S-Nitrosylation of Calcium-Handling Proteins in Cardiac Adrenergic Signaling and Hypertrophy


Rationale: The regulation of calcium (Ca\(^{2+}\)) homeostasis by β-adrenergic receptor (βAR) activation provides the essential underpinnings of sympathetic regulation of myocardial function, as well as a basis for understanding molecular events that result in hypertrophic signaling and heart failure. Sympathetic stimulation of the βAR not only induces protein phosphorylation but also activates nitric oxide–dependent signaling, which modulates cardiac contractility. Nonetheless, the role of nitric oxide in βAR-dependent regulation of Ca\(^{2+}\) handling has not yet been explicated fully.

Objective: To elucidate the role of protein S-nitrosylation, a major transducer of nitric oxide bioactivity, on βAR-dependent alterations in cardiomyocyte Ca\(^{2+}\) handling and hypertrophy.

Methods and Results: Using transgenic mice to titrate the levels of protein S-nitrosylation, we uncovered major roles for protein S-nitrosylation, in general, and for phospholamban and cardiac troponin C S-nitrosylation, in particular, in βAR-dependent regulation of Ca\(^{2+}\) homeostasis. Notably, S-nitrosylation of phospholamban consequent upon βAR stimulation is necessary for the inhibitory pentamerization of phospholamban, which activates sarcoplasmic reticulum Ca\(^{2+}\)-ATPase and increases cytosolic Ca\(^{2+}\) transients. Coincident S-nitrosylation of cardiac troponin C decreases myocardial sensitivity to Ca\(^{2+}\). During chronic adrenergic stimulation, global reductions in cellular S-nitrosylation mitigate hypertrophic signaling resulting from Ca\(^{2+}\) overload.

Conclusions: S-Nitrosylation operates in concert with phosphorylation to regulate many cardiac Ca\(^{2+}\)-handling proteins, including phospholamban and cardiac troponin C, thereby playing an essential and previously unrecognized role in cardiac Ca\(^{2+}\) homeostasis. Manipulation of the S-nitrosylation level may prove therapeutic in heart failure. (Circ Res. 2015;117:793-803. DOI: 10.1161/CIRCRESAHA.115.307157.)

Key Words: beta adrenergic ■ calcium ■ heart failure ■ myocardial contraction ■ nitric oxide ■ receptors
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>Ad</td>
<td>adenooviral</td>
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<tr>
<td>βAR</td>
<td>β-adrenergic receptor</td>
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<td>cTnC</td>
<td>cardiac Troponin C</td>
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<tr>
<td>GSNO</td>
<td>S-nitrosoglutathione</td>
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<td>GSNOR</td>
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<td>GSNOR-Tg</td>
<td>cardiomyocyte-specific GSNOR overexpression</td>
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<td>ISO</td>
<td>isoproterenol</td>
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kinase II that also phosphorylates additional Ca\(^{2+}\)-handling proteins.\(^2\) Phosphorylation of cTnC and PLN promotes relaxation of cardiomyocytes by accelerating Ca\(^{2+}\) dissociation from cTnC and Ca\(^{2+}\) reuptake into the SR by SERCA2a, respectively.\(^3\) Combined, activated βAR induces positive inotropic and lusitropic effects. In contrast, chronic βAR activation and sustained increase of intracellular Ca\(^{2+}\) induces left ventricular (LV) hypertrophy and dysfunction,\(^4\) which ultimately leads to the development of heart failure.

βAR stimulation activates nitric oxide (NO) synthases 1 (NOS1) and NOS3 to produce NO, which regulates cardiac function.\(^5,6\) For example, NOS1-derived NO S-nitrosylates and activates the ryanodine receptor, resulting in cytosolic Ca\(^{2+}\) release and enhanced catecholamine-stimulated contractility.\(^7\) On the other hand, NOS3-derived NO increases LV compliance and attenuates βAR-induced inotropy,\(^8\) presumably by activation of soluble guanylate cyclase and subsequent production of cyclic guanosine monophosphate. The existence of multiple downstream signaling mechanisms and different sources of NO has lead to conflicting data regarding the specific contribution of NO and its effectors to the regulation of myocardial function.\(^8\)

Accumulating evidence suggests that protein S-nitrosylation (SNO) is a major effector of NO signaling. By analogy to the role of phosphatases, which regulate the levels of protein phosphorylation, amounts of NO are set by enzymes that metabolize S-nitrosothiols (denitrosylases). Reduction of S-nitrosothiols via S-nitrosoglutathione (GSNO) by S-nitrosoglutathione reductase (GSNOR) controls the intracellular levels of protein SNO because GSNO and protein S-nitrosothiols are in equilibrium.\(^9\) Recent studies suggest that signaling through the βAR is regulated by GSNOR-dependent denitrosylation.\(^10\) Nonetheless, the impact of protein SNO on cardiomyocyte function and its regulation by adrenergic signaling is incompletely understood. Given the central role of Ca\(^{2+}\) in cardiomyocyte function, we hypothesized that GSNOR regulates cardiomyocyte function via controlling SNO levels of Ca\(^{2+}\)-handling proteins after βAR stimulation. Here, we report that βAR stimulation induces SNO of Ca\(^{2+}\)-handling proteins in cardiomyocytes. Our results suggest that, in addition to phosphorylation, SNO of key Ca\(^{2+}\)-handling proteins is required for the proper transduction of βAR signaling in cardiomyocytes. Detrimental effects of Ca\(^{2+}\) mishandling induced by chronic hemodynamic stress may be mitigated by enhancing protein denitrosylation.

Methods

Animal Studies

The generation of mice deficient in GSNOR (GSNOR\(^{-/-}\)) and PLN (PLN\(^{-/-}\)), as well as mice with cardiomyocyte-specific GSNOR overexpression (GSNOR-Tg), has been described elsewhere.\(^11,12\) All protocols and experimental procedures were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and performed in accordance with the guidelines of the National Institutes of Health for the use of animals in research.

Isolated Cardiomyocytes and Langendorff-Perfused Hearts

Isolation of ventricular cardiomyocytes of adult mice and high-speed imaging of Δ[Ca\(^{2+}\)] and sarcomere length (SL) shortening (ie, twitch contraction) was performed as previously described with minor modifications.\(^14,15\) Myocardial Ca\(^{2+}\) responsivness was examined in intact cardiomyocytes, as estimated by the ratio of percent SL shortening/Δ[Ca\(^{2+}\)], as well as in permeabilized cardiomyocytes.\(^15\) The LTCC current (I\(_{\text{LTCC}}\)) was measured in isolated cardiomyocytes using the whole-cell voltage-clamp technique, as previously described.\(^16\) SR Ca\(^{2+}\) load, SR Ca\(^{2+}\) leak, and sodium–calcium exchanger (NCX) activity were measured as previously described.\(^15\) All experiments in isolated cardiomyocytes were conducted at 37°C, unless otherwise specified. LV contractility was measured in isolated perfused mouse hearts as described previously.\(^17\)

In Vivo Chronic βAR Stimulation

ni-Isoproterenol (ISO) was administered to mice for 14 days using surgically implanted mini-osmotic pumps (Alzet-20; Durect Corporation, CA). Based on pilot studies, we used a high dose of ISO (60 mg/kg/d) to examine the impact of GSNOR overexpression and a low dose of ISO (30 mg/kg/d) to examine the effects of GSNOR deficiency. After removal of the pumps, mice were observed for an additional 14 days. In vivo cardiac function was evaluated by transthoracic echocardiography, as described previously.\(^17\) At the end of the study, hearts were harvested for histological examination and analysis of protein expression.

Analysis of Protein SNO

A modified biotin switch assay was performed as previously described with minor modifications. Detailed experimental protocols of organic mercury resin–assisted capture of S-nitrosylated proteins and peptides have been published elsewhere.\(^18\) After purification, specific nitrosylated proteins were detected using standard immunoblot techniques.

Adenovirus Specifying Mutant PLN and cTnC

Adenoviral (Ad) constructs were generated encoding wild-type (WT) PLN (C36/C41/C46), single cysteine-to-alanine PLN mutants (C36A, C41A, C46A, respectively), triple PLN mutant (C36A/C41A/C46A), WT cTnC (C35/C84), single cysteine-to-serine cTnC mutants (C35S, C84S), and double cTnC mutant (C35S/C84S) protein. Ad.PLN solution was injected in vivo into the myocardium at the LV apex, and Ad.cTnC was used to infect cultured adult mouse cardiomyocytes.

Statistics

All data are presented as mean±SEM. For isolated cardiomyocyte experiments, measurements of all cells from 1 mouse were averaged, and the average value for each mouse was used for statistical analysis.
enhanced denitrosylation alters the intracellular calcium (Ca\textsuperscript{2+}) response to β-adrenergic receptor (βAR) stimulation. A. Left, Representative continuous recordings of sarcomere length and Ca\textsuperscript{2+} transients (Δ[Ca\textsuperscript{2+}]) in isolated cardiomyocytes before and during infusion of isoproterenol (ISO) at 10 nmol/L. Right, Representative traces of sarcomere length and Δ[Ca\textsuperscript{2+}] after different doses of ISO in wild-type (WT) cardiomyocytes and cardiomyocytes overexpressing GSNOR (TG) paced at 2 Hz. B. Dose-dependent changes of sarcomere shortening and Δ[Ca\textsuperscript{2+}] in response to ISO in isolated WT and TG cardiomyocytes. n=4 mice (13–18 cells per mouse) per group. C. Calcineurin activity in isolated WT and TG hearts treated with or without ISO (10 μmol/L). n=5 mice per group. D. Sarcomere shortening and Δ[Ca\textsuperscript{2+}] in response to ISO at 10 nmol/L with or without carboxy-PTIO (C-PTIO, 300 μmol/L) in isolated WT cardiomyocytes, n=4 mice (11–15 cells per mouse) per group. **P<0.01 and ###P<0.001 (vs baseline); *P<0.05, **P<0.01, and ***P<0.001. Carboxy-PTIO indicates 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxide potassium salt; and GSNOR, S-nitrosoglutathione reductase.

Results

GSNOR Modulates Ca\textsuperscript{2+} Handling in Cardiomyocytes

After stimulation with the βAR agonist ISO, twitch contraction was increased to the same extent in WT and GSNOR-Tg cardiomyocytes (Figure 1A and 1B), but attenuated in GSNOR−/− cardiomyocytes at higher doses of ISO (Online Figure 1A and 1B). In WT cardiomyocytes, the ISO-induced increases in contraction correlated with increases in Δ[Ca\textsuperscript{2+}] (Figure 1B), whereas the attenuated contraction in GSNOR−/− cardiomyocytes was associated with an impaired increase of Δ[Ca\textsuperscript{2+}] to ISO stimulation. In contrast, GSNOR-Tg cardiomyocytes showed no change in Δ[Ca\textsuperscript{2+}], despite a sustained contractile response to ISO stimulation (Figure 1B). In isolated perfused hearts, ISO enhanced LV inotropy in both GSNOR-Tg and WT hearts (Online Figure 1C). However, calcineurin, a Ca\textsuperscript{2+}-dependent enzyme, was activated only in WT hearts, suggesting suppressed Δ[Ca\textsuperscript{2+}] in GSNOR-Tg hearts during βAR stimulation (Figure 1C). The NO scavenger carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxide potassium salt) also prevented the ISO-induced increase in Δ[Ca\textsuperscript{2+}] in WT cardiomyocytes without affecting inotropy (Figure 1D). These observations suggest that SNO mediates both an augmentation of Δ[Ca\textsuperscript{2+}] and a decrease in myocardial sensitivity to Ca\textsuperscript{2+} during βAR stimulation.

Enhanced Denitrosylation Prevents LV Hypertrophy and Dysfunction During Chronic βAR Stimulation

Increased Δ[Ca\textsuperscript{2+}] activates calcineurin, leading to the translocation of nuclear factor of activated T-cells and the activation of downstream hypertrophic signaling. Because GSNOR-Tg inhibited ISO-induced increases in both Δ[Ca\textsuperscript{2+}] and calcineurin activity (Figure 1B and 1C), we surmised that denitrosylation may mitigate LV remodeling in response to adrenergic stimulation. Infusion of ISO (60 mg/kg/d) in WT mice for 2 weeks resulted in marked LV and cardiomyocyte hypertrophy and myocardial fibrosis, associated with the nuclear translocation of nuclear factor of activated T-cells-c3 and the associated LV remodeling and dysfunction. These observations suggest that S-nitrosothiol homeostasis plays an important role in limiting pathological hypertrophy by titrating Δ[Ca\textsuperscript{2+}].

GSNOR Overexpression or NOS Inhibition Prevents SNO of Ca\textsuperscript{2+}-Handling Proteins After βAR Stimulation

We used both the modified biotin switch assay and phenyl mercury resin–assisted capture method to identify Ca\textsuperscript{2+}-handling proteins that undergo SNO after βAR stimulation in lysates of isolated WT and GSNOR-Tg cardiomyocytes incubated with ISO (10 nmol/L). Stimulation by ISO increased the SNO of PLN, NCX, and cTnC, but not ryanodine receptor, SERCA2a, LTCC, cTnI, tropomyosin, or actin, in WT cardiomyocytes, as detected by modified biotin switch (Figure 3; Online Figure 3). GSNOR-Tg or inhibition of NO synthesis with L-NG-nitroarginine-methylrestore blocked ISO-induced SNO of PLN, NCX, and cTnC (Figure 3). In contrast, we found that all Ca\textsuperscript{2+}-handling proteins probed were S-nitrosylated in ISO-treated WT, but not in GSNOR-Tg, cardiomyocytes, as determined by mercury resin–assisted capture (Online Figure 4). Thus, βAR stimulation induces GSNOR-coupled, reversible SNO of multiple Ca\textsuperscript{2+}-handling proteins, including PLN, NCX, and cTnC.
Phosphorylation of PLN Is Insufficient for βAR-Induced PLN Pentamerization

The amount of Ca\(^{2+}\) released into the cytosol is predominantly determined by the function of LTCC and SR. Because GSNOR-Tg did not affect LTCC current with or without ISO (Online Figure V), we tested the effect of GSNOR on SR function. βAR stimulation increased SR Ca\(^{2+}\) load, SR Ca\(^{2+}\) leak, and fractional Ca\(^{2+}\) release in a dose-dependent manner in WT, but not in GSNOR-Tg, cardiomyocytes (Figure 4A). These results suggest that ISO failed to activate SERCA2a in GSNOR-Tg myocytes. The monomeric form of PLN inhibits SERCA2a, whereas the pentamic form is functionally inactive.\(^2\) Under basal conditions, the PLN pentamer/monomer ratio was similar in WT and GSNOR-Tg hearts (Figure 4B). In WT cardiomyocytes, ISO markedly increased the PLN pentamer/monomer ratio, whereas in GSNOR-Tg myocytes or in WT cardiomyocytes treated with carboxy-PTIO, ISO failed to alter the PLN pentamer/monomer ratio (Figure 4B). Of note, the levels of ISO-induced PLN phosphorylation at Ser\(^{16}\) and Thr\(^{17}\) did not differ between WT and GSNOR-Tg cardiomyocytes (Figure 4C), indicating that protein kinase A and Ca\(^{2+}\)/calmodulin-dependent protein kinase II–mediated phosphorylation of PLN is not altered by GSNOR-Tg and that it is not sufficient to cause pentamerization without concomitant SNO.

SNO of PLN at Cys\(^{36}\) and Cys\(^{41}\) Is Required for SERCA2a Activation

PLN has 3 cysteine residues (Cys\(^{36}\), Cys\(^{41}\), and Cys\(^{46}\)), which are located in the hydrophobic C-terminal transmembrane region (Online Figure VIA–VIC) and likely modulate oligomerization of PLN.\(^2\) To elucidate the role of βAR-induced SNO of PLN on Ca\(^{2+}\) handling, we constructed recombinant adenovirus vectors encoding mutant PLN in which cysteines are replaced by alanines (Online Figure VIF and VIE). In human embryonic kidney 293 cells expressing Ad.PLN, mutation of all cysteines to alanines (C36A/C41A/C46A) abolished SNO of PLN by exogenous S-nitrosocysteine, a cell-permeable S-nitrosothiol (Online Figure VIF). Moreover, in human umbilical vein endothelial cells (with intact βAR) expressing Ad.PLN, ISO nitosylated WT PLN, but not mutant PLN\(^{C36A/C41A/C46A}\) (Online Figure VIG). We examined the effects of expressing SNO-resistant PLN mutants on Ca\(^{2+}\) handling in PLN\(^{−/−}\) cardiomyocytes. As reported previously, ISO failed to augment ∆[Ca\(^{2+}\)] and SL shortening or to accelerate sarcomere relaxation and ∆[Ca\(^{2+}\)] decay in PLN\(^{−/−}\) cardiomyocytes transfected with empty adenovirus encoding the red fluorescent protein marker alone (Figure 5).\(^2\) Ad-mediated expression of WT PLN or PLN\(^{C36A}\), but not PLN\(^{C36A}\), PLN\(^{C41A}\), or PLN\(^{C36A/C41A/C46A}\), restored the ability of ISO to enhance ∆[Ca\(^{2+}\)] and contraction in PLN\(^{−/−}\) cardiomyocytes.
Denitrosylation Activates NCX

Although ISO-induced SERCA2a activation was impaired in GSNOR-Tg cardiomyocytes, GSNOR-Tg did not affect the rates of \( \Delta \left[ \text{Ca}^{2+} \right] \) decay and SL relengthening at baseline or after \( \beta \text{AR} \) stimulation (Online Figure VIIA and VIIIB). This suggests that an alternative mechanism of \( \text{Ca}^{2+} \) removal compensates for the lack of SERCA2a activity in GSNOR-Tg cardiomyocytes treated with ISO. We found that ISO dose-dependently increased NCX activity in GSNOR-Tg, but not in WT cardiomyocytes (Online Figure VIIIC and VIID). Inhibition of NCX with SEA0400 (2-[4-[(2,5-difluorophenyl) methoxy]phenoxy]-5-ethoxyaniline) slowed \( \Delta \left[ \text{Ca}^{2+} \right] \) decay more markedly in GSNOR-Tg than in WT cardiomyocytes (Online Figure VIIID and VIIIE). Together with the lack of ISO-induced SNO of NCX in GSNOR-Tg cardiomyocytes (Figure 3C), these results suggest that \( \beta \text{AR} \)-induced upregulation of NCX activity (presumably because of protein kinase A–induced phosphorylation) is counteracted by SNO, resulting in minimal changes in NCX activity in WT hearts. The increased NCX activity in GSNOR-Tg hearts may therefore be an underlying mechanism contributing to the accelerated \( \Delta \left[ \text{Ca}^{2+} \right] \) decay during \( \beta \text{AR} \) stimulation, at least partially taking over the \( \text{Ca}^{2+} \) removal function normally performed by SERCA2a.

Protein SNO Is Required for the Ability of \( \beta \text{AR} \) Stimulation to Reduce Myocardial \( \text{Ca}^{2+} \) Responsiveness

Increments in extracellular \([\text{Ca}^{2+}]\) concentration-dependently increased contraction in GSNOR-Tg and WT cardiomyocytes, whereas GSNOR-Tg markedly attenuated the ability of extracellular \([\text{Ca}^{2+}]\) to increase \( \Delta \left[ \text{Ca}^{2+} \right] \), suggesting an increased myocardial \( \text{Ca}^{2+} \) responsiveness in GSNOR-Tg (Online Figure VIIIA and VIIIB). To assess the myofilament \( \text{Ca}^{2+} \) sensitivity, we measured \( \text{Ca}^{2+} \)-induced changes in SL in unloaded permeabilized cardiomyocytes. At baseline, \( \text{Ca}^{2+} \) sensitivity was similar between WT and GSNOR-Tg cardiomyocytes (Figure 6A). Incubation with ISO decreased myofilament \( \text{Ca}^{2+} \) sensitivity in WT cardiomyocytes, whereas ISO increased \( \text{Ca}^{2+} \) sensitivity in GSNOR-Tg cardiomyocytes (Figure 6A). Of note, phosphorylation of cTnI at Ser22/23, an important regulator of \( \text{Ca}^{2+} \) sensitivity, was increased to a similar extent by ISO in WT and GSNOR-Tg hearts (Figure 6B). These results suggest that protein SNO is required for the ability of ISO to reduce myofilament \( \text{Ca}^{2+} \) sensitivity.

Enhanced Denitrosylation Augments the Temperature-Dependent Increase of Myocardial \( \text{Ca}^{2+} \) Responsiveness

To define the mechanisms responsible for the increased myocardial \( \text{Ca}^{2+} \) sensitivity in ISO-treated GSNOR-Tg cardiomyocytes, we examined the impact of temperature on myocardial \( \text{Ca}^{2+} \) responsiveness with or without ISO, as assessed by the ratio of percent SL shortening/\( \Delta \left[ \text{Ca}^{2+} \right] \), during twitch contraction in intact cardiomyocytes. Myocardial \( \text{Ca}^{2+} \) responsiveness was similar at 37°C and 25°C in WT cardiomyocytes with or without ISO, although there was a trend toward lower \( \text{Ca}^{2+} \) responsiveness with ISO at both temperatures (Table). Increase of temperature from 25°C to 37°C markedly increased \( \text{Ca}^{2+} \) responsiveness in GSNOR-Tg cardiomyocytes (Table). ISO increased \( \text{Ca}^{2+} \) responsiveness at 37°C, but not at 25°C, in GSNOR-Tg cardiomyocytes. Because increase in \( \text{Ca}^{2+} \) responsiveness with temperature is largely determined by the temperature dependence of cross-bridge cycling rates, these results suggest that enhanced denitrosylation increases myocardial \( \text{Ca}^{2+} \) responsiveness during \( \beta \text{AR} \) stimulation by accelerating cross-bridge cycling at physiological temperature.
SNO of cTnC at Cys84 Is Required for the βAR-Induced Reduction of Myocardial Ca2+ Sensitivity

To examine the impact of SNO of cTnC on myocardial Ca2+ responsiveness, we constructed recombinant Ad vectors encoding mutant cTnC, in which cysteines (Cys35 and Cys84) are replaced with serines (C35S/C84S) to abolish SNO of cTnC by exogenous S-nitrosocysteine (Online Figure VIIA). In human embryonic kidney 293 cells expressing cTnC, mutation of both cysteines to serines (C35S/C84S) abolished SNO of cTnC at Cys84 Is Required for the reduction of myocardial Ca2+ sensitivity as the basis of the effects of C84S mutation (cTnC C84S; Figure 6E). Notably, mutation of cTnC did not alter the responses to ISO in GSNOR-Tg cardiomyocytes, which show impaired SNO (Figure 6D). Expression of mutant cTnC did not affect sarcomere relaxation or Δ[Ca2+] decay in either genotype (Online Figure IVA and IVB). These results suggest that βAR-induced SNO of cTnC-Cys84 is required for the reduction of myocardial Ca2+ sensitivity in normal hearts stimulated with ISO.

Discussion

The current observations uncover the existence of previously unrecognized signaling events dependent on SNO of key Ca2+-handling proteins during βAR stimulation. Although multiple Ca2+-handling SNO proteins, including PLN, have been previously reported,24 our findings reveal a far more important role for SNO in transduction of adrenergic activation and hypertrophic signaling than previously thought. Phosphorylation and SNO in fact work in concert to control Ca2+ homeostasis (Figure 7).

As is the case for phosphorylation, SNO is pervasive. Therefore, many proteins are potential targets for both SNO and phosphorylation.25 Using 2 distinct enrichment techniques
The central role of PLN in myocardial Ca\(^{2+}\) handling has been established by studies using PLN\(^{-/-}\) mice.\(^{26}\) Previous studies using a nonphosphorylatable mutant of PLN showed that phosphorylation of PLN at Ser\(^{16}\) is necessary for mediating the maximal myocardial response to βAR stimulation.\(^{3,22}\) However, the SNO status of PLN SNO was not addressed in that study. The current results show that βAR stimulation induces both phosphorylation and SNO of PLN. Moreover, when SNO of PLN was prevented by GSNOR-Tg or NO scavenging, βAR stimulation failed to induce PLN pentamerization and SERCA2a activation, despite intact phosphorylation of PLN. These results indicate that phosphorylation is necessary but not sufficient for PLN pentamerization and activation of SERCA2a during βAR stimulation. It has been postulated that cysteine residues in the transmembrane domain of PLN play a role in pentamer formation and stability.\(^{27}\) Whether SNO-PLN leads to disulfide bond formation between PLN monomers remains to be determined.\(^{28}\)

βAR stimulation exerts inotropic effects as a net consequence of increased Δ[Ca\(^{2+}\)], reduced thin filament Ca\(^{2+}\) sensitivity, and enhanced cross-bridge cycling (Figure 7). Although the relative contribution of these 3 components to βAR-induced inotropy is still debated, mathematical modeling suggests that enhanced cross-bridge cycling plays a central role in the βAR-induced twitch responses.\(^{29}\) Without it, the Δ[Ca\(^{2+}\)] is only able to offset the intrinsic negative inotropic effect of ISO-induced reduction in thin-filament Ca\(^{2+}\) sensitivity. In the current study, we observed that GSNOR-Tg abolished the ability of ISO to increase Δ[Ca\(^{2+}\)] without impairing inotropic twitch responses. Similarly, cTnCC\(^{84S}\) mutation further augmented ISO-induced inotropy without augmenting Δ[Ca\(^{2+}\)]. These observations suggest that GSNOR-Tg and cTnCC\(^{84S}\) either prevented the reduction of thin filament Ca\(^{2+}\) sensitivity or augmented the cross-bridge cycling in response to ISO.

βAR signaling is transduced through the cTnI N-terminus (residues 1–30), which is thought to be bound to the N-domain of cTnC when unphosphorylated and to dissociate on phosphorylation at Ser\(^{22/23}\).\(^{30}\) The dissociation of cTnN N-terminus decreases the affinity of the cTnI switch region (residues 147–163) to the N-domain of cTnC when unphosphorylated and to dissociate on phosphorylation at Ser\(^{22/23}\).\(^{30}\) The dissociation of cTnN N-terminus decreases the affinity of the cTnI switch region (residues 147–163) to the N-domain of cTnC, thus decreasing the Ca\(^{2+}\) sensitivity of thin filaments.\(^{31}\) The crystal structure of cTnC shows that Cys\(^{84}\) is located in the hydrophobic core of the D-helix of the N-domain that interacts with the switch region of cTnI (Online Figure IXC).\(^{31,32}\) It has been reported that Cys\(^{84}\) contributes to the formation of intramolecular (within cTnC) or intermolecular (between cTnC and cTnI) disulfide bonds, which may affect the cTnC–cTnI interaction.\(^{33}\) These data support the idea that βAR-induced nitrosylation of cTnC at Cys\(^{84}\) may regulate cTnC–cTnI interactions, thereby influencing myofilament Ca\(^{2+}\) sensitivity. Indeed, a missense mutation of cTnC (cTnC\(^{50S}\)) has been found to be associated with hypertrophic cardiomyopathy and to increase the Ca\(^{2+}\) sensitivity of reconstituted skinned fibers, indicating an important role of Cys\(^{84}\) in modulating Ca\(^{2+}\) sensitivity.\(^{34}\)
Alternatively, SNO may alter myocardial Ca\textsuperscript{2+} sensitivity by modulating cross-bridge cycling kinetics. It is known that physiological temperature (eg, 37°C) increases myocardial Ca\textsuperscript{2+} responsiveness primarily because of increased cross-bridge cycling rates compared with lower temperatures (eg, 15–25°C). We observed that the temperature-dependent increase of Ca\textsuperscript{2+} responsiveness was markedly exaggerated in ISO-treated GSNOR-Tg cardiomyocytes. These results suggest that ISO-induced twitch inotropy in GSNOR-Tg cardiomyocytes is at least partly mediated by enhanced cross-bridge cycling. This hypothesis is consistent with a previous report that NO reduces myocardial Ca\textsuperscript{2+} sensitivity by slowing cross-bridge cycling rates. The temperature dependency of the effects of SNO–myofilament proteins also explains why the effects of SNO to reduce myofilament Ca\textsuperscript{2+} sensitivity were not observed in most previous studies where myofilament Ca\textsuperscript{2+} sensitivity was typically measured only at room temperatures (eg, 15°C). In addition to its well-documented role in myocardial contractile function, Ca\textsuperscript{2+} plays an important role in programming myocardial hypertrophy, including the calcineurin–nuclear factor of activated T-cells signaling. Because GSNOR-Tg attenuated ISO-induced increases in \(\Delta[Ca^{2+}]\), hypertrophic signaling, and LV dysfunction, whereas GSNOR\textsuperscript{−/−} worsened LV remodeling after chronic ISO infusion, we suggest that protein denitrosylation protects against pathological LV remodeling and heart failure in response to

### Table. Percent SL Shortening (%ΔL/L\textsubscript{0}), \(\Delta[Ca^{2+}]\), and Ratio of %ΔL/L/\(\Delta[Ca^{2+}]\) (Ratio) in WT and GSNOR-Tg (TG) Cardiomyocytes at Baseline (Base) and After ISO Stimulation at 25°C or 37°C

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\(n=14\) to 18 cells. \(\Delta[Ca^{2+}]\) indicates calcium transients; GSNOR-Tg, cardiomyocyte-specific GSNOR overexpression; ISO, isoproterenol; SL, sarcomere length; and WT, wild-type.* \(P<0.0001\) (versus baseline).† \(P<0.001\) (versus 25°C).‡ \(P<0.05\) (versus 25°C).§ \(P<0.01\) (versus baseline).
chronic βAR activation. Collectively, these results suggest that cellular levels of S-nitrosothiols may determine the fate of cardiomyocytes under chronic adrenergic stimulation by modulating Ca²⁺ handling.

Protein SNO may exert harmful or beneficial effects depending on the cellular context. GSNOR −/− mice were shown to have increased tissue damage and mortality after sepsis, whereas GSNOR-Tg prevented sepsis-induced LV dysfunction in mice. Consistent with our current results, βAR-induced hyper-SNO of ryanodine receptor was shown to increase SR Ca²⁺ leak and depress the myocardial response to βAR stimulation in GSNOR −/− mice. On the other hand, GSNOR deficiency prevented oxidative stress–induced increase in SR Ca²⁺ leak and reduced myocardial injury after permanent left coronary artery ligation. Although the current study showed that enhanced denitrosylation has protective effects against LV remodeling in response to chronic βAR stimulation, the effects of GSNOR on the development of LV dysfunction remain to be determined in more clinically relevant models of heart failure.

It has been reported that NOS1 deficiency depresses SERCA2a function at baseline and blunts positive inotropic response to βAR stimulation. Because NOS1 is localized to the SR, PLN SNO is likely impaired in NOS1 −/− cardiomyocytes, in line with our results in GSNOR-Tg mice in which ISO failed to activate SERCA2a and nitrosylate PLN. Nonetheless, the phenotype of GSNOR-Tg cardiomyocytes is unlikely to be the same as that of NOS1 −/− or NOS1/NOS3 double knockout mice. GSNOR only denitrosylates a subset of the nitrosoproteome, whereas SNO depletion may be more complete in NOS1/NOS3 double knockout cardiomyocytes. In fact, we observed no difference in the SNO levels of Ca²⁺-handling proteins at baseline between WT and GSNOR-Tg cardiomyocytes. In addition, deficiency of NOS abolishes both cyclic guanosine monophosphate–dependent and –independent effects (ie, SNO) of NO, whereas GSNOR-Tg attenuates protein SNO and leaves cyclic guanosine monophosphate–dependent effects intact. These differences may explain the discrepancies in Ca²⁺-handling parameters obtained in our current studies in GSNOR-Tg mice and previous studies using NOS-deficient mice.

Calcium is the primary determinant of cardiac force production. Alterations in phosphorylation levels of Ca²⁺-handling proteins downstream of the βAR not only control myocardial contraction and relaxation but also represent molecular signatures of failing hearts. Our observations reveal previously unrecognized roles for SNO of Ca²⁺-handling proteins, particularly PLN and cTnC, in transducing adrenergic and hypertrophic responses of the heart. S-Nitrosylation may thus operate in parallel with protein phosphorylation to exert widespread control over cardiac function, and both may go awry in disease. Denitrosylation-based approaches may represent a new therapeutic strategy to improve cardiac function in settings of increased nitrosative stress, including heart failure, myocardial ischemia, and sepsis.

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Disclosures

None.

References


**What Is Known?**

- Regulation of calcium (Ca$^{2+}$) homeostasis by β-adrenergic receptor (βAR) activation is a fundamental mechanism of sympathetic regulation of myocardial function, as well as a basis for understanding molecular events that result in hypertrophic signaling and heart failure.
- Sympathetic activation of βAR not only induces protein phosphorylation but also stimulates nitric oxide production.
- Nitric oxide–dependent protein S-nitrosylation modulates myocardial function.

**What New Information Does This Article Contribute?**

- S-nitrosylation provides an essential parallel pathway that acts in concert with phosphorylation to regulate cardiac Ca$^{2+}$ homeostasis.
- βAR stimulation–induced S-nitrosylation of phospholamban is necessary for its pentamerization, which increases Ca$^{2+}$ transients, whereas S-nitrosylation of cardiac troponin C (cTnC) is required to decrease myocardial Ca$^{2+}$ sensitivity.
- Enhanced denitrosylation prevents pathological left ventricular hypertrophy induced by chronic βAR activation by limiting Ca$^{2+}$ overload.

Sympathetic activation critically regulates myocardial function via βAR-dependent signaling. Activation of βAR modulates Ca$^{2+}$ handling of cardiac myocytes typically via protein phosphorylation. Although nitric oxide modulates cardiac function by S-nitrosylating proteins, how S-nitrosylation affects βAR signaling was incompletely understood. Using transgenic mice to titrate the levels of protein S-nitrosylation, we uncovered unanticipated roles for protein S-nitrosylation, in general, and of phospholamban and cTnC, in particular, in the βAR-dependent regulation of Ca$^{2+}$ homeostasis. Notably, S-nitrosylation of phospholamban consequent upon βAR activation is necessary to augment Ca$^{2+}$ release from sarcoplasmic reticulum during contraction. In parallel, S-nitrosylation of cTnC is required to decrease myocardial Ca$^{2+}$ sensitivity in response to βAR stimulation. Global inhibition of cellular S-nitrosylation prevents hypertrophic signaling in response to chronic adrenergic stimulation, whereas selective inhibition of cTnC S-nitrosylation recapitulates the impact of naturally occurring mutation (cTnCC84Y). Thus, S-nitrosylation provides a previously unrecognized parallel pathway, at least in part through phospholamban and cTnC, which acts in concert with phosphorylation to regulate cardiac Ca$^{2+}$ homeostasis. Inhibition of S-nitrosylation of specific protein targets may prove to be a therapeutic option for the treatment of heart failure.
S-Nitrosylation of Calcium-Handling Proteins in Cardiac Adrenergic Signaling and Hypertrophy


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SUPPLEMENTAL MATERIAL

S-nitrosylation of calcium-handling proteins in cardiac adrenergic signaling and hypertrophy

Irie, et al.

DETAILED METHODS

Mice and drugs
All protocols and experimental procedures were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and performed in accordance with the guidelines of the National Institutes of Health for the use of animals in research. The generation of PLN deficient (PLN−/−) mice, mice with cardiomyocyte-specific GSNOR overexpression (GSNOR-Tg) and GSNOR deficient (GSNOR−/−) mice has been described elsewhere.1-3 PLN−/− and GSNOR−/− mice were backcrossed on to FVB/N and C57BL6 background, respectively, for more than 10 generations. Wild-type (WT) FVB/N and C57BL6 mice were used as WT controls for PLN−/− and GSNOR−/− mice, respectively. Wild-type littermate mice were used as controls for GSNOR-Tg. Male WT, GSNOR-Tg, and GSNOR−/− mice (2- to 4-months-old) were age-and weight-matched throughout the study. Unless otherwise noted, all chemicals were obtained from Sigma-Aldrich (Saint-Louis, MI).

Cardiomyocyte isolation
Isolation of adult mouse ventricular cardiomyocytes was carried out as previously described with minor modifications.4,5 Briefly, the animals were heparinized (8,000 U/kg, i.p.) and deeply anesthetized with an overdose of pentobarbital (300 mg/kg, i.p.). The heart was rapidly excised and the aorta was cannulated. The heart was retrogradely perfused with calcium-free cell isolation buffer (CIB) containing (in mmol/L) 0.4 EGTA, 130 NaCl, 5.4 KCl, 0.5 MgCl2, 0.33 NaH2PO4, 22 glucose, 25 HEPES-NaOH (pH 7.4), and 50 µU/mL bovine insulin for 3 min at 37°C. The perfusate was then switched to the enzyme solution, which consisted of CIB supplemented with 0.3 mmol/L CaCl2, 1.2 mg/mL each of collagenase B and D (Roche), 0.08 mg/mL trypsin and 0.08 mg/mL protease. The ventricular tissue was then minced and filtered. The cell suspension was rinsed several times, with stepwise increases in the Ca2+ concentration and finally resuspended in Tyrode solution, containing (in mmol/L) 140 NaCl, 5.4 KCl, 1.8 CaCl2, 0.5 MgCl2, 0.33 NaH2PO4, 11 glucose and 5 HEPES-NaOH (pH 7.4).

Measurement of contractility and calcium concentration in isolated cardiomyocytes
Cardiomyocytes were loaded with the Ca2+-sensitive fluorescent dye Fura-2/acetoxymethylester (Invitrogen, Carlsbad, CA) at a concentration of 1.5 µmol/L in Tyrode buffer for 20 min at 25 °C. Cardiomyocytes were superfused with Tyrode buffer at 37 °C and paced at 2 Hz by a MyoPacer Field Stimulator (IonOptix, Milton, MA), and sarcomere length and Fura-2 fluorescence were recorded simultaneously using a dual-excitation fluorescence photomultiplier system (Hyperswitch Light Source; IonOptix). The ratio of photon live count detected by the excitation at 340 nm compared with 380 nm represents the Fura-2 fluorescence ratio, which is a measure of the intracellular Ca2+ concentration. Sarcomere length and Δ[Ca2+] data were analyzed with IonWizard software. To assess the acute effects of βAR stimulation, continuous recording was performed. After 30 sec recording of sarcomere shortening and Δ[Ca2+] baseline, Tyrode buffer was rapidly switched to Tyrode buffer containing DL-isoproterenol (ISO) at 10 nmol/L and measurements were continued until a steady-state was reached. For the dose-response measurements, isolated cardiomyocytes were pretreated with different concentrations of ISO for 10 min before measurement of
contractility and $\Delta[Ca^{2+}]$. To assess the effect of scavenging NO, isolated cardiomyocytes were incubated with 300 µmol/L carboxy-PTIO for 10 min.

**Measurements of LV contractility with isolated perfused mouse hearts**
LV contractility was measured in isolated perfused mouse hearts, as described previously. Briefly, after pentobarbital anesthesia (50 mg/kg i.p.), the heart was excised and transferred to ice-cold perfusion buffer (a modified Krebs-Henseleit buffer, containing, in mmol/L: NaCl 118.5; NaHCO$_3$ 25; glucose 11; KCl 4; MgSO$_4$ 1.2; KH$_2$PO$_4$ 1.2; pyruvate 1; CaCl$_2$ 1.8; gassed and equilibrated with 95% O$_2$ and 5% CO$_2$ at 37°C). The aorta was cannulated and the heart was mounted on the Langendorff apparatus and retrogradely perfused at an initial pressure of 70 mmHg. Coronary flow rate was measured using an N1 in-line flow probe and a T106 flow meter (Transonic Systems, Ithaca, NY) and maintained at a constant rate (2.0–3.5 ml/min). After stabilization, increasing concentrations of ISO (10, 100, 1000 nmol/L) were infused. The LV diastolic pressure was initially set at 5–10 mmHg using a fluid-filled balloon inserted into the LV, which also contained the tip of a Millar SPR-671 pressure transducer (ADInstruments, Colorado Springs, CO). LV pressure was constantly measured, and maximum and minimum rate of LV pressure change (dP/dt$_{max}$ and dP/dt$_{min}$, respectively) were calculated from the LV pressure signal using a Powerlab 8/30 data acquisition system and Chart Pro software (ADInstruments, Colorado Springs, CO).

**Calcineurin activity assay**
After perfusion with ISO (1000 nmol/L) during the Langendorff experiments, heart samples were collected. Baseline hearts were perfused with perfusion buffer only. Calcineurin activity was measured by using Calcineurin Cellular Activity Assay Kit (BML-AK816, Enzo life science, Farmingdale, NY) following the manufacturer’s instructions.

**Chronic isoproterenol infusion**
ISO (60 mg/kg/day for the comparison between WT and GSNOR-Tg or 30 mg/kg/day for the comparison between WT and GSNOR$^{1-}$) was administered to mice for 14 days using mini-osmotic pumps (Alzet-20; Durect Corporation, CA). The pumps were implanted in the interscapular region under 2-3% inhaled isoflurane anesthesia. At the end of the treatment period, pumps were surgically removed. After each surgical procedure, cefazolin (100mg/kg i.p.) was administered. Bupivacaine (0.25%, 2 mg/kg infiltration around incision) and Buprenorphine (0.1 mg/kg s.c.) were administered 30 min before the procedure and 12 and 24 hours after to minimize discomfort.

**Echocardiography**
In vivo cardiac function was evaluated before and 7, 14, and 28 days after implantation of mini-osmotic pumps by transthoracic echocardiography, as described previously. Mice were lightly anesthetized with ketamine (20 mg/kg), and images were collected using a 13.0-MHz linear probe (Vivid 7; GE Medical System, Milwaukee, WI). Interventricular septum thicknesses in diastole (IVSTd) and M-mode images were obtained from a parasternal short-axis view at the midventricular level with a clear view of the papillary muscle. Left ventricular (LV) internal diameters at end-diastole (LVIDd) and end-systole (LVIDs) and LV posterior wall thickness in diastole (LVPWTd) were measured. The fractional shortening, ejection fraction, heart rate and LV mass were calculated on an EchoPAC workstation (GE Healthcare, Wauwatosa, WI). LV mass was calculated according to the Troy equation:

\[
\text{LV mass (mg) } = 1.05([\text{LVIDd} + \text{LVPWTd} + \text{IVSTd}]^3 - [\text{LVIDd}]^3) \text{(mm)}
\]

**Histology**
Mice were weighed, and hearts were dissected 28 days after ISO pump implantation. The hearts were perfused first with sterile cold PBS, and weighed. Hearts were fixed in 4% paraformaldehyde in PBS for 24 hours. After fixation, hearts were embedded in paraffin. Sections (4 µm) were prepared at 200 µm intervals. Hematoxylin and eosin staining (HE staining) and Masson’s Trichrome staining (MT staining) were performed by standard methods. Cardiomyocyte hypertrophy was assessed by measuring the cross-sectional area of on average 207±12 cardiomyocytes per HE stained section. Cardiac fibrosis was determined by calculating the percentage of MT stained area of interstitial fibrosis per total area of cardiac tissue in 11 randomly selected fields. Histological observations were performed with a fluorescence microscope (Nikon ECLIPSE-TE2000-S). Histological images were analyzed using NIH ImageJ software (version 1.43). Measurements and analysis were performed by an investigator blinded to the identity of the samples.

**Protein immunodetection**

Hearts dissected 28 days after osmotic mini-pump implantation were homogenized and separated in nuclear and cytoplasmic fractions using a Nuclear Extraction Kit (Active Motif; Carlsbad, CA) according to the manufacturer's protocol. Protein extracts from isolated cardiomyocytes with or without pretreatment with ISO (10 nmol/L) were prepared using lysis buffer containing (in mmol/L): 50 Tris-HCl (pH 7.4), 150 NaCl, 1 EDTA, 0.1 PMSF, 1% NP-40, 0.1 % SDS, 1:100 phosphatase inhibitor cocktail, and 1:100 protease inhibitor cocktail. Samples were diluted to 0.6 µg/µl with HEN buffer containing (in mmol/L) 250 Hepes, 1 EDTA, and 0.2 neocuproine, and free thiol residues were then blocked by adding 100 mmol/L N-ethylmaleimide and incubated for 30 min at 50°C with frequent vortexing. Next, the protein S-nitrosocysteine were reduced with L-ascorbate (30 mmol/L) and labeled with N-[6-(biotinamido) hexyl]-3′-(2′-pyridyldithio) propionamide (0.2 mmol/L). Biotinylated proteins were then purified using streptavidin-agarose beads. As a positive control, isolated cardiomyocytes were incubated with CysNO (100 µmol/L) for 15 min before homogenization. For negative control samples, L-ascorbate was omitted during the biotin labeling step. Specific proteins were detected in the streptavidin-purified mixture using standard immunoblot techniques as described above.

**Modified biotin switch assay to detect protein S-nitrosylation**

Biotin switch assay was performed as previously described with minor modifications. Briefly, isolated cardiomyocytes were homogenized in lysis buffer containing (in mmol/L) 25 HEPES (pH 7.4), 50 NaCl, 1 EDTA, 0.2 neocuproine, 1 PMSF, 1 % (v/v) NP-40 and 1:100 protease inhibitor cocktail. Samples were diluted to 0.6 µg/µl with HEN buffer containing (in mmol/L) 250 HEPES (pH 7.4), 1 EDTA and 0.2 neocuproine, and free thiol residues were then blocked by adding 100 mmol/L N-ethylmaleimide and incubated for 30 min at 50°C with frequent vortexing. MMTS-blocked protein suspensions were loaded onto chromatographic columns containing activated phenylmercury resin and left to react for 60 min at 25°C. Nonspecific interactions were
eliminated with extensive washes and bound proteins were eluted with 50 mM b-mercaptoethanol. Bound fractions were processed for western blot based identification of the proteins of interest using specific antibodies.

**Measurement of L type Ca\(^{2+}\) current**

The L-Type Ca\(^{2+}\) channel (LTCC) current \((I_{\text{Ca,L}})\) was measured in isolated cardiomyocytes using the whole-cell voltage-clamp technique, as previously described.\(^{11}\) Depolarizations of 500 ms from -80 mV to various membrane potentials were applied every 4 seconds and were preceded by a 100-ms prepulse to -40 mV to inactivate the Na\(^+\) current. The external solution was K\(^+\)-free (to eliminate inward K\(^+\) currents) and contained (in mmol/L) 140 NaCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 5 HEPES and 10 glucose. For selective measurement of \(I_{\text{Ca,L}}\), the pipette solution contained (in mmol/L) 110 CsCl, 5 MgATP, 10 HEPES, 0.4 MgCl\(_2\), 5 glucose, 20 tetraethylammonium (TEA), and 5 BAPTA, pH 7.2. Cs\(^+\) and TEA inhibited outward K\(^+\) currents, and BAPTA was used to buffer Ca\(^{2+}\). Cells were superfused with the physiological solution at 37 °C.

**Measurement of SR Ca\(^{2+}\) load, SR Ca\(^{2+}\) leak and sodium-calcium exchanger activity**

SR calcium load \(((\text{Ca}^{2+})_{\text{SRT}})\) and SR calcium leak \((\text{SR}[\text{Ca}^{2+}]_{\text{leak}})\) measurements were performed as previously described \(^{12}\) with minor modifications. Briefly, SR was loaded by pacing at 2 Hz in normal Tyrode buffer for 30 seconds, followed by a rapid switch to 0 Na\(^+\), 0 Ca\(^{2+}\) Tyrode buffer (containing 140 mmol/L Li\(^+\) and 10 mmol/L EGTA instead of Na\(^+\) and Ca\(^{2+}\)) with or without 1 mmol/L tetracaine. After at least 30 seconds, \([\text{Ca}^{2+}]_{\text{SRT}}\) was measured as the amount of Ca\(^{2+}\) released after addition of 10 mmol/L caffeine. Fractional SR calcium release was calculated as the ratio of the twitch calcium transient to the caffeine-stimulated calcium transient.\(^{13}\) NCX activity was assessed by first superfusing myocytes with Tyrode buffer and measuring the intracellular Ca\(^{2+}\) transient after addition of 10 mmol/L caffeine, and subsequently repeating this measurement during superfusion with the 0 Na\(^+\), 0 Ca\(^{2+}\) Tyrode buffer to inhibit NCX activity. The difference in the decay rate of the caffeine-induced calcium transient between the two traces was taken to represent NCX activity.\(^{14}\) All experiments were performed at 37 °C.

**Measurement of PLN monomer and pentamer**

After excision of the heart from pentobarbital (50 mg/kg i.p.) anesthetized mice, the aorta was cannulated immediately and hearts were perfused retrogradely for 10 min, maintaining a perfusion pressure of 60-80 mmHg with a modified Krebs-Henseleit buffer with or without ISO (10 nmol/L) as described above. After 10 min perfusion, the heart was frozen immediately in liquid nitrogen. Heart samples were homogenized in lysis buffer containing (in mmol/L) 10 Tris (pH 7.5), 315 Sucrose, 1 EDTA, 1 PMSF and 1:100 protease inhibitor cocktail. Protein concentrations were quantified by BCA assay (Pierce Biotechnology). Concentrated (4X) SDS-sample buffer containing (in mmol/L) 8% (w/v) SDS, 600 NaCl, 40% (w/v) glycerol, 0.1% bromophenol blue was added and samples were either kept on ice or boiled for 5 minutes. DTT (62 mmol/L) was added to a portion of the samples that were boiled to reduce any disulfide bonds. PLN monomers and pentamers were then detected using standard immunoblot techniques as described above.

**Generation of mutant PLN adenovirus**

We used the GeneStrings gene synthesis service (Invitrogen, Carlsbad, CA) to generate DNA encoding wild-type and mutant PLN. We generated DNAs encoding single cysteine-to-alanine mutations at positions 36, 41, and 46 in the PLN protein (C36A, C41A, C46A, respectively), as well as a triple mutant (C36A/C41A/C46A) and a wild-type control (C36/C41/C46). These DNAs were then cloned into the pAdTrace-Tox vector, which was a kind gift of Dr. He
(University of Chicago Medical Center). The vector contains a CMV-driven monomeric red fluorescent protein (mRFP1) marker, a CMV-flanked insertion site for the gene of interest, and two regions of sequence homology to a vector carrying the adenoviral backbone.

The plasmids encoding wild-type and mutant PLN were used to transform the AdEasier-1 bacterial strain (Addgene, Cambridge, MA), which contains a plasmid encoding the E1/E3-deficient adenoviral backbone with homology to the pAdTrace-TOX shuttle vector. The AdEasier-1 cells are based on the recBC sbc BJ5183 strain, which allows for homologous recombination of the shuttle vector with the adenoviral backbone, leading to generation of the final adenoviral DNA construct containing the gene of interest. After confirmation of correct recombination, the adenoviral vector was used to transform recombination-defective TOP10 bacteria (Invitrogen) to avoid further unwanted recombination during plasmid amplification. The adenoviral vector was linearized and transfected into the AD-293 cell line (Agilent Technologies, Santa Clara, CA) using Lipofectamine 2000 (Invitrogen). Successful transfection was confirmed by verifying mRFP1 expression of AD-293 cells using fluorescence microscopy. Adenovirus was harvested from these cells and further amplified to reach a titer of \(~10^{11}-10^{12}\) pfu/ml using procedures described by Luo et al.15

**In vivo gene transfer with adenovirus encoding wild-type or mutant PLN**

Mice were anesthetized with 5 % isoflurane in 100 % oxygen, intubated, ventilated mechanically (Mini–Vent; Harvard Apparatus, Holliston, MA, USA) and maintained with 2.0 % isoflurane in the right lateral decubitus position. Cefazolin (40 mg/kg) was administered intraperitoneally before the surgical procedure. Core body temperature was maintained at 37 °C throughout the procedure. A transverse 10-mm incision of the skin was made under the axilla and the chest was opened at the level of 4th intercostal space. A subsequent incision was made into the pericardium to reveal the left ventricular wall. Virus solution was injected into the myocardium at the left ventricular apex in a total volume of 40 to 50 µL. After hemostasis was achieved, the chest and skin were closed with 5-0 monofilament suture, mice were extubated, and returned to their cages. Bupivacaine (0.25%, 2mg/kg) was infiltrated around the incisional wound 2h after surgery before mice were allowed to wake up, and buprenorphine (0.1 mg/kg, i.p.) was administered before and 12h after the procedure to minimize discomfort.

**Measurement of Ca\(^{2+}\) responsiveness in intact cardiomyocytes**

Changes in the extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{ex}\))–shortening relation were examined in intact cardiomyocytes as previously described. Baseline variables were collected from individual myocytes for 1.5 min. Concentration–response curve measurements were subsequently performed during superfusion with Tyrode buffer containing 0.5, 1, 2 and 4 mmol/L CaCl\(_2\). Data were then acquired for 1.5 min after establishment of a new steady state.

**Measurement of myofilament Ca\(^{2+}\) sensitivity in permeabilized cardiomyocytes**

After isolated cardiomyocytes were pretreated with 10 nmol/L DL-isoproterenol (ISO) for 15 min, WT and GSNOR-Tg cardiomyocytes were dissociated and permeabilized according to the method described previously. Briefly, isolated cardiomyocytes were skinned in relaxing solution containing (in mmol/L) 10 EGTA, 5.9 MgAc, 5.9 Na\(_2\)ATP, 10 creatine phosphate, 40 imidazole, 70 potassium propionate, 5 NaN\(_3\), 0.5 PMSF, and 0.004 leupeptin; pH 7.0 with 1% Triton X-100. Cardiomyocytes were perfused using an eight-channel perfusion system that allowed quick switching and exclusive perfusion of a single cardiomyocyte with 8 different pCa (=–log [Ca\(^{2+}\)]) solutions. Sarcomere length was measured in each pCa solution using the IonOptix system. Cells were excluded from the study if sarcomere length in relaxing solution was <1.80 µm. Data were summarized from 10-12 cardiomyocytes from 4 mice in each group.
Generation of cTnC recombinant adenovirus

A cDNA encoding mouse cardiac troponin C (cTnC) was obtained from Thermo Scientific (clone ID: MMM1013-202842467). PCR primers were designed to introduce cysteine-to-serine mutations, accompanied by silent mutations to introduce new restriction sites for identification of mutant clones. DNAs encoding cTnC with single mutations of Cys35 (C35S) and Cys84 (C84S) residues, as well as a double mutant of both residues (C35S/C84S) were generated. A PCR-based mutation strategy using 5'-phosphorylated internal primers and non-phosphorylated outer primers combined with T4 DNA ligase-mediated ligation of the mutated products was used as described previously. In addition, 5' and 3' adapter primers were synthesized, which incorporated a 5' c-myc tag into the cTnC sequence and introduced unique 5' and 3' restriction sites (KpnI and HindIII, respectively) to directionally clone the mutated PCR products into the pAdTrack-CMV shuttle vector (Addgene, Cambridge, MA). This vector is analogous to the pAdTrack-TOX vector described above, with the exception that a CMV-driven EGFP marker replaces the mRFP1 marker in pAdTrace-TOX. After successful ligation of DNAs encoding wild-type or mutant cTnC into the EGFP-expressing shuttle vector, adenoviral DNA was generated using the pAdEasy system as described above for the mutant PLN adenoviruses. Virus titers were determined by plaque assay.

Adult mouse cardiomyocyte culture and infection with recombinant adenovirus

Cardiomyocyte isolation was performed as described above in sterile conditions. Adult mouse cardiomyocyte culture was performed as previously described with modifications. Briefly, after isolation, cardiomyocytes were resuspended in plating medium (MEM with Hank's salts (Invitrogen) containing 50 µU/ml insulin, 100 U/ml penicillin, 50 µg/ml gentamicin, 0.2% bovine serum albumin, 2 mmol/l glutamine, 25µmol/ml S-(-)-blebbistatin and 5% fetal bovine serum). Cardiomyocytes were incubated in this medium for 2hr and were then plated on 25 mm square cover slips that had previously been coated with 100 µl/coverslip of 56 nmol/l mouse laminin (Invitrogen) for 2hr. After 1 hr of incubation for cell attachment, cardiomyocytes were infected with adenoviral vector encoding WT cTnC (C35/C84) or mutant cTnC (C35S, C84S or C35S/C84S) at 200–400 multiplicity of infection. After 1 hr of incubation for viral infection, plating medium was replaced with culture medium, which is identical to the plating medium but lacks fetal bovine serum. Cardiomyocytes were subsequently incubated at 32°C. Successful infection of cardiomyocytes was confirmed by detection of EGFP using direct fluorescence microscopy.

Detection of exogenous cTnC in cardiomyocytes after adenovirus transfection

Cultured cardiomyocytes were collected 48 hr after infection with adenovirus. Protein extracts were prepared and cTnC was detected by immunoblot. Transfected myc-cTnC was differentiated from endogenous cTnC because myc-cTnC migrated more slowly than wild-type protein in the polyacrylamide gel.

Detection of S-nitrosylated PLN or TnC in HEK-293 cells

Human embryonic kidney (HEK)-293 cells were infected with adenoviral vector encoding wild-type PLN or mutant PLN (C36A/C41A/C46A) at 50 multiplicity of infection. Similarly, HEK-293 cells were infected with adenoviral vector encoding wild-type cTnC or mutant cTnC (C35S/C84S) at 50 multiplicity of infection. After 36 h, cells were incubated with 100µM CysNO for 10 minutes. Cells were lysed in 500 µL lysis buffer (25mM Hepes (pH7.4), 50mM NaCl, 1mM EDTA, 0.2mM neocuproine, 1mM PMSF, 1% (v/v) NP-40 and protease inhibitor cocktail) and the biotin switch assay was then performed.

Detection of S-nitrosylated PLN or TnC in HUVEC cells
Human umbilical vein endothelial (HUVEC) cells were cultured in EGM growth medium (Lonza) containing EGM-2 supplement. Experiments were performed at passages 4-5. HUVEC cells were infected with adenoviral vector encoding wild-type PLN or mutant PLN (C36A/C41A/C46A) at 50 multiplicity of infection. Similarly, HUVEC cells were infected with adenoviral vector encoding wild-type cTnC or mutant cTnC (C35S/C84S) at 50 multiplicity of infection. After 36 h, cells were stimulated with 10µM ISO for 10 minutes. Cells were lysed in 500 µL lysis buffer (25mM Hepes (pH7.4), 50mM NaCl, 1mM EDTA, 0.2mM neocuproine, 1mM PMSF, 1%(v/v) NP-40 and protease inhibitor cocktail) and the biotin switch assay was then performed.

Measurement of sarcomere shortening and Ca\textsuperscript{2+} transient in cultured cardiomyocytes
48 hours after infection with adenovirus, cover slips containing cardiomyocytes were transferred to S-(-)-Blebbistatin-free tyrode solution containing Fura2-AM 2 µmol/L and Pluronic F-175 0.04% (W/V). After S-(-)-Blebbistatin was washed out by perfusion for 20 min, cover slips were placed on the IonOptix microscope system as described above. Cultured cardiomyocytes were paced at 2 Hz and EGFP expression was confirmed using an FITC 485/505 nm exposure filter. Sarcomere length and Fura-2 fluorescence were recorded in EGFP-positive cardiomyocytes.

Statistics
All data are presented as mean ± standard error of the mean (s.e.m.), and the number of samples per group (N) is indicated for each experiment. For isolated cardiomyocyte experiments, measurements of all cells from one mouse were averaged and the average value for each mouse was used for statistical comparison between groups, unless otherwise specified. Data were analyzed using Prism 5 software package (GraphPad Software, La Jolla, CA). Statistical differences among groups were evaluated using the appropriate test as indicated in the text. P values smaller than 0.05 were considered significant.
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### ONLINE TABLE I. Antibodies used throughout the study.

<table>
<thead>
<tr>
<th>Sample/application</th>
<th>Antibody</th>
<th>Dilution</th>
<th>Source</th>
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<td>Cardiac nuclear fraction</td>
<td>NFAT&lt;sub&gt;C3&lt;/sub&gt;</td>
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<td>SCB</td>
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<tr>
<td></td>
<td>Histone</td>
<td>1:10,000</td>
<td>CST</td>
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<td>Cardiac cytoplasmic fraction</td>
<td>β-tubulin</td>
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<td>AB</td>
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<tr>
<td></td>
<td>BNP</td>
<td>1:1,000</td>
<td>PP</td>
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<tr>
<td></td>
<td>GAPDH</td>
<td>1:20,000</td>
<td>CST</td>
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<tr>
<td></td>
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<tr>
<td></td>
<td>Thr&lt;sup&gt;17&lt;/sup&gt; phospho-PLN</td>
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<tr>
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<td>Total cTnI</td>
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<td>Actin</td>
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<td>CST</td>
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<tr>
<td></td>
<td>NCX</td>
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IRES: immunoreaction enhancer solution (Cosmo Bio USA, Carlsbad, CA). Suppliers: AB: Abcam, Cambridge, MA; BA: Badrilla Ltd, Leeds, UK; CST: Cell Signaling Technology, Danvers, MA; MM: Merck Millipore, Billerica, MA; NB: Novus Biologicals, Littleton, CO; PB: Pierce Biotechnology, Rockford, IL; PP: Phoenix Pharmaceuticals Inc, Burlingame, CA; PRO:
Promega, Madison, WI; SCB: Santa Cruz Biotechnology, Dallas, TX; UG: Upstate Group LLC, Charlottesville, VA
ONLINE FIGURE LEGENDS

Online Figure I. Dose-dependent changes of sarcomere shortening (A) and Δ[Ca^{2+}] (B) in response to ISO in isolated WT and GSNOR\(^{-/-}\) (KO) cardiomyocytes. n=4 mice (8-12 cells/mouse). (C) Maximum and minimum rate of LV pressure change (dP/dt\(_{max}\) and dP/dt\(_{min}\), respectively) in response to ISO in isolated Langendorff perfused WT and TG hearts. n=5 mice per group.

Online Figure II. GSNOR deficiency worsened LV remodeling in response to low dose isoproterenol infusion. (A) Representative echocardiography images (Top), heart images (middle) and histological images of hematoxylin and eosin staining for cardiomyocyte area (upper histological image) and Masson’s Trichrome staining for interstitial fibrosis (lower histological image) at baseline and 28 days after starting low dose chronic ISO infusion (30mg/kg/day) for 14 days in WT and GSNOR\(^{-/-}\) (KO) mice. Fractional shortening (B) and LV mass (C) calculated by echocardiography at baseline, and day 7, 14, and 28 after the start of ISO infusion in WT and GSNOR\(^{-/-}\) (KO) mice. n=5 mice per group. Heart weight/body weight ratio (D) and heart weight/tibia length ratio (E) at baseline and day 28 in WT and GSNOR\(^{-/-}\) (KO) mice. N=5 mice per group. (F) Mean cardiomyocyte area in LV section at baseline and day 28 in WT and GSNOR\(^{-/-}\) (KO) mice. N=5 mice per group (an average of 260 cells +/- 89 were measured from 20 fields per mouse). (G) Interstitial fibrosis in LV sections at baseline and day 28 in WT and GSNOR\(^{-/-}\) (KO) mice. N=4-7 mice per group (the fibrotic area was measured from 11 fields per mouse). *P<0.05, **P<0.001 and ###P<0.001 (vs baseline) as determined by two-way or two-way repeated measures ANOVA with Bonferroni’s post hoc test.

Online Figure III. Impact of GSNOR overexpression on S-nitrosylation of Ca\(^{2+}\) handling proteins measured by modified biotin switch method. Representative biotin switch assay blots and summary of S-nitrosylation levels of ryanodine receptor (RyR2) (A), SERCA2a (B), LTCC (C), cTnI (D), tropomyosin (TPM) (E), and actin (F) in WT and TG cardiomyocytes lysates without (Base) and with ISO treatment (10nM). n=5 mice per group. No significant difference was found between experimental groups by two-way ANOVA with Bonferroni’s post hoc test.

Online Figure IV. Impact of GSNOR overexpression on S-nitrosylation of Ca\(^{2+}\) handling proteins measured by phenyl mercury resin-assisted capture method. Representative immunoblots after enrichment of S-nitrosylated proteins with phenyl mercury resin-assisted capture. S-nitrosylated and total protein levels of PLN, NCX, cTnC, actin, TPM, cTnI, LTCC, SERCA2a, RyR2, and myosin binding protein C (MYBPC3) in WT and TG cardiomyocytes lysates without and with ISO treatment (10nM).

Online Figure V. Lack of effects of GSNOR on L-type Ca\(^{2+}\) channel current density (A) L-type Ca\(^{2+}\) channel (LTCC) current density as a function of voltage in WT and GSNOR-Tg (TG) cardiomyocytes without (base) or with isoproterenol treatment (ISO, 10nM for 30 minutes). N=3-4 mice (8-14 cells per mouse). ###P<0.01, ####P<0.001 vs. baseline. (B) Peak LTCC currents of WT and TG cardiomyocytes at baseline or after ISO treatment. N=3-4 mice **P<0.01.

Online Figure VI. Generation of and cardiac infection with recombinant adeno virus encoding mutant PLN protein. Molecular modeling of the structure of the unphosphorylated (PBD ID: 1ZLL) (A) and Ser\(^{16}\) phosphorylated (PBD ID: 2M3B) (B) PLN, showing the location of Ser\(^{16}\) (black arrow heads) and Cys\(^{36}\), Cys\(^{41}\), and Cys\(^{46}\) (white arrows). (C) Molecular
modeling of adjacent Ser\textsuperscript{16} phosphorylated PLN monomers that shows the location of Ser\textsuperscript{16} (S16) and Cys\textsuperscript{36} (C36), Cys\textsuperscript{41} (C41), and Cys\textsuperscript{46} (C46) based on the PDB 2M3B. (D) Schematic representation of the pAdTrace-Tox shuttle vector containing the coding sequence for the different PLN cysteine-to-alanine mutants. CMV: cytomegalovirus, mRFP1: monomeric red fluorescent protein, BGH: bovine growth hormone. Recombination of this vector with the viral backbone present in AdEasier-1 bacteria leads to the generation of a functional adenovirus encoding both mRFP1 and the desired PLN mutant variant (Ad.PLN.mRFP1). (E) Expression levels of endogenous (left) PLN in WT and PLN\textsuperscript{+} cardiomyocytes, and virally-encoded (right) PLN mutants in PLN\textsuperscript{−} cardiomyocytes infected with Ad.PLN.mRFP1. (F) Representative modified biotin switch assay blots showing CysNO-induced S-nitrosylation of PLN in HEK293 cells expressing wild-type PLN but not C36A/C41A/C46A mutant PLN. (G) Representative modified biotin switch assay blots showing ISO-stimulated S-nitrosylation of PLN in HUVEC cells expressing wild-type PLN but not C36A/C41A/C46A mutant PLN.

**Online Figure VII. βAR stimulation leads to increased NCX activity in GSNOR-Tg cardiomyocytes.** (A) Representative Ca\textsuperscript{2+} transient recordings in WT and GSNOR-Tg (TG) before and after ISO (10 nmol/L) stimulation. (B) Time constant of intracellular Ca\textsuperscript{2+} concentration decay in WT and TG cardiomyocytes at different doses of ISO. N=4 mice (5-10 cardiomyocytes per mouse). (C) Representative normalized Ca\textsuperscript{2+} tracings recorded in WT and TG cells exposed to different concentrations of ISO and challenged with 10 mmol/L caffeine. (D) NCX activity calculated from subsequent caffeine stimulation of WT and TG cardiomyocytes in normal Tyrode buffer and 0 Na\textsuperscript{+}, 0 Ca\textsuperscript{2+} Tyrode buffer during treatment with different doses of ISO. N=4 mice (5-10 cardiomyocytes per mouse). ###P<0.001 (vs baseline), **P<0.01, ***P<0.001. Normalized Ca\textsuperscript{2+} transient recordings in WT and TG cardiomyocytes at baseline and after 10 mmol/L ISO stimulation, without (E) or with (F) the NCX inhibitor SEA-400. N=4 mice (8-10 cardiomyocytes per mouse). ###P<0.001 vs baseline, ***P<0.001 vs WT.

**Online Figure VIII. Generation and cardiomyocyte infection with recombinant adenovirus encoding mutant cTnC protein.** Sarcomere length shortening (A) and Δ[Ca\textsuperscript{2+}] (B) in response to increasing extracellular Ca\textsuperscript{2+} concentration in isoproterenol (ISO)-stimulated intact WT and GSNOR-Tg cardiomyocytes. n=4 mice (10-17 cells per mouse) per group. (C) Schematic representation of the pAdTrack-CMV shuttle vector containing the coding sequence for the different cTnC cysteine-to-serine mutants. CMV: cytomegalovirus, EGFP: enhanced green fluorescent protein. Recombination of this vector with the viral backbone present in AdEasier-1 bacteria leads to the generation of a functional adenovirus encoding both EGFP and the desired cTnC mutant variant (Ad.cTnC.EGFP). (D) Expression levels of endogenous and virally-encoded cTnC proteins in WT cardiomyocytes infected with Ad.cTnC.EGFP. Virally encoded cTnC carries an extra 5′ c-Myc tag, allowing it to be distinguished from the shorter endogenous cTnC on Western blot. The % replacement of endogenous cTnC by viral cTnC was calculated as the relative ratio of c-Myc tagged cTnC versus non-tagged cTnC. (E) Representative modified biotin switch assay blots showing CysNO-induced S-nitrosylation of cTnC in HEK293 cells expressing wild-type cTnC but not C35S/C84S mutant cTnC. (F) Representative modified biotin switch assay blots showing ISO-stimulated S-nitrosylation of cTnC in HUVEC cells expressing wild-type cTnC but not C35S/C84S mutant cTnC.

**Online Figure IX. Effects of mutant cTnC on cardiomyocyte relaxation and Ca\textsuperscript{2+} decay**

Time constants of sarcomere relaxation (A) and intracellular Ca\textsuperscript{2+} decay (B) at baseline and after ISO (10nM) stimulation in WT and GSNOR-Tg (TG) cultured cardiomyocytes infected with empty Ad.EGFP or mutant Ad.cTnC.EGFP viruses. N=24-36 cardiomyocytes per group.
***: P<0.001 vs baseline as determined by two-way ANOVA with Bonferroni’s post hoc test.

(C) Core structure model (PDB file 1MXL) of Ca$^{2+}$-saturated human cTnC N-domain in complex with the switch region of cTnl (residue 147-163) shows the location of Cys$^{84}$. 
Online Figure I
Online Figure II
Online Figure III
Online Figure IV
Online Figure V

A

Voltage (mV)

-30 -20 -10 0 10 20 30 40 50 60

$I_{Ca}$ (pA / pF)

WT Base

WT ISO

TG Base

TG ISO

B

Peak L-type $Ca^{2+}$ currents

$I_{Ca}$ (pA / pF)

WT

TG

**
Online Figure VI
Online Figure VII
Online Figure VIII