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Neuronal nitric oxide synthase signaling within cardiac myocytes targets phospholamban

Honglan Wang, Mark J. Kohr, Christopher J. Traynham, Debra G. Wheeler, Paul M. L. Janssen, and Mark T. Ziolo

Department of Physiology and Cell Biology, Davis Heart and Lung Research Institute, The Ohio State University, Columbus, Ohio

Abstract

Studies have shown that neuronal nitric oxide synthase (nNOS, NOS1) knockout mice (NOS1^{-/-}) have increased or decreased contractility, but consistently have found a slowed rate of intracellular Ca²⁺ ([Ca²⁺]_i) decline and relengthening. Contraction and [Ca²⁺]_i decline are determined by many factors, one of which is phospholamban (PLB). The purpose of this study is to determine the involvement of PLB in the NOS1-mediated effects. Force-frequency experiments were performed in trabeculae isolated from NOS1^{-/-} and wild-type (WT) mice. We also simultaneously measured Ca²⁺ transients (Fluo-4) and cell shortening (edge detection) in myocytes isolated from WT, NOS1^{-/-}, and PLB^{-/-} mice. NOS1^{-/-} trabeculae had a blunted force-frequency response and prolonged relaxation. We observed similar effects in myocytes with NOS1 knockout or specific NOS1 inhibition with *S*-methyl-L-thiocitrulline (SMLT) in WT myocytes (i.e., decreased Ca²⁺ transient and cell shortening amplitudes and prolonged decline of [Ca²⁺]_i). Alternatively, NOS1 inhibition with SMLT in PLB^{-/-} myocytes had no effect. Acute inhibition of NOS1 with SMLT in WT myocytes also decreased basal PLB serine16 phosphorylation. Furthermore, there was a decreased SR Ca²⁺ load with NOS1 knockout or inhibition, which is consistent with the negative contractile effects. Perfusion with FeTPPS (peroxynitrite decomposition catalyst) mimicked the effects of NOS1 knockout or inhibition. β-Adrenergic stimulation restored the slowed [Ca²⁺]_i decline in NOS1^{-/-} myocytes, but a blunted contraction remained, suggesting additional protein target(s). In summary, NOS1 inhibition or knockout leads to decreased contraction and slowed [Ca²⁺]_i decline, and this effect is absent in PLB^{-/-} myocytes. Thus NOS1 signaling modulates PLB serine16 phosphorylation, in part, via peroxynitrite.

Keywords

NOS1; peroxynitrite; force-frequency response

It is well established that nitric oxide (NO) is an important regulator of cardiac contractility in normal and diseased hearts (41). Two isozymes of NO synthase are constitutively expressed within cardiac myocytes: neuronal NO synthase (nNOS, NOS1) and endothelial NO synthase (eNOS, NOS3). Although NO is a highly diffusible signaling molecule, recent studies have shown that signaling via NOS1 and NOS3 is compartmentalized. NOS1 is localized to the sarcoplasmic reticulum (SR), whereas NOS3 is localized to the caveolae (2,37), and both modulate cardiac contraction differently (2).

Contraction of cardiac myocytes occurs by excitation-contraction coupling (EC coupling) (4). EC coupling is initiated by Ca^{2+} entry through the L-type Ca^{2+} channel. This Ca^{2+} induces a larger Ca^{2+} release through the SR Ca^{2+} release channel (ryanodine receptor, RyR). During relaxation, Ca^{2+} is resequestered into the SR via the sarco(endo)plasmic reticulum Ca^{2+} -ATPase (SERCA)-phospholamban (PLB) complex. The rate of $[\text{Ca}^{2+}]_i$ decline, and thus relaxation, is mainly determined by SR Ca^{2+} uptake in isolated, unloaded myocytes. This is especially true in mouse myocytes, where $[\text{Ca}^{2+}]_i$ decline is ~92% dependent on SERCA (5). SERCA activity can be regulated by many factors, one of which is the SERCA/PLB ratio, such that an increase in the SERCA/PLB ratio should result in an enhanced rate of relaxation (24). $\text{NOS1}^{-/-}$ hearts have an increase in the SERCA/PLB ratio (20,30). However, studies consistently observed that NOS1 knockout (or acute inhibition) led to a slower $[\text{Ca}^{2+}]_i$ decline and/or myocyte relengthening (1,20,30). As with $[\text{Ca}^{2+}]_i$ decline, an increase in the SERCA/PLB ratio should also result in an enhanced SR Ca^{2+} load (23), which is an important influence on cardiac contractility (3,16,31). That is, the more Ca^{2+} that is stored in the SR, the more Ca^{2+} that will be released, leading to a larger contraction. However, studies have observed a reduced SR Ca^{2+} load, which contributes to a blunted force-frequency response (14,20).

Besides decreased SERCA activity, slowed $[\text{Ca}^{2+}]_i$ decline may theoretically be due to increased RyR activity (5). It has been found that the effects of NOS1 knockout are, in part, via increased oxidation of RyR leading to increased RyR open probability (14). We suggest that reduced SR Ca^{2+} uptake may also be involved in $\text{NOS1}^{-/-}$ mice and with NOS1 inhibition in wild-type (WT) mice. In addition to changes in SERCA/PLB ratio, SERCA activity can be acutely regulated by PLB, such that PLB inhibits SERCA function. However, phosphorylation of PLB relieves this inhibition. For example, stimulation of the β -AR pathway leads to positive inotropy and lusitropy (6). These effects are mainly via cAMP-dependent protein kinase (PKA) phosphorylation of PLB at serine16, which leads to increased SR Ca^{2+} load, contraction, and an increased rate of relaxation (9). Thus we propose that the slowed $[\text{Ca}^{2+}]_i$ decline and blunted contraction are due to, in part, decreased SR Ca^{2+} uptake. Therefore, the purpose of this study is to investigate the role of PLB in the NOS1 -mediated alterations in Ca^{2+} handling.

MATERIALS AND METHODS

Force-frequency experiment

Right ventricular ultra-thin trabeculae were isolated from NOS1 knockout ($\text{NOS1}^{-/-}$) and corresponding WT (C57BL/6J) (Jackson Laboratories, Bar Harbor, ME) mice as previously described (33). Briefly, the heart was rapidly excised and immediately perfused with Krebs-Henseleit solution containing (in mM) 137 NaCl, 5 KCl, 1.2 NaH_2PO_4 , 20 NaHCO_3 , 10 glucose, and 0.25 CaCl_2 ; 20 mM 2,3-butanedione monoxime was added to minimize cutting damage and to arrest the heart (28). The buffer solution was maintained in equilibrium with 95% O_2 -5% CO_2 . After the blood was flushed out of the hearts, the right ventricle was carefully opened and unbranched ultrathin trabeculae were carefully dissected and mounted onto the experimental setup. Baseline conditions were set at 37°C, 1.5 mM extracellular Ca^{2+} , at a stimulation frequency of 4 Hz. The muscles were allowed to stabilize for ~15–30 min, and then the muscle's length was stretched until developed force was maximal or an increase in developed force was accompanied by a disproportional increase in diastolic force. This length corresponded to an enddiastolic sarcomere length of ~2.2 μm . Data were obtained at stimulation frequencies of 4, 6, 8, 10, 12, and 14 Hz after force had stabilized at each frequency. All the animal protocols and procedures were performed in accordance with National Institutes of Health guidelines and approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.

Isolation of ventricular myocytes

Ventricular myocytes were isolated from NOS1^{-/-}, their corresponding WT, and PLB knockout (PLB^{-/-}) mice, as previously described (36). Briefly, the heart was mounted on a Langendorff apparatus and perfused with modified MEM (Sigma, St. Louis, MO, 37°C, bubbled with 95% O₂-5% CO₂). Blendzyme type IV (0.077 mg/ml) (Roche Applied Science, Indianapolis, IN) was then added to the perfusate. After 7–20 min, the heart was taken down, the ventricles minced, and myocytes dissociated by trituration. Subsequently the myocytes were filtered, centrifuged, and resuspended in MEM containing 200 μM Ca²⁺. Myocytes were used within 6 h after isolation.

Simultaneous measurement of Ca²⁺ transients and myocyte shortening

Myocytes were loaded at 22°C with Fluo-4 AM (10 μM, Molecular Probes, Eugene, OR) for 30 min and washed out, and then an additional 30 min were allowed for intracellular deesterification. The instrumentation used for cell fluorescence measurement was a Cairn Research (Faversham, UK) epifluorescence system. Myocytes were stimulated via platinum electrodes connected to a Grass S48 stimulator at a frequency of 0.5 Hz. [Ca²⁺]_i was measured by Fluo-4 epifluorescence with excitation at 480 ± 20 nm and emission at 535 ± 25 nm. The illumination field was restricted to collect the emission of a single cell. Data were expressed as F/F₀, where F was the fluorescence intensity and F₀ was the intensity at rest. Simultaneous measurement of shortening was also performed by use of an edgedetection system (Crescent Electronics, Sandy, UT). Data were expressed as percentage of resting cell length (%RCL). Measurements were performed at room temperature.

SR Ca²⁺ load

SR Ca²⁺ load was measured by rapid application of 10 mM caffeine for 10 s. The amplitude of the caffeine-induced Ca²⁺ transient was used as an index of SR Ca²⁺ load (34).

Western blot

Western blots were performed on isolated myocytes or whole heart homogenates. Total PLB and phosphorylated PLB were measured by using antibodies to PLB (Zymed, Invitrogen; Carlsbad, CA), phosphorylated serine16 and threonine17 (Badrilla, Leeds, UK). We also measured the expression of NOS1 (BD Biosciences, San Diego, CA) in PLB^{-/-} and WT (CF1) cardiac homogenates.

Solution and drugs

Normal Tyrode solution consists of (in mM) 140 NaCl, 4 KCl, 1 MgCl₂, 1 CaCl₂, 10 glucose, 5 HEPES, 1 L-arginine, pH 7.4 adjusted with HCl. S-methyl-L-thiocitrulline (SMLT, Calbiochem, La Jolla, CA), a specific NOS1 inhibitor; isoproterenol (Iso, 1 μM), a nonselective β-AR agonist; 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ, 25 μM), an inhibitor of NO stimulation of guanylate cyclase; and 5,10,15,20-tetrakis (4-sulfonatophenyl) prophyrinato iron (III) (FeTPPS, 50 μM, Calbiochem), a peroxynitrite decomposition catalyst, were prepared fresh each experimental day. All chemicals were from Sigma except where indicated.

Statistics

Results were expressed as means ± SE. Statistical significance ($P < 0.05$) was determined by ANOVA (followed by Newman-Keuls test) for multiple groups. Paired or unpaired *t*-tests were used for comparison between two groups.

RESULTS

Effect of NOS1 knockout on force-frequency response

We performed force-frequency experiments on trabeculae isolated from WT and NOS1 knockout (NOS1^{-/-}) mouse hearts. Shown in Fig. 1A are representative force traces at various stimulation frequencies in a WT and NOS1^{-/-} trabeculae. Data are summarized in Fig. 1B. WT trabeculae showed the typical positive force-frequency response (4 Hz: 14.0 ± 0.8 mN/mm²; 14 Hz: 15.4 ± 1.6 mN/mm²). However, trabeculae from NOS1^{-/-} hearts displayed a negative force-frequency response (4 Hz: 14.5 ± 0.4 mN/mm²; 14 Hz: 9.1 ± 0.3 mN/mm²). In addition, trabeculae from NOS1^{-/-} hearts exhibited a slower relaxation observed as an increased time to 50% relaxation (RT₅₀). Thus these data show that NOS1^{-/-} trabeculae have a blunted force-frequency response as well as slowed relaxation.

Effects of NOS1 knockout or inhibition on basal cardiac function

Since we observed a decrease in force production and slowed relaxation in trabeculae, we investigated the mechanism in isolated myocytes. Contractile function was tested in WT and NOS1^{-/-} myocytes by simultaneously measuring Ca²⁺ transients and cell shortening. Shown in Fig. 2A are representative examples of cell shortening and Ca²⁺ transient traces in a NOS1^{-/-} and a WT ($\pm$ SMLT) myocyte. Data are summarized in Fig. 2B. In NOS1^{-/-} vs. WT myocytes, we found a significantly decreased basal cardiac function observed as a decrease in cell shortening amplitude (3.6 ± 0.7 vs. $7.0 \pm 1.0\%$ RCL; $P < 0.05$), Ca²⁺ transient amplitude (0.31 ± 0.04 vs. 0.66 ± 0.10 $\Delta F/F_0$; $P < 0.05$), and slower decline in [Ca²⁺]_i measured as RT₅₀ (409 ± 29 vs. 252 ± 17 ms; $P < 0.05$). These results are analogous to our trabeculae data. We also measured basal cardiac function in WT myocytes in the presence of SMLT (300 nM), a specific NOS1 inhibitor (29). Figure 2 shows that, similar to NOS1 knockout, acute NOS1 inhibition with SMLT in WT myocytes significantly decreased shortening amplitude ($3.2 \pm 0.4\%$ RCL; $P < 0.05$ vs. WT), decreased Ca²⁺ transient amplitude (0.44 ± 0.06 $\Delta F/F_0$; $P < 0.05$ vs. WT), as well as slowed the decline in [Ca²⁺]_i (374 ± 23 ms; $P < 0.05$ vs. WT). In addition, SMLT had no effect in NOS1^{-/-} mice (data not shown). Thus these data show that NOS1 knockout or inhibition has negative inotropic and lusitropic effects.

Effect of NOS1 inhibition in PLB^{-/-} mice

Myocyte contraction and the rate of [Ca²⁺]_i decline are determined by many factors, one of which is PLB function. We investigated the effects of NOS1 signaling on PLB by performing functional studies in PLB^{-/-} myocytes in the presence or absence of the specific NOS1 inhibitor SMLT. Shown in Fig. 3A are our summary data. Acute NOS1 inhibition with SMLT in PLB^{-/-} myocytes did not change basal contractility compared with control PLB^{-/-} myocytes (cell shortening amplitude, 10.4 ± 1.2 vs. $12.2 \pm 1.2\%$ RCL, Ca²⁺ transient amplitude, 2.6 ± 0.2 vs. 2.3 ± 0.2 $\Delta F/F_0$, Ca²⁺ transient RT₅₀, 126 ± 7 vs. 113 ± 5 ms). The effects of NOS1 inhibition in WT vs. PLB^{-/-} can be more clearly seen in Fig. 3B. Since there were increased baseline features with PLB knockout, we compared the effect of SMLT on cell shortening amplitude, Ca²⁺ transient amplitude, and Ca²⁺ transient RT₅₀, as the percent of control (without SMLT). The effects of SMLT were only observed in WT myocytes compared with PLB^{-/-} myocytes on cell shortening amplitude (-54 ± 7 vs. $-13 \pm 13\%$ change from -SMLT), Ca²⁺ transient amplitude ($-33\% \pm 13$ vs. $13 \pm 16\%$ change from -SMLT), and [Ca²⁺]_i decline (48 ± 14 vs. $12 \pm 8\%$ change from -SMLT). This lack of effect of SMLT was not due to a change in NOS1 expression, since our Western blot data showed that in PLB^{-/-} hearts, compared with WT, there were no differences in the expression of NOS1 (144 ± 5 vs. 130 ± 5 AU, $n = 4$ hearts). Thus these data suggest that PLB is an important end target of NOS1 signaling.

Effect of NOS1 on PLB phosphorylation

Since our data revealed that PLB is an important end target of NOS1 to regulate myocyte contraction and $[Ca^{2+}]_i$ decline, we investigated the basal phosphorylation status of PLB in isolated myocytes. We examined PLB phosphorylation at the PKA site serine16 (normalized to total PLB) in WT myocytes ($\pm$ SMLT). Figure 4A shows that acute NOS1 inhibition decreased basal serine16 PLB phosphorylation (phosphorylated serine16/total PLB: 0.62 ± 0.04 vs. 0.87 ± 0.11 , $P < 0.05$). We also investigated the phosphorylation status of PLB at the Ca^{2+} /CaM-dependent protein kinase site, threonine17. Figure 4B shows that acute NOS1 inhibition with SMLT did not modify the phosphorylation at Thr17 [phosphorylated Thr17/total PLB: 0.35 ± 0.05 vs. 0.36 ± 0.05 , $P = \text{not significant (NS)}$]. These results suggest that NOS1 mediates PLB function via alterations in its phosphorylation status at serine16. Thus the acute NOS1 inhibition-induced decrease in PLB serine16 phosphorylation should decrease myocyte contraction and slow $[Ca^{2+}]_i$ decline, which we did observe.

Effects of NOS1 on SR Ca^{2+} load

The decrease in PLB serine16 phosphorylation should lead to a decrease in SR Ca^{2+} load. Thus SR Ca^{2+} load was measured by the caffeine-induced Ca^{2+} transient amplitude in NOS1^{-/-} and WT ($\pm$ SMLT) myocytes. Shown in Fig. 5, specific NOS1 inhibition with SMLT in WT myocytes led to a significant decrease in SR Ca^{2+} load vs. WT myocytes (1.7 ± 0.2 vs. $2.8 \pm 0.3 \Delta F/F_0$, $P < 0.05$). NOS1^{-/-} myocytes also had a decreased SR Ca^{2+} load ($2.0 \pm 0.2 \Delta F/F_0$, $P < 0.05$ vs. WT). In addition, SMLT had no effect on NOS1^{-/-} myocyte SR Ca^{2+} load ($1.6 \pm 0.2 \Delta F/F_0$). Our data indicate that NOS1 signaling is able to increase SR Ca^{2+} load. Thus, under our experimental conditions, the negative force-frequency response and the decrease in shortening and Ca^{2+} transient amplitudes with NOS1 knockout and/or inhibition are likely due to the decreased SR Ca^{2+} load.

The majority of the caffeine-induced Ca^{2+} transient decline is due to Ca^{2+} extrusion via Na⁺/Ca²⁺ exchanger (NCX) and can be used as an indirect measure of NCX activity. We observed no change in the caffeine-induced Ca^{2+} transient RT₅₀ between NOS1^{-/-} and WT myocytes ($2,228 \pm 237$ vs. $2,050 \pm 108$ ms, $P = \text{NS}$). These data suggest that NOS1 signaling does not regulate NCX function.

Effect of NOS1 knockout on the response to β -AR stimulation

We next examined whether increasing PLB serine16 phosphorylation via β -adrenergic (β -AR) stimulation can reverse the effects of NOS1 knockout. The effects of β -AR stimulation were tested in NOS1^{-/-} and WT myocytes by measuring Ca^{2+} transients and cell shortening at 1 Hz. After reaching steady state, myocytes were perfused with Iso (β -AR agonist, 1 μ M). Data are summarized in Fig. 6. We found that NOS1^{-/-} myocytes had a significantly decreased β -AR-stimulated Ca^{2+} transient amplitude compared with WT myocytes (0.56 ± 0.05 vs. $1.01 \pm 0.17 \Delta F/F_0$, $P < 0.05$). Cell shortening (data not shown) paralleled the Ca^{2+} transient data. Interestingly, the Iso-stimulated Ca^{2+} transient RT₅₀ in NOS1^{-/-} myocytes was similar to that of WT myocytes (107 ± 4 vs. 110 ± 4 ms, $P = \text{NS}$). These data suggest that NOS1^{-/-} myocytes have a decreased Ca^{2+} transient and cell shortening response to β -AR stimulation, but Iso abolished the slower Ca^{2+} decline in NOS1^{-/-} myocytes.

NOS1 signaling pathway

We investigated the NOS1 signaling pathway (cGMP dependent or independent) by using ODQ (an inhibitor of NO stimulation of guanylate cyclase) (12). ODQ (25 μ M) did not have any effect in WT, NOS1^{-/-}, or WT+SMLT myocytes (Fig. 7A; WT: Ca^{2+} transient amplitude 0.60 ± 0.06 vs. $0.60 \pm 0.07 \Delta F/F_0$; Ca^{2+} transient RT₅₀ 226 ± 12 vs. 226 ± 3 ms; NOS1^{-/-}: Ca^{2+} transient amplitude 0.40 ± 0.06 vs. $0.39 \pm 0.04 \Delta F/F_0$, Ca^{2+} transient RT₅₀ 278 ± 10 vs.

279 ± 11 ms; WT+SMLT: Ca²⁺ transient amplitude 0.42 ± 0.04 vs. 0.44 ± 0.04 ΔF/F₀, Ca²⁺ transient RT₅₀ 284 ± 10 vs. 283 ± 8 ms; all *P* = NS). Cell shortening (data not shown) paralleled the Ca²⁺ transient data. These data suggest that NOS1 signaling regulating myocyte contraction and [Ca²⁺]_i decline is via the cGMP-independent pathway.

We further examined the cGMP-independent pathway by using FeTPPS, a peroxynitrite decomposition catalyst (27). Perfusion of myocytes with FeTPPS (50 μM) resulted in decreased cell shortening amplitude (data not shown), decreased Ca²⁺ transient amplitude, and slowed [Ca²⁺]_i decline in WT myocytes (Fig. 7B; Ca²⁺ transient amplitude, 0.64 ± 0.04 vs. 0.75 ± 0.05 ΔF/F₀, Ca²⁺ transient RT₅₀, 255 ± 11 vs. 230 ± 11 ms, all *P* < 0.05). However, FeTPPS did not have any effect in NOS1^{-/-} or WT+SMLT myocytes (NOS1^{-/-}: Ca²⁺ transient amplitude, 0.36 ± 0.03 vs. 0.37 ± 0.03 ΔF/F₀; Ca²⁺ transient RT₅₀, 280 ± 7 vs. 279 ± 7 ms; WT+SMLT: Ca²⁺ transient amplitude, 0.40 ± 0.06 vs. 0.42 ± 0.06 ΔF/F₀; Ca²⁺ transient RT₅₀, 293 ± 9 vs. 295 ± 9 ms; all *P* = NS). Thus FeTPPS mimicked the effects of NOS1 knockout or inhibition on myocyte contraction and [Ca²⁺]_i decline, which suggests that NOS1 signals, in part, via the formation of peroxynitrite.

We also examined the effects of FeTPPS on PLB serine16 phosphorylation (normalized to total PLB) in WT myocytes (±SMLT). Figure 7C shows that FeTPPS decreased basal serine16 PLB phosphorylation (phosphorylated serine16/total PLB: 0.42 ± 0.10 vs. 0.92 ± 0.20, *P* < 0.05) in WT myocytes. However, in WT myocytes with acute NOS1 inhibition, FeTPPS had no effect on serine16 PLB phosphorylation (phosphorylated serine16/total PLB: 0.55 ± 0.17 vs. 0.78 ± 0.12, *P* = NS). These data indicate that peroxynitrite decomposition by FeTPPS decreases PLB serine16 phosphorylation, which could account for the decreased myocyte contraction and slowed [Ca²⁺]_i decline in WT myocytes with FeTPPS.

DISCUSSION

Our results demonstrate that NOS1 knockout blunts the force-frequency response manifested as decreased force production and slowed relaxation. Similar results were obtained in isolated myocytes. Specifically, NOS1 knockout or inhibition decreased shortening and Ca²⁺ transient amplitudes and slowed the decline of [Ca²⁺]_i. This was associated with a reduced SR Ca²⁺ load. However, in PLB^{-/-} mice, there was no effect of NOS1 inhibition. Acute NOS1 inhibition also decreased PLB serine16 phosphorylation. Peroxynitrite decomposition by FeTPPS in WT myocytes mimicked the functional effects of NOS1 knockout or inhibition (i.e., decreased cell shortening and Ca²⁺ transient amplitudes and slowed the decline of [Ca²⁺]_i). FeTPPS also decreased PLB serine16 phosphorylation. Therefore, these findings demonstrate that NOS1 signals, in part, by the formation of peroxynitrite, which modulates PLB phosphorylation leading to changes in inotropy and lusitropy.

Functional effects of NOS1 knockout or inhibition

Previous studies have found conflicting results on cardiac myocyte function with NOS1 knockout or inhibition. It has been reported that specific NOS1 knockout or inhibition leads to an increase in myocyte basal contractility and the response to β-AR stimulation (1,26,30, 38). However, other studies have shown that NOS1 knockout or inhibition decreases the response to β-AR stimulation and decreases myocyte contraction evidenced as a blunted force-frequency response (2,14,20). These discrepancies may arise due to the use of unloaded myocytes and/or variations in frequency range.

Hence, we performed experiments on trabeculae isolated from WT and NOS1^{-/-} mice. Using trabeculae, we were able to study NOS1 in experiments in which we can impose a load on the muscle and use stimulation frequencies that encompass the physiological range of the mouse and perform the protocol at body temperature (37°C). Our data indicate that trabecula from

NOS1^{-/-} have a blunted force-frequency response (Fig. 1) compared with WT trabeculae, which performed normally compared with previous studies (15,18,33). Even though previous studies have found opposite effects of NOS1 knockout or inhibition on myocyte contraction, these studies have consistently found a slowed rate of [Ca²⁺]_i decline or myocyte relengthening (1,20,26,38). Consistent with these findings, our NOS1^{-/-} trabeculae also exhibited a slowed decline in force (Fig. 2). Interestingly, it has been found that increasing developed force generally results in a slowed relaxation (17). Thus with the increased force production in WT trabeculae we should a priori have observed a slower RT₅₀ (vs. NOS1^{-/-}), which was not the case. Thus, considering this force-dependent factor, our data suggest that the isolated effect of NOS1 knockout alters relaxation to an even greater extent than quantified in our experiments. However, few studies have investigated the mechanism(s) responsible for the decreased inotropy and lusitropy in NOS1 knockout mice.

We used isolated myocytes from WT and NOS1^{-/-} hearts to investigate the mechanisms of the NOS1-induced effects on cardiac function. Consistent with our trabeculae data, we found decreased basal contraction (shortening and Ca²⁺ transient amplitudes) and slowed [Ca²⁺]_i decline (Fig. 2).

We also observed similar functional effects in our WT myocytes with acute inhibition of NOS1 with SMLT as with NOS1^{-/-} myocytes (Fig. 2). Under our experimental conditions SMLT was a specific inhibitor of NOS1, since we did not observe an effect of SMLT on NOS1^{-/-} myocytes. Also, we did not observe any upregulation of NOS3 expression in NOS1^{-/-} myocytes (data not shown), which confirms previously published results (1,2,21). Thus we confirm here that NOS1 signaling regulates myocyte contraction and [Ca²⁺]_i decline.

Effects of NOS1 inhibition in PLB^{-/-} myocytes

Since PLB is an important regulator of cardiac contractility (25), we examined the role of PLB in NOS1 signaling. Specific inhibition of NOS1 with SMLT had no effect on PLB^{-/-} myocyte shortening amplitude, Ca²⁺ transient amplitude, or Ca²⁺ transient RT₅₀ (Fig. 3A). Figure 3B shows that NOS1 inhibition only had an effect in WT myocytes compared with PLB^{-/-} myocytes. A previous study also found that acute NOS1 inhibition in PLB^{-/-} myocytes did not affect [Ca²⁺]_i decline, but no data on the effects on shortening and Ca²⁺ transient amplitudes were reported (38). We have extended this initial observation by showing that there is also no effect of acute NOS1 inhibition on shortening and Ca²⁺ transient amplitudes. Thus our data suggest that PLB is an end target of NOS1 signaling leading to increased myocyte contraction and faster [Ca²⁺]_i decline. Previous studies have shown a decrease in PLB expression in NOS1^{-/-} mice (20,30,38), and we suggest that the decrease in PLB expression may be compensatory to try to increase myocyte contraction. Accordingly, the change in SERCA/PLB ratio should have increased the Ca²⁺ affinity and SR Ca²⁺ transport, leading to accelerated relaxation and increased contraction in NOS1^{-/-} mice, but neither was observed in previous studies or in our present results.

NOS1 and PLB phosphorylation

Since PLB is an end target of NOS1 signaling, we examined the effect of NOS1 inhibition on PLB phosphorylation levels. Our data showed that acute NOS1 inhibition in WT myocytes reduced basal serine16 phosphorylation (Fig. 4). We believe that these changes in PLB phosphorylation can translate into significant effects on the [Ca²⁺]_i decline and relengthening. In mice, SERCA is responsible for ~92% of the [Ca²⁺]_i decline (5). PLB inhibits SERCA activity, and when phosphorylated, this inhibition is relieved. Thus any changes to PLB phosphorylation will have effects on the decline of the Ca²⁺ transient. In addition, the NCX is responsible for ~7% of the Ca²⁺ transient decline (5). The decline of the caffeine-induced

Ca²⁺ transient data, an indirect measure of NCX, indicated that NOS1 knockout does not cause a change in NCX activity.

NOS1 effects on SR Ca²⁺ load

Decreased PLB serine16 phosphorylation, which decreases SR Ca²⁺ uptake, should result in decreased SR Ca²⁺ load. A major determinant of cardiac contractility is the SR Ca²⁺ load (3, 16,31). Our data showed a reduced SR Ca²⁺ load in NOS1^{-/-} myocytes and WT myocytes with NOS1 inhibition (Fig. 5), which is consistent with the slowed [Ca²⁺]_i decline and decreased PLB serine16 phosphorylation. Consistent with our observed decrease in SR Ca²⁺ load, a previous study found that NOS1^{-/-} cardiac homogenates had decreased SR Ca²⁺ uptake compared with WT cardiac homogenates (39). Thus, under our experimental conditions, the blunted force-frequency response in trabeculae as well as decreased myocyte shortening and Ca²⁺ transient amplitudes can be explained by a reduced SR Ca²⁺ load.

NOS1 effects on the response to β -AR stimulation

Since we observed a decrease in PLB phosphorylation at the PKA site serine16, we investigated whether increasing PLB serine16 phosphorylation could reverse the effects of NOS1 knockout. Thus NOS1^{-/-} and WT myocytes were perfused with the nonspecific β -AR agonist Iso. Our data show that NOS1^{-/-} myocytes have a blunted response to β -AR stimulation. This was observed as a decrease in shortening (data not shown) and Ca²⁺ transient amplitudes (Fig. 6). Interestingly, however, with β -AR stimulation the Ca²⁺ transient RT₅₀ were similar in NOS1^{-/-} and WT myocytes. It has been previously shown that PLB serine16 phosphorylation and [Ca²⁺]_i decline were similar in β -AR-stimulated NOS1^{-/-} and WT myocytes (38). This study also showed that there was no difference in β -AR-stimulated myocyte shortening between NOS1^{-/-} and WT myocytes. Our results were somewhat different in that although our [Ca²⁺]_i decline was similar between the two groups, the NOS1^{-/-} myocytes still had a blunted response (shortening and Ca²⁺ transient amplitudes) to β -AR stimulation. These data suggest that there is another target of NOS1 signaling. It has been reported that the SR Ca²⁺ release channel (RyR) is altered in NOS1^{-/-} myocytes (14), which could contribute to our observed blunted β -AR response. We may not have observed the effects of NOS1 signaling on RyR in the PLB^{-/-} myocytes because of their supercontractile phenotype and compensatory adaptations (i.e., reduced RyR levels) (8), which may have obscured the effects of NOS1 signaling on RyR.

NOS1 signaling pathway

NO can signal via two general pathways: cGMP dependent or independent (40). We first determined which general pathway NOS1 signals through by using ODQ, an inhibitor of NO stimulation of guanylate cyclase. ODQ did not have an effect on shortening amplitude, Ca²⁺ transient amplitude, or [Ca²⁺]_i decline in WT myocytes, NOS1^{-/-} myocytes, and WT myocytes with acute NOS1 inhibition (Fig. 7). These data suggest that NOS1 signals via the cGMP-independent pathway, consistent with a previous study (38). NOS1 coimmunoprecipitates with xanthine oxidoreductase (XOR) (19). XOR is a major source of superoxide radicals, which can react with NO to form peroxynitrite. We further tested the NOS1 signaling pathway by using FeTPPS, a peroxynitrite decomposition catalyst. Our data showed that FeTPPS in WT myocytes resulted in decreased shortening and Ca²⁺ transient amplitudes and slowed decline of [Ca²⁺]_i (Fig. 7). However, FeTPPS had no effect in NOS1^{-/-} myocytes or WT myocytes with acute NOS1 inhibition (Fig. 7). Furthermore, perfusion with FeTPPS in WT myocytes decreased PLB serine16 phosphorylation. The effects of FeTPPS mimicked the effects of NOS1 knockout or inhibition. Thus our data suggest that NOS1 signals through the cGMP-independent pathway via, in part, the formation of peroxynitrite. These data are consistent with our previous study that showed that low levels of exogenous peroxynitrite (produced from

SIN-1) increased basal Ca^{2+} transient and shortening amplitude in murine myocytes. Interestingly, similar to NOS1 inhibition, the contractile effects of SIN-1 were absent in $\text{PLB}^{-/-}$ myocytes (22). However, it still remains to be determined whether NO itself or other reactive nitrogen species are also part of the NOS1 signaling pathway.

A previous study (38) also found NOS1 knockout or inhibition decreased PLB serine16 phosphorylation similar to our study. These authors concluded that the reduced PLB phosphorylation was responsible for the slowed $[\text{Ca}^{2+}]_i$ decline. Upon further investigation they demonstrated that there was increased protein phosphatase 1 (PP1) and 2a (PP2a) activity in $\text{NOS1}^{-/-}$ myocytes, which was responsible for the decreased PLB phosphorylation and slowed relaxation. The redox environment in NOS1 knockout (or with acute inhibition) is conducive to phosphatase activation. Specifically, our study demonstrated that NOS1 signals, in part, via peroxynitrite formation; it has been shown that low levels of peroxynitrite can inhibit phosphatase activity (10). Also, superoxide, which is increased in $\text{NOS1}^{-/-}$ myocytes (19,21), increases phosphatase activity (32). Zhang et al. (38) observed increased myocyte shortening and Ca^{2+} transient amplitudes. Their previous study showed that this was due to an increase in SR Ca^{2+} load resulting from enhanced L-type Ca^{2+} current (30). We may not have observed this positive inotropic effect of NOS1 knockout in our trabeculae and isolated myocyte studies owing to different experimental conditions. Yet, consistent with our data, other studies have shown that increased PP1 or PP2A activity, which decreased PLB serine16 phosphorylation, leads to decreased myocyte shortening, Ca^{2+} transient amplitude, and prolonged relaxation (7,13). In addition to the enhanced phosphatase activity, PLB can have decreased phosphorylation because of reduced PKA activity. Zhang et al. observed that PKA inhibition slowed myocyte relaxation in WT myocytes but had no effect in $\text{NOS1}^{-/-}$ myocytes, suggesting decreased PKA activity in $\text{NOS1}^{-/-}$ myocytes. It has been demonstrated within cardiac myocytes that *S*-nitroso-*N*-acetyl-DL-penicillamine (nitrosylating agent) is able to stimulate adenylate cyclase, increase cAMP production, and activate PKA, resulting in positive inotropic effect (35). It is known that PKA is a redox-sensitive protein (11). Thus NOS1 signaling may also directly activate PKA. Alterations in any of these pathways will change PLB phosphorylation levels. Further studies are needed to identify other NOS1-mediated pathways that would modify PLB phosphorylation.

In summary, our results demonstrate that NOS1 knockout or inhibition blunted the force-frequency response, decreased shortening and Ca^{2+} transient amplitudes, and prolonged relaxation and $[\text{Ca}^{2+}]_i$ decline. This was associated with a decrease in PLB serine16 phosphorylation and a reduced SR Ca^{2+} load. FeTPPS mimicked the functional effects of NOS1 knockout or inhibition. In addition, acute NOS1 inhibition had no effect in $\text{PLB}^{-/-}$ myocytes. Thus NOS1 signaling, in part, via peroxynitrite targets PLB phosphorylation regulating the inotropic and lusitropic state of the myocyte.

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Fig. 1.

Neuronal nitric oxide synthase (NOS1) knockout blunts the force-frequency response in trabeculae. *A*: representative force traces at different stimulation frequencies in a wild-type (WT; *left*) and NOS1 knockout (NOS1^{-/-}; *right*) trabeculae. Numbers (4–14) correspond to stimulation frequency (Hz). *B*: summary data (means $\pm$ SE) of force (*left*) and time to 50% relaxation (RT₅₀; *right*). **P* < 0.05 vs. WT at similar stimulation frequency; *n* = 3 trabeculae/3 hearts for WT, *n* = 4 trabeculae/4 hearts for NOS1^{-/-}.

Fig. 2.

NOS1 knockout or inhibition decreases basal shortening and Ca²⁺ transient amplitudes and slows intracellular Ca²⁺ ([Ca²⁺]_i) decline. *A*: individual, steady-state cell shortening (*top*) and Ca²⁺ transient (*bottom*) traces measured in WT (solid line), WT + *S*-methyl-L-thiocitrulline (SMLT) (dashed line), and NOS1 knockout (shaded line) myocytes. *F*, fluorescence intensity; *F*₀, fluorescence intensity at rest. *B*: summary data (means ± SE) of the effects of NOS1 knockout (NOS1^{-/-}) or specific inhibition with SMLT (300 nM) in wild-type (WT + SMLT) myocytes on shortening (*top*) and Ca²⁺ transient (*middle*) amplitudes, and [Ca²⁺]_i decline measured as time to 50% relaxation (RT₅₀) (*bottom*). **P* < 0.05 vs. WT; *n* = 22 cells/8 hearts for WT, *n* = 21 cells/6 hearts for WT + SMLT, *n* = 18 cells/7 hearts for NOS1^{-/-}.

Fig. 3.

No effect of NOS1 inhibition in phospholamban (PLB) knockout myocytes. *A*: summary data (means $\pm$ SE) of the effects of NOS1 inhibition (SMLT, 300 nM) on cell shortening (*left*) and Ca²⁺ transient (*middle*) amplitudes, and [Ca²⁺]_i decline measured as RT₅₀ (*right*). RCL, resting cell length. *B*: summary data (means $\pm$ SE), expressed as a % change from myocytes without SMLT, of the effects of NOS1 inhibition (SMLT) on WT (solid bars) and PLB knockout (PLB^{-/-}, shaded bars) myocytes on cell shortening (*left*) and Ca²⁺ transient (*middle*) amplitudes, and Ca²⁺ transient RT₅₀ (*right*); *n* = 26 cells/4 hearts for PLB^{-/-}, *n* = 27 cells/4 hearts for PLB^{-/-} + SMLT.

Fig. 4.

NOS1 inhibition decreases PLB phosphorylation. *A*: summary data (means $\pm$ SE) for PLB serine16 (PLB_{Ser16}) phosphorylation normalized to total PLB in isolated WT myocytes $\pm$ SMLT. *Inset* is a representative Western blot, serine16 phosphorylation (*top*) and total PLB (*bottom*). *B*: summary data (means $\pm$ SE) for PLB threonine17 phosphorylation normalized to total PLB in isolated WT myocytes $\pm$ SMLT. *Inset* is a representative Western blot, threonine17 phosphorylation (*top*) and total PLB (*bottom*). * $P < 0.05$ vs. -SMLT, $n = 8$ hearts.

Fig. 5. NOS1 knockout or inhibition decreases sarcoplasmic reticulum (SR) Ca²⁺ load. Summary data (means ± SE) of the effects of NOS1 knockout or specific inhibition with SMLT (300 nM) in wild-type (WT + SMLT) myocytes on SR Ca²⁺ load. **P* < 0.05 vs. all other groups; *n* = 32 cells/10 hearts for WT, *n* = 46 cells/8 hearts for WT + SMLT, *n* = 34 cells/8 hearts for NOS1^{-/-}, *n* = 29 cells/6 hearts for NOS1^{-/-} + SMLT.

Fig. 6.

Effects of NOS1 knockout on the response to β -adrenergic (β -AR) stimulation. *Left*: summary data (means $\pm$ SE) of Ca^{2+} transient amplitude before and after β -AR stimulation [isoproterenol (Iso)] in WT (open bars) and NOS1^{-/-} (solid bars) myocytes. *Right*: summary data (means $\pm$ SE) of $[\text{Ca}^{2+}]_i$ decline measured as RT₅₀ before and after β -AR stimulation (Iso) in WT (open bars) and NOS1^{-/-} (solid bars) myocytes. * $P < 0.05$ vs. WT control (CONT), ** $P < 0.05$ vs. WT Iso; n 23 cells/11 hearts for WT, n 28 cells/13 hearts for NOS1^{-/-}.

Fig. 7.

NOS1 signals, in part, via peroxynitrite. **A:** summary data (means $\pm$ SE) of the effects of 1H-[1,2,4] oxadiazolo [4,3-a] quinoxalin-1-one (ODQ) on myocyte Ca²⁺ transient amplitude (*left*) and RT₅₀ (*right*) in WT, NOS1^{-/-}, and WT + SMLT myocytes. **B:** summary data (means $\pm$ SE) of the effects of 5,10,15,20-tetrakis (4-sulfonatophenyl) prophyrimato iron (III) (FeTPPS) on myocyte Ca²⁺ transient amplitude (*left*) and RT₅₀ (*right*) in WT, NOS1^{-/-}, and WT + SMLT myocytes. **C:** summary data (means $\pm$ SE) for PLB serine16 phosphorylation normalized to total PLB in isolated WT myocytes $\pm$ FeTPPS. *Inset* is a representative Western blot, serine16 phosphorylation (*top*) and total PLB (*bottom*). * P < 0.05 vs. WT CONT (paired t -test); ** P < 0.05 vs. WT CONT (unpaired t -test). ODQ experiments: n = 13 cells/3 hearts for WT, n = 11

cells/3 hearts for NOS1^{-/-}, $n = 15$ cells/3 hearts for WT + SMLT. FeTPPS experiments: $n = 23$ cells/6 hearts for WT, $n = 29$ cells/5 hearts for NOS1^{-/-}, $n = 15$ cells/6 hearts for WT+SMLT. PLB phosphorylation experiments: $n = 8$ hearts.