogous scenario is proposed by Marks et al41 for the manner in which the RyR responds to phosphorylation; physiologically activating phosphorylation is limited, whereas in disease conditions, phosphorylation is accentuated leading to increased channel activity and leak.

It is important to note that SERCA2a upregulation in combination with PLB downregulation may also contribute to a blunted FFR. Increasing contraction frequency leads to decreased PLB inhibition of SERCA2a and, subsequently, a positive FFR.9 However, if PLB is already downregulated, as in the PLB-deficient mouse42 or NOS1−/− and NOS3−/−, then hearts may potentially lack a cardiac reserve mechanism for increased contraction at higher frequencies.

Our study of NOS3−/− mice was designed to confirm the importance of NOS1 regulation of the FFR. Indeed, mice lacking NOS3, the isoform localized to the sarcolemma, had a positive FFR indistinguishable from WT in isolated myocytes and similar to WT in vivo. Moreover, SR Ca\(^{2+}\) stores and release from NOS3−/− myocytes were similar to WT. Interestingly, although SERCA2a was unchanged, PLB expression was reduced in NOS3−/−. The lack of a depressed FFR in NOS3−/− is consistent with the known gene dosage effect for PLB on myocardial contractility.43 Alternatively, increased Ca\(^{2+}\) influx through the L-type channel44 in NOS3−/− has the potential to offset reduced PLB and preserve the frequency effect. It is important to note that if PLB was not downregulated in NOS3−/−, the FFR could potentially be enhanced in the absence of this isoform, as has been observed in some NOS inhibitor studies.25,26 Further studies are underway to address these questions.

**Implications for Heart Failure**

In heart failure (HF), the FFR is depressed.44,45 Understanding the mechanism of enhancement of SR Ca\(^{2+}\) cycling in response to increasing stimulation frequency in healthy myocytes might offer insight into HF pathophysiology. For example, SERCA2a overexpression in failing cardiac myocytes restores Ca\(^{2+}\) stores and enhances the frequency response.46 Recently, alterations in RyR activity are implicated as contributing to depressed FFR in HF.47 Our demonstration that NOS1 deficiency inhibits the FFR in myocytes and intact hearts, suggests that its inactivation may play a role in HF pathogenesis. Interestingly, cardiac NOS1 has recently been shown to be upregulated in experimental models of myocardial infarction48 and hypertension.49 Future work will be required to establish the precise functional versus compensatory role played by this phenomenon.

**Limitations**

Several technical issues and limitations warrant mention. We measured the FFR in intact open chest mice at rates of conscious exercising mice50 and with baseline contractility under anesthesia resembling that of conscious animals.51 In addition, we have previously shown that hearts from both NOS1 and NOS3 develop cardiac hypertrophy.4 To address this potential confounding factor, we studied isolated myocytes from 2-month-old mice, an age which preceded any cardiac structural changes. It was not possible to study intact mice at this age because of small size, and 5-month-old animals were studied. The concordance between the findings from the intact heart and isolated myocyte studies suggests that cardiac hypertrophy likely does not explain the diminished FFR in NOS1−/− mice. Although the frequencies used for isolated myocyte studies were below those for the intact studies, they are comparable to most published murine myocyte studies, and the results from these studies qualitatively resembled those obtained in intact animals. Finally, although the NOS1−/− and NOS3−/− mice used were both backcrossed to C57BL/6 background (and are therefore considered to be 99.6% C57BL/6), the originating SV129 ES
cells used to create these mice were of different subtypes. Accordingly, although unlikely, we cannot rule-out an influence of residual SV129-related impact on our protein-abundance findings.

Summary

These data demonstrate that NOS1, as opposed to NOS3, preferentially influences the cardiac FFR, a central mechanism of cardiac contractile reserve. This is consistent with the evolving concept that intracrine signaling pathways are spatially compartmentalized with unique effector molecules in subcellular microdomains. Thus, the same intracrine signaling molecule can exert different and in some cases opposite effects on cellular/organ level function. In addition, these results call into question the notion that net NO production is the primary determinant of its intracrine signaling effect. Together these findings have important implications for cardiac physiology and pathophysiology and demonstrate that NO must be added to the list of key modulators of SR Ca\(^{2+}\) cycling.

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