Interaction Between Neuronal Nitric Oxide Synthase Signaling and Temperature Influences Sarcoplasmic Reticulum Calcium Leak
Role of Nitroso–Redox Balance

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Rationale: Although nitric oxide (NO) signaling modulates cardiac function and excitation–contraction coupling, opposing results because of inconsistent experimental conditions, particularly with respect to temperature, confound the ability to elucidate NO signaling pathways. Here, we show that temperature significantly modulates NO effects.

Objective: To test the hypothesis that temperature profoundly affects nitroso–redox equilibrium, thereby affecting sarcoplasmic reticulum (SR) calcium (Ca\(^{2+}\)) leak.

Methods and Results: We measured SR Ca\(^{2+}\) leak in cardiomyocytes from wild-type (WT), NO/redox imbalance (neuronal nitric oxide synthase–deficient mice-1 [NOS1\(^{-/-}\)], and hyper S-nitrosoglutathione reductase–deficient (GSNOR\(^{-/-}\)) mice. In WT cardiomyocytes, SR Ca\(^{2+}\) leak increased because temperature decreased from 37°C to 23°C, whereas in NOS1\(^{-/-}\) cells, the leak suddenly increased when the temperature surpassed 30°C. GSNOR\(^{-/-}\) cardiomyocytes exhibited low leak throughout the temperature range. Exogenously added NO had a biphasic effect on NOS1\(^{-/-}\) cardiomyocytes; reducing leak at 37°C but increasing it at subphysiological temperatures. Oxypurinol and Tempol diminished the leak in NOS1\(^{-/-}\) cardiomyocytes. Cooling from 37°C to 23°C increased reactive oxygen species generation in WT but decreased it in NOS1\(^{-/-}\) cardiomyocytes. Oxypurinol further reduced reactive oxygen species generation. At 23°C in WT cells, leak was decreased by tetrahydrobiopterin, an essential NOS cofactor. Cooling significantly increased SR Ca\(^{2+}\) content in NOS1\(^{-/-}\) cells but had no effect in WT or GSNOR\(^{-/-}\).

Conclusions: Ca\(^{2+}\) leak and temperature are normally inversely proportional, whereas NOS1 deficiency reverses this effect, increasing leak and elevating reactive oxygen species production because temperature increases. Reduced denitrosylation (GSNOR deficiency) eliminates the temperature dependence of leak. Thus, temperature regulates the balance between NO and reactive oxygen species which in turn has a major effect on SR Ca\(^{2+}\).

Key Words: calcium signaling • induced hypothermia • nitric oxide • nitric oxide synthase • nitroso-redox imbalance • reactive oxygen species • 5,6,7,8-tetrahydrobiopterin
of focal cerebral ischemia. Temperature modulates NO production in vitro, directly affecting NOS activity. Thus, changes in temperature, by affecting NO production and therefore the nitroso–redox (NO/redox) balance in the heart, may be closely related to ryanodine receptor (RyR2)-mediated SR Ca²⁺ leak. Post-translational modifications, such as S-nitrosylation, of RyR2 are influenced by cellular NO/re- dox state and can both positively and negatively affect SR Ca²⁺ leak. Signaling defects in Ca²⁺ handling, such as Ca²⁺ leak, contribute to impaired contractility in the failing heart, thus depleting SR Ca²⁺ storage as a consequence of the leak and leading to impaired contractile function of the heart.

This study focused on the effect of temperature on SR Ca²⁺ leak in isolated cardiomyocytes from WT mice or mouse models of aberrant S-nitrosylation, NOS1−/− and S-nitrosoglutathione reductase–deficient (GSNOR−/−) mice, and shows that reactive oxygen/nitrogen species signaling are affected by temperature.

### Methods

#### Animal Models

We studied age-matched C57BL/6J mice (WT; n=26) and mice with a homozygous deletion of NOS1 (B6;129S4-Nos1tm1Plh; n=20; Jackson Laboratories, Bar Harbor, ME; stock number 002633); or tm1Plh/a homozygous deletion of NOS1 (B6;129S4-Nos1tm1Plh; n=10). All protocols and experimental procedures were approved by the Animal Care and Use Committee of the University of Miami and followed the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85–234, revised 2011).

#### Myocyte Isolation

Cardiac myocytes were isolated and prepared from hearts as previously described. Briefly, hearts were harvested and perfused retrogradely in a modified Langendorff system (constant flow, 2 mL/min) with an isolation solution (see Online Data Supplement), bubbled with 5% CO₂ and 95% O₂, for 15 minutes. Once cleaned, the hearts were perfused with collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ) 315 U/mL and protease type XIV (Sigma–Aldrich, St. Louis, MO) 5.2 U/mL for 10 minutes. After digestion, myocytes were released by gentle mechanical disruption. The extracellular Ca²⁺ was restored by sequential additions of CaCl₂ in a Ca²⁺-free Tyrode solution (see Online Data Supplement). Cardiomyocytes were resuspended in a 1.8 CaCl₂ Tyrode solution at room temperature and then loaded with Fura-2. Myocytes were stimulated at 0.5 or 1 Hz.

#### Intracellular Ca²⁺ Measurement

Intracellular Ca²⁺ was measured using the Ca²⁺-sensitive dye Fura-2 (Molecular Probes, Eugene, OR) and a dual-excitation spectrophotometer (IonOptix LLC, Milton, MA), excited with a xenon lamp at wavelengths of 340 and 380 nm. The emission fluorescence was reflected through a barrier filter (510±15 nm) to a photomultiplier tube. The in vivo calibration was performed superfusing a free Ca²⁺ and then a Ca²⁺ saturating (5 mmol/L) solution both containing 10 μmol/L ionomycin (Sigma–Aldrich) until reaching a minimal (Rₙₐ₉) or a maximal (Rₘₐₓ) ratio values, respectively. [Ca²⁺], was calculated as described previously.

### Measurement of SR Ca²⁺ Leak and SR Ca²⁺ Content

SR Ca²⁺ leakage was assessed with 1 mmol/L tetracaine (Sigma–Aldrich) as described by Shannon et al. The observed decrease in the Fura-2 ratio in presence of tetracaine compared with the nontetra- caine-treated condition was considered the Ca²⁺ leak for a particular myocyte (Online Figure I). After assessing Ca²⁺ leak, tetracaine was washed out by superfusing fresh 0Na/0Ca²⁺ Tyrode solution and SR Ca²⁺ content was assessed as described by Bassani et al by a caffeine challenge.

SR Ca²⁺ contents were calculated considering that SR represents 3.5% and cytosol 65% of the myocyte volume as previously described. SR leak–SR load pairs were grouped by similar SR Ca²⁺ load and expressed as a leak–load relationship fitted by an exponential growth function using the Graph Pad Prism software (version 4.02). Measurements were mostly performed at 23°C, 25°C, 30°C, 34°C, or 37°C.

#### Treatments

Cardiomyocytes loaded with Fura-2 were preincubated for 20 minutes with the following compounds (unless otherwise is specified): Tempol (100 μmol/L; Calbiochem, Calbiochem/EMD Biosciences, San Diego, CA); Oxypurinol (100 μmol/L; Sigma–Aldrich); (±)-S-Nitroso-N-acetylpenicillamine (SNAP; 1 or 50 μmol/L; Santa Cruz, Santa Cruz, CA); N⁶-(1-Imino-3-butenyl)-L-ornithine (L-NAME; 100 μmol/L; Enzo Life Sciences, Plymouth Meeting, PA); Hydrogen peroxide (H₂O₂; 100 μmol/L; Sigma–Aldrich) (6R)-5,6,7,8-Tetrahydrobiopterin dihydrochloride (BH₄; 300 μmol/L; Sigma–Aldrich). Experiments in control condition (no treatment) were run in parallel to each type of pharmacological intervention for each batch of cardiomyocytes.

### Detection of Reactive Oxygen Species

Reactive oxygen species (ROS) were measured by using the sensitive probe 2′,7’-dichlorodihydrofluoresceine diacetate and the background fluorescence (Fᵣ) was acquired and then, cardiomyocytes were incubated during 30 minutes at 23°C or 37°C with 2’-7’-dichlorodihydrofluorescein diacetate and washed. The initial fluorescence (Fᵣ) and a second measure after 5 minutes (Fₐ) were acquired. Myocytes were stimulated at 1 Hz and ROS expressed as:

\[
\text{ROS} = \left( \frac{F - F_0}{F_{max} - F_0} \right)
\]

Alternatively, control or 100 μmol/L oxypurinol-treated cardiomyocytes were loaded with 2’-7’-dichlorodihydrofluorescein diacetate for 15 minutes on polylysine-coated microscope slides at 23°C or 37°C. After 10 minutes washing with Tyrode solution, cardiomyocytes were fixed with 2% p-formaldehyde in cold phosphate-buffered saline and then washed once with phosphate-buffered saline. Cardiomyocytes treated with 1 mmol/L H₂O₂ at either 23°C or 37°C, from each mouse model were used as positive control for ROS and were used as maximal fluorescence signal for normalization of each group (Fₘₐₓ). Fluorescence (Fᵣ) was captured with an excitation wavelength of 488 and 525 nm emission. Images were quantified by ImageJ (National Institutes of Health) software and results were expressed as:

\[
F_{DCF} = \left( \frac{F - F_0}{F_{max} - F_0} \right)
\]

where F₀ is background.
Nitric Oxide Measurement

Isolated WT mouse cardiomyocytes were loaded with the NO-sensitive dye 4,5-diaminofluorescein diacetate (5 μmol/L; Calbiochem/EMD Biosciences) for 20 minutes at room temperature. Cells were then loaded with the NO-sensitive dye and then collected in RNA later lysis buffer (Qiagen, Valencia, CA). Total RNA was extracted from cells using PureLink Micro-to-Midi Total RNA Purification System (Qiagen) and reverse-transcribed using high capacity cDNA reverse transcription Kit (Applied Biosystems, Foster City, CA). All samples were treated with TurboTM DNase (Ambion, Austin, TX). Quantitative real-time polymerase chain reaction (PCR) was performed in triplicate using a 20-μL reaction mixture containing 10 ng of cDNA, TaqMan Universal PCR Master Mix (Roche, Branchburg, NJ) and primer/probe sets for nitric oxide synthase 1 (NOS1, Mm00435171_m1), NOS2 (Mm00440502_m1), and NOS3 (Mm00435197_g1; TaqMan Gene Expression Assay, Applied Biosystems, Foster City, CA). As an internal control glucoronidase, β (GUSB, Mm01197698_m1) was determined in each reaction. Reaction conditions were performed according to manufacturer instructions: 1 cycle of 50°C for 2 minutes, 1 cycle of 90°C for 10 minutes, and 40 cycles of 95°C for 15 s and 60°C for 1 minute. Software from q5 multicolor real-time PCR detection system (Bio-Rad, Hercules, CA) was used for PCR analyses. mRNA relative expression was calculated by 2^ΔΔCt method. Quantitative PCR data reflects 4 different experiments each done at 23°C, 30°C, and 37°C.

Statistical Analysis

Data are expressed as means±SEM. For leak–load relationship, an exponential growth fit, which compares independent fits with a global shared fit was applied. Two or more groups of data were considered to fit different curves if P<0.05. For comparisons of 2 groups, Student t test was used. For comparison of 2 groups, 1- or 2-way ANOVA was performed. Two-way ANOVA was used when a second variable was involved. Newman–Keuls or Bonferroni post hoc tests were used as appropriate by the GraphPad Prism version 4.02 (GraphPad Prism Software Corporation San Diego, CA). A P<0.05 was considered significant.

Results

Leak–Load Relationship is Critically Affected by Temperature

We measured SR Ca2+ leak (Online Figure I) in cardiomyocytes from WT and NOS1−/− (which have depressed S-nitrosylation and oxidative stress) mice over a broad temperature range from 23°C to 37°C. The SR Ca2+ leak response to changes in temperature was different in these 2 models (Figure 1A). The pattern of SR Ca2+ leak–temperature, at matched SR Ca2+ load, shows that leak slightly increases (P=0.038) when the temperature is reduced from physiological temperature through 34°C, 30°C, 25°C, and 23°C in WT cardiomyocytes, with the leak at 23°C, being higher than at 37°C (Figure 1B; P=0.014 and 2A). In marked contrast, the temperature-dependent change in leak in NOS1−/− cardiomyocytes was lower compared with WT at low temperature (Figure 1A and 1B), in agreement with Wang et al.3 However, NOS1−/− cardiomyocytes exhibited a significant increase in leak when the temperature surpassed 30°C (Figure 1A) as we previously showed.9

Reactive Oxygen Species are Associated With the SR Ca2+ Leak

ROS signaling plays a role in the regulation of RyR channel gating by promoting redox post-translational modifications.3,14,25 In particular, oxidative stress induces RyR2 to leak Ca2+ from the SR.19 We found that SR Ca2+ leak at either 23°C or 37°C (Figure 2A) correlated with the rate of ROS production (Figure 2B, as measured with dichlorofluoresceine in an IonOptix system) in both WT and NOS1−/− cardiomyocytes. ROS was increased at 23°C compared with 37°C in WT, whereas in NOS1−/− the leak was lower at 23°C compared with physiological temperature (Figure 2B). To verify that ROS mediates SR Ca2+ leak–temperature dependence, we first tested the effect of the superoxide scavenger Tempol on Ca2+ leak at 23°C, 30°C, and 37°C. Tempol eliminated the Ca2+ leak in both WT (P<0.0001) and NOS1−/− (P<0.0001) cardiomyocytes within the range of temperatures (Figure 2C–2E), confirming the importance of ROS in the gating of RyR2 channels in both models. We further investigated the involvement of xanthine oxidoreductase (XOR), a superoxide-generating enzyme. As
shown above, ROS is primarily elevated in NOS1−/− at 37°C and WT at 23°C. Oxyypurinol reduced ROS in XOR-treated cells (Figure 3A and 3B) and Ca2+ leak in NOS1−/− cardiomyocytes (Figure 3C). The SR Ca2+ leak–load relationship was virtually identical in NOS1−/− under control conditions or oxyypurinol between 23°C and 30°C (K+Oxy=0.0089±0.0045 versus K−Oxy=0.0048±0.0024, P=0.475; Figure 3D), whereas at 34°C and 37°C oxyypurinol-treated NOS1−/− cardiomyocytes exhibited a leak–load curve that was less steep than NOS1−/− control (K+Oxy=0.0030±0.0025 versus K−Oxy=0.0117±0.0015, P=0.0481 at 37°C; Figure 3E).

Involvement of NO in the Temperature-Mediated Changes in SR Ca2+ Leak
To test whether defective NO production was the only cause for the biphasic response of SR Ca2+ leak to changes in temperature, we supplemented the bathing buffer with the NO donor SNAP. At temperatures >30°C, 1 μmol/L SNAP decreased the leak in NOS1−/− and were equivalent to the levels observed in WT cardiomyocytes. As predicted by our hypothesis, at the lower temperatures (23°C and 25°C), 1 μmol/L SNAP increased the leak in NOS1−/− to the WT values (Figure 4A). Surprisingly, 50 μmol/L SNAP did not significantly affect the leak in NOS1−/− cardiomyocytes (Online Figure II). Thus, only 1 μmol/L SNAP was able to restore the normal SR Ca2+ leak–temperature pattern in NOS1−/− cardiomyocytes, suggesting that regulation of SR Ca2+ in diastole is extremely sensitive to the level of NO, at least under this particular condition of exogenous supply of NO by SNAP. Next, we used 100 μmol/L l-VNIO to specifically inhibit NOS1 in WT cardiomyocytes. This approach allowed us to test whether acute inhibition of this isoform was sufficient to mimic the leak–temperature pattern observed in the genetically modified model. Consistently, l-VNIO reduced the leak at 23°C and increased it at 37°C as expected (Figure 4B).

Reduced SR Ca2+ Leak is Associated With Low ROS Levels in a GSNO Reductase Deficiency Model
As shown above, aberrant S-nitrosylation and the redox state of cardiomyocytes affects SR Ca2+ leak. Deficiency of GSNO also induced an aberrant NO/redox state with hyper S-nitrosylation of a broad spectrum of proteins. 26 S-nitrosylation is thought to exert a protective role against an oxidative environment, thus the levels of ROS in GSNO−/− cardiomyocytes were lower than those in WT at 23°C but not at 37°C (Figure 5A and B). Consistent with the results shown above, where the extent of the leak is proportional to the amount of ROS, the GSNO−/− model exhibited lower
leak compared with WT cardiomyocytes at subphysiological temperatures (Figure 5C; \(P=0.001\); at matched SR Ca\(^{2+}\) load, insert). Importantly, the leak at 37°C in GSNOR\(^{-/-}\) was not different, regardless of the lower ROS level, compared with WT, agreeing with our previous findings.\(^{21}\) Surprisingly, the treatment of GSNOR\(^{-/-}\) cardiomyocytes with 100 \(\mu\)mol/L hydrogen peroxide (H\(_2\)O\(_2\)), an oxidant agent that induces cardiomyocyte oxidative stress, did not change SR Ca\(^{2+}\) leak in this model (Figure 5C), suggesting that the supposed protective role of hyperS-nitrosylation might be in part because of enhanced cellular antioxidant mechanisms.

**SR Ca\(^{2+}\) Stores are Primarily Affected by Elevated Leak Under NO/Redox Imbalance**

The SR Ca\(^{2+}\) content of cardiomyocytes paced at 1 Hz was estimated by caffeine challenge. WT and GSNOR\(^{-/-}\) cardiomyocytes exhibited a relatively stable Ca\(^{2+}\) content throughout the studied range of temperatures. Surprisingly, despite the different behavior of leak in GSNOR\(^{-/-}\) compared with WT cardiomyocytes when the temperature drops, the average Ca\(^{2+}\) loads were not different throughout the broad range of studied temperatures (Figure 6A). In contrast, the SR Ca\(^{2+}\) content in NOS1\(^{-/-}\) was higher at 23°C to 25°C than at 34°C to 37°C. At the low range of temperature, SR Ca\(^{2+}\) load was elevated in NOS1\(^{-/-}\) cardiomyocytes compared with WT or GSNOR\(^{-/-}\). However, at a physiological range of temperatures (34°C–37°C), the Ca\(^{2+}\) load was significantly reduced in NOS1\(^{-/-}\) cells (Figure 6A). Thus, there was a significant correlation between the SR Ca\(^{2+}\) leak and the resulting average SR Ca\(^{2+}\) content in NOS1\(^{-/-}\) (Figure 6B; \(R^2=0.9014\)) but not in WT (\(R^2=0.0189\)) or GSNOR\(^{-/-}\) (\(R^2=0.3553\)) cardiomyocytes. These results suggest that Ca\(^{2+}\) stores in the NO/redox imbalance model are highly dependent on the leak of Ca\(^{2+}\) from the SR.

**Coupling of NO Synthase and NO Production**

NO production was assessed in cardiomyocytes from WT mice to verify that intracellular NOS-derived NO generation is dependent on temperature. NO levels increased because the temperature increased from 23°C to 37°C (Figure 7A) as described previously for activity of NOS1, NOS2, and NOS3 in vitro.\(^{14}\) NO production at the different temperatures was inversely correlated with the Ca\(^{2+}\) leak (at matched SR Ca\(^{2+}\) load) in WT cardiomyocytes (\(R^2=0.9014\)) but not in WT (\(R^2=0.0189\)) or GSNOR\(^{-/-}\) (\(R^2=0.3553\)) cardiomyocytes. These results suggest that Ca\(^{2+}\) stores in the NO/redox imbalance model are highly dependent on the leak of Ca\(^{2+}\) from the SR.

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**Figure 3. Xanthine oxidase-derived superoxide mediates the increase of leak in cardiomyocytes.**

A, Representative images of 2′,7′-dichlorodihydrofluorescein in response to reactive oxygen species (ROS) in wild-type (WT) or neuronal nitric oxide synthase–deficient mice-1 (NOS1\(^{-/-}\)) cardiomyocytes in the presence or the absence of 100 \(\mu\)mol/L oxypurinol at 23°C or 37°C (Bar, 10 \(\mu\)m). B, Averaged ROS production in WT or NOS1\(^{-/-}\) cardiomyocytes in the presence or the absence of oxypurinol 100 \(\mu\)mol/L at 23°C or 37°C. Values were normalized by the maximal signal for each strain in the presence of H\(_2\)O\(_2\) (catalase-treated cardiomyocytes were used as negative control). \(*P<0.05, **P<0.01, and ***P<0.001\) (−)Oxy- vs +Oxy-treated cardiomyocytes, Student t test. C, Dependence of sarcoplasmic reticulum (SR) Ca\(^{2+}\) leak with temperature in NOS1\(^{-/-}\) cardiomyocytes in the absence or the presence of 100 \(\mu\)mol/L oxypurinol (at matched SR Ca\(^{2+}\) load ≈75 \(\mu\)mol/L; **\(P<0.01\) (−)Oxy- vs +Oxy-treated NOS1\(^{-/-}\); 2-way ANOVA). D, SR Ca\(^{2+}\) load–leak relationship in nontreated and oxypurinol-treated NOS1\(^{-/-}\) cardiomyocytes at 23°C and (E) at 37°C (†\(P<0.05\) (−)Oxy vs +Oxy NOS1\(^{-/-}\); exponential growth fitting). H\(_2\)DCF indicates 2′,7′-dichlorodihydrofluoresceine.

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contributes to maintain the functional quaternary structure (dimer) of the enzyme. Interestingly, there was a time-dependent reduction in leak ($P=0.066$ with 2 minutes preincubation and $P=0.011$ with 20 minutes preincubation; Figure 7C), suggesting that the elevated leak at room temperature is mediated by NOS1 uncoupling. In addition, we examined the relationship between temperature and expression of NOS1, NOS2, and NOS3 in WT cardiomyocytes exposed to 23°C, 30°C, and 37°C for 30 minutes. No differences were seen in the amount of mRNA of each isoform in response to temperature change (Figure 7D), further suggesting that dependence of NO production on temperature is regulated by the activity rather than expression of NOS isoforms.

## Discussion

These findings reconcile a central, 15-year-old controversy in NO cardiobiology; temperature profoundly affects SR Ca$^{2+}$ leak. These results explain why experiments on isolated myocytes when performed at room temperature yield different findings to those performed at physiological temperatures.6,9

We previously showed that leak was increased in the absence of NOS1 at 37°C, whereas Wang et al8 showed a reduced leak at room temperature compared with WT control cardiomyocytes. Therefore, we have highlighted that the thermal dependence of the cardiomyocyte metabolism tightly affects the redox state of the cell, which modifies RyR2 activity and thereby SR Ca$^{2+}$ leak. At low temperatures, RyR2 gating is favored,10,11 and may be associated with reduced NOS activity.14 It has been proposed that XOR and NOS1 colocalize in the SR in close proximity to RyR2. Thus, NOS1-mediated NO production exerts a powerful inhibitory effect on myocardial XOR and tightly controls RyR2 channel activity. Therefore, a decrease or absence of NOS1 activity leads to a rise in superoxide anion and dysregulation of RyR2-mediated Ca$^{2+}$ release. We consistently found increased ROS generation in WT cardiomyocytes when cooled from 37°C to 23°C. This effect was primarily XOR-mediated because inhibition of this enzyme with oxypurinol prevented the rise of ROS at 23°C. We speculate that the limited availability of NOS1-derived NO in this microenvironment is still sufficient to account for the differences in the leak between WT and NOS1−/− at 23°C in the presence of ROS. The interaction of NO with superoxide yields peroxynitrite, a highly reactive molecule that is more RyR2-damaging than ROS.15,28

Consistent with observations in NOS1−/− cardiomyocytes that the complete absence of NOS1-derived NO is associated with greatly enhanced XOR activity,16,27 the reduction in ROS by oxypurinol was associated with reduced SR Ca$^{2+}$ leak. In addition, the complete elimination of ROS by Tempol,
suggested that enhanced
activity, would not affect leak at physiologi-
cal temperature. In contrast to WT, decreasing temperature
abolished the leak–temperature dependence either in WT or
NOS1−/− cardiomyocytes, confirming the key role of ROS in
this process. Other sources of ROS might play a signaling
role, affecting the Ca2+ leak control. For instance, mitochon-
drial ROS release and ERK activation in ventricular myocytes.30 Moreover, additional evidence that ROS are associated with leak is the fact that, at subphysiological temperatures, where the leak is reduced in GSNOR−/− compared with WT cardiomyocytes, there is also less ROS. In this regard, RyR2 is a highly redox-sensitive Ca2+ channel and its activity is increased by oxidizing agents and decreased by reducing agents. However, its regulation is even more complex. Thus, the intracellular NO/redox equilibrium is a critical factor determining the responsiveness to Ca2+ of the RyR2 channel. Post-translational modifications of RyR2, such as S-glutathionylation31 or S-nitrosylation,18,19 are considered protective against irreversible oxidation of redox-sensitive cysteine residues on this channel. As shown previously,21 the leak in GSNOR−/− cardiomyocytes was not different from WT at 37°C, consistent with there being no difference in ROS, and suggesting that enhanced S-nitrosylation caused by deficient denitrosylation activity, would not affect leak at physiological temperature. In contrast to WT, decreasing temperature did not increase the leak in GSNOR−/− cells, which could indicate a redox-protective effect of S-nitrosylation at subphysiological temperatures. Further evidence for this hypothesis is the fact that the exposure of GSNOR−/− cardiomyocytes to an oxidative stimulus was not sufficient to break down this redox-protective shield mediated by hyper S-nitrosylation.

We mentioned above that a lack of NOS1-derived NO pro-
duction may be the underlying cause of the atypical SR Ca2+
leak–temperature pattern in cardiomyocytes from NOS1−/− mice. We have shown that restoring the NO supply in this model rescues the pattern of high leak at room temperature and low leak at the physiological range of temperatures as ob-
erved in WT cardiomyocytes. However, there is a complexity
to NO signaling which is evidenced in this work by the ability of 1 μmol/L but not 50 μmol/L of SNAP to elicit such an effect. We previously showed a triphasic response of SR Ca2+ leak to increasing doses of nitroglycerin in NOS1−/− car-
diomyocytes,9 thus the effects of exogenously added NO are highly dependent on the concentration and the nature of the NO donor. Accordingly, the idea that reduced NOS1 activity promotes a local microenvironment for this particular behavior of SR Ca2+ leak, explains the specific inhibition of NOS1 in WT cardiomyocytes yielding results consistent with those in the NOS1 knockout model. As expected, the high leak at 23°C in WT was abolished and the low leak at 37°C was increased by 1-l-VNIO. However, the effect was less robust at the physi-
ological temperature because this increase was only half the level of the leak observed in NOS1−/− at this temperature. We speculate that the intracellular metabolism of the drug might be different at 37°C and therefore, the effective concentration of 1-l-VNIO might not be sufficient to efficiently inhibit NOS1. Alternatively, the time of exposure to the inhibitor may not be adequate to generate the characteristic NO/redox imbalance as seen by the chronic deletion of NOS1. The risk of using a higher dose of 1-l-VNIO is the loss of specificity. Thus, we confirmed that defective NOS1-derived NO production leads to an NO/redox imbalance that differentially affects SR Ca2+ leak at physiological or subphysiological temperatures in isolated cardiomyocytes.

Reduced activity of NOS1 at low temperature may be the cause of increased XOR-mediated ROS in WT cardiomyo-
cytes. Moreover, this redox dysregulation would uncouple
NOS1, further contributing to ROS production (see the pro-
posed working model in Figure 8). Thus, recoupling of NOS1, as mediated by BH4 treatment, restores the redox balance and leads to low levels of SR Ca2+ leak. It was recently shown that BH4 supplementation restores NO production and S-
nitrosylation of proteins, in cardiomyocytes from a model of dystrophic cardiomyopathy exhibiting oxidative stress.20

It has been demonstrated previously that NO-mediated S-
nitrosylation of RyR2 enhances its activity.3,32 However, it is important to differentiate activation of the channel from a leaky channel. RyR2 activity is regulated by many factors, including Ca2+, phosphorylation, S-nitrosylation, oxidation, etc. Despite activation, the channel might be more or less stabilized, exhibiting different levels of diastolic Ca2+ leak. Recent studies have associated low S-nitrosylation levels with increased leak measured at 37°C in different models,16–20 suggest-
ging that NO-mediated S-nitrosylation reduces leak. Here, we propose that temperature is a variable that, by affecting
the NO/redox balance, may change the response of RyR2 to exogenous NO.

Ca²⁺ stores in NOS1−/− cardiomyocytes are highly dependent on the leak of Ca²⁺ from the SR, because increased leak at higher temperature is associated with depleted SR and vice versa. In addition to the increased leak at 37°C, a slow Ca²⁺ decay (Online Figure III) also contributes to the depletion of SR Ca²⁺ stores at this temperature in NOS1−/−. However, Ca²⁺ decay is not different from WT at 23°C (Online Figure III), thus the decreased Ca²⁺ leak would be enough to explain the augmented Ca²⁺ content at low temperature. This dependence allows us to speculate that SR Ca²⁺ stores in this model of NO/redox imbalance are poorly regulated. There was no obvious association between SR Ca²⁺ content and leak within the range of temperatures studied in WT myocytes, suggesting that despite reduced Ca²⁺ decay with decreasing temperature (Online Figure III), it may be enough to compensate for the increased leak in normal mice. Similarly, SR Ca²⁺ content was not significantly affected by leak in GSNOR−/− cardiomyocytes. We would expect an enhanced SR filling in GSNOR−/− because they exhibit not only lower leak at subphysiological temperatures but also faster Ca²⁺ reuptake than WT (Online Figure III).

Paradoxically, we did not observe such a response. We do not have a logical explanation for these results, but just speculations of a tight set point for the SR filling as a consequence of the hyper S-nitrosylation phenotype. Further investigations of this model of aberrant S-nitrosylation should elucidate the underlying mechanisms.

Potential Implications

Several studies have investigated the effect of hypothermic preconditioning or hypothermia on ischemia–reperfusion and myocardial infarction. These studies demonstrate that therapeutic hypothermia profoundly prevents the deleterious effects induced by these experimental models of myocardial injury. Therapeutic hypothermia is broadly tested in clinical trials and protocols are still being optimized with the main goal of reducing neurological impairment after cardiac arrest. However, the understanding of its benefits on cardioprotection is limited. Reducing temperature in a normal healthy heart increases the Ca²⁺ leak, which would be detrimental to the myocardium. Although this effect of cooling does not seem to be protective for healthy cardiomyocytes, the perspective changes when the scenario switches to a highly aggressive condition, such as ischemia–reperfusion, where a burst of ROS is released and the NO/redox equilibrium is disrupted. Indeed, the latter situation is better represented by the NO/redox imbalance described for NOS1−/− hearts at physiological temperature. On reperfusion, the ischemic myocardium undergoes oxidative stress, leading to Ca²⁺ handling abnormalities, including SR Ca²⁺ leak, which is characteristic in cardiomyocytes with NOS1 deletion. Therefore, inhibition of NOS1 during therapeutic hypothermia could avoid peroxynitrite formation, and might yield better results in terms of reducing spontaneous SR Ca²⁺ release into the cytosol, thereby improving cardioprotection and the

Figure 7. Nitric oxide (NO) synthase uncoupling increases leak at low temperature in wild-type (WT) cardiomyocytes. A, NO generation in WT cardiomyocytes measured by detection of diaminofluorescein (DAF-2) at 23°C, 25°C, 30°C, 34°C, or 37°C. Fluorescence was expressed as relative fluorescence units (F/F₀) by normalization of the DAF-2 signal at each temperature (F) to the fluorescence at 23°C (F₀; n=4 mice; *P<0.05 vs 23°C; 1-way ANOVA). B, Linear correlation between sarcoplasmic reticulum (SR) Ca²⁺ leak and NO production in WT cardiomyocytes at all studied temperatures. C, SR Ca²⁺ leak (at matched SR Ca²⁺ load ≈75 μmol/L) in WT cardiomyocytes in the absence (baseline [BL]) or the presence of 100 μmol/L tetrahydrobiopterin (BH₄) for 2 or 20 minutes (*P<0.05; Student t test). D, mRNA content of nitric oxide synthase (NOS)-1, NOS2, and NOS3 in WT cardiomyocytes exposed to 23°C, 30°C, and 37°C for 30 minutes (n=4; Student t test).
outcomes of this powerful intervention. Alternatively, according to our data, inhibition of GSNOR during hyperthermia may also add substantial improvements to this protocol because Ca\textsuperscript{2+} leak is kept low during cooling in the GSNOR-deficient model.

**Conclusion**

Here, we show that Ca\textsuperscript{2+} leak increases when the temperature drops, coinciding with an XOR-mediated increase in ROS production and NOS1 uncoupling. In contrast, the leak rises at temperatures >30°C in the absence of NOS1 activity and is also associated with elevated ROS. Deficient denitrosylation has a protective role by maintaining low leak levels independent of the temperature. These results suggest that Ca\textsuperscript{2+} leak from the SR is regulated by a crucial interaction between temperature and NO/redox balance.

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**Disclosures**

None.

**References**


The response to temperature of cardiomyocytes lacking neuronal NO synthase is dependent on temperature and is strongly associated with temperature on myocardial protection conferred by ischaemic preconditioning or hypothermia in patients undergoing reperfusion postischemia or myocardial infarction, might be beneficial in preventing a temperature-dependent increase in calcium leak, thereby favoring cardioprotection and improving clinical outcomes.

What New Information Does This Article Contribute?

- Calcium leak is dependent on temperature and is strongly associated with NO and reactive oxygen species.
- The response to temperature of cardiomyocytes lacking neuronal NO synthase activity (neuronal nitric oxide synthase-deficient mice-1 [NOS1−/−] mouse model) is opposite that of wild-type cardiomyocytes, whereas deficient denitrosylation (GSNOR−/− mouse model) protects against the cooling-induced rise in reactive oxygen species and calcium leak.

Using pharmacological interventions on isolated cardiomyocytes derived from NOS1−/−, GSNOR−/−, and wild-type mouse models, we demonstrate that calcium leak exhibits changes that are regulated by an interaction between temperature and the NO/redox balance. This work highlights that temperature is a crucial factor in NO cardiobiology, and reconciles controversial findings in this field. Moreover, these results support the proposed cross-talk between xanthine oxidase and neuronal NO synthase in the regulation of the calcium channel gating in the heart. Decrease in temperature toward subphysiological conditions disrupts this cross-talk favoring a rise of reactive oxygen species and therefore increasing calcium leak. Hence, either inhibition of neuronal NO synthase or preventing denitrosylation during therapeutic hypothermia in patients undergoing reperfusion postischemia or myocardial infarction, might be beneficial in preventing a temperature-dependent increase in calcium leak, thereby favoring cardioprotection and improving clinical outcomes.
Interaction Between Neuronal Nitric Oxide Synthase Signaling and Temperature Influences Sarcoplasmic Reticulum Calcium Leak: Role of Nitroso–Redox Balance
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**SUPPLEMENTAL MATERIAL**

**Expanded Methods**

**Animal Models**

We studied age matched C57BL/6J mice (wild-type, WT; n=26) and mice with a homozygous deletion of NOS1 (B6;129S4-Nos1<tm1Pleo/J; n=20; Jackson Laboratories, Bar Harbor, ME); or S-nitroso glutathione reductase (GSNOR<sup>−/−</sup>) (n=10). All protocols and experimental procedures were approved by the Animal Care and Use Committee of the University of Miami and followed the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-234, revised 2011).

**Myocyte Isolation**

Cardiac myocytes were isolated and prepared from hearts as previously described<sup>1</sup>. Briefly, mice were sacrificed by cervical dislocation. Hearts were harvested and perfused retrogradely in a modified Langendorff system (constant flow 2 mL/min) with an isolation solution containing (in mmol/L): NaCl 120, KCl 5.4, MgSO<sub>4</sub> 1.4, NaH<sub>2</sub>PO<sub>4</sub> 1.2, NaHCO<sub>3</sub> 20, 2,3-butanediene monoxime (BDM, Sigma-Aldrich Co. Saint Lois, MO) 10, taurine (Sigma-Aldrich Co. Saint Lois, MO) 5, glucose 5.6, bubbled with 5% CO<sub>2</sub>–95% O<sub>2</sub> for at least 15 minutes. Once cleaned, the hearts were perfused with collagenase type 2 (Worthington Biochemical Corporation, Lakewood, NJ) ~315 U/mL and protease type XIV (Sigma-Aldrich Co.) 5.2 U/mL for 10 minutes. After digestion, the hearts were quickly removed and cut into several chunks and myocytes were released by gentle mechanical disruption. The supernatant containing the dispersed myocytes was filtered through a 500 µm pore mesh into a 15 mL Falcon tube and centrifuged at 800 rpm for 1 minute. The cell pellet was resuspended in a Ca<sup>2+</sup>–free Tyrode solution (in mmol/L, 144 NaCl, 1 MgCl<sub>2</sub>, 10 HEPES, 5.6 glucose, 5 KCl adjusted to a pH 7 with NaOH) containing 0.5% bovine serum albumin to stop enzymatic digestion. The extracellular Ca<sup>2+</sup> was restored by three sequential additions of CaCl<sub>2</sub> (0.125, 0.25 and 0.5 mmol/L). Finally, the cardiomyocytes were resuspended in a 1.8 CaCl<sub>2</sub> Tyrode solution at room temperature and then loaded with Fura-2. Myocytes were stimulated at 0.5 or 1 Hz.

**Intracellular Ca<sup>2+</sup> Measuring**

Intracellular Ca<sup>2+</sup> was measured using the Ca<sup>2+</sup>-sensitive dye Fura-2 (Molecular Probes, Eugene, OR, USA) and a dual-excitation spectrofluorometer (IonOptix LLC, Milton, MA, USA), excited with a xenon lamp at wavelengths of 340 and 380 nm. The emission fluorescence was reflected through a barrier filter (510 ± 15 nm) to a photomultiplier tube. The “in vivo” calibration was performed superfusing a free Ca<sup>2+</sup> and then a Ca<sup>2+</sup> saturating (5 mmol/L) solution both containing 10 µmol/L ionomycin (Sigma-Aldrich, St. Louis, MO) until reaching a minimal (R<sub>min</sub>) or a maximal (R<sub>max</sub>) ratio values, respectively. [Ca<sup>2+</sup>]<sub>i</sub> was calculated using the following equation, as described previously<sup>2</sup>:

\[
[Ca^{2+}]_i = \frac{K_d \cdot (S_f^2/S_b^2) \cdot (R-R_{min})}{(R_{max}-R)}
\]

K<sub>d</sub> (dissociation constant) in adult myocytes was taken as 224 nmol/L, R<sub>min</sub>=0.479 and R<sub>max</sub>=2.920 were measured experimentally. The scaling factors S<sub>f</sub>=4608 and S<sub>b</sub>=2865 were extracted from calibration as described by Grynkiewicz et al<sup>3</sup>. Experiments were then repeated in the conditions detailed below (see Treatments). Δ[Ca<sup>2+</sup>]<sub>i</sub> amplitude was considered as: peak [Ca<sup>2+</sup>]<sub>i</sub> – resting [Ca<sup>2+</sup>]<sub>i</sub>.

**SR Ca<sup>2+</sup> Leak and SR Ca<sup>2+</sup> Load Measuring**

SR Ca<sup>2+</sup> leakage was assessed with tetracaine (Sigma-Aldrich, St. Louis, MO, USA) as described by Shannon et al<sup>4</sup>. Briefly, after pacing was stopped, a fast switch to a 0Na<sup>+</sup>/0Ca<sup>2+</sup> Tyrode solution (Na<sup>+</sup> was replaced by an equimolar amount of Li<sup>+</sup>) was performed. After 60 seconds, as described by Bassani et
al.\textsuperscript{5}, a rapid switching to \(0\text{Na}^+/0\text{Ca}^{2+}\) solution containing 20 mM caffeine to assess SR \(\text{Ca}^{2+}\) content was applied. Following recovery of the cell, the same pacing protocol was assessed. After stop pacing, a switch to \(0\text{Na}^+/0\text{Ca}^{2+}\) Tyrode solution containing 1 mmol/L tetracaine was performed. The observed decrease in the Fura-2 ratio in presence of tetracaine compared to the non-tetracaine treated condition was considered the \(\text{Ca}^{2+}\) leak for a particular myocyte. After assessing \(\text{Ca}^{2+}\) leak, tetracaine was washed out by superfusing fresh \(0\text{Na}^+/0\text{Ca}^{2+}\) Tyrode solution and SR \(\text{Ca}^{2+}\) content was assessed by caffeine challenge.\textsuperscript{5} SR \(\text{Ca}^{2+}\) contents were calculated considering that SR represents 3.5\% and cytosol 65\% of the myocyte volume as previously described.\textsuperscript{2} The following equation from Shannon et al.\textsuperscript{4} was used:

\[
[\text{Ca}^{2+}]_{\text{SR}} = [\text{Ca}^{2+}]_{\text{caff}} + (\beta_{\text{max-SR}} *[\text{Ca}^{2+}]_{\text{caff}})/([\text{Ca}^{2+}]_{\text{caff}} + K_d\text{-SR})
\]

\([\text{Ca}^{2+}]_{\text{SR}}\) is the SR \(\text{Ca}^{2+}\) content, \([\text{Ca}^{2+}]_{\text{caff}}\) is the SR \(\text{Ca}^{2+}\) released by caffeine, \(\beta_{\text{max-SR}}\) and \(K_d\text{-SR}\) are the usual Michaelis parameters for SR \(\text{Ca}^{2+}\) binding. SR leak-SR load pairs were grouped by similar SR \(\text{Ca}^{2+}\) load and expressed as a leak-load relationship fitted by an exponential growth function using the Graph Pad Prism software (version 4.02). Measurements were mostly carried out at 23°C, 25°C, 30°C, 34°C or 37°C.

**Treatments**

Cardiomyocytes loaded with Fura-2 were pre-incubated for 20 minutes with the following compounds (unless otherwise is specified): Tempol (100 \(\mu\text{mol/L}, \text{Calbiochem, Calbiochem/EMD Biosciences, San Diego, CA, USA}); Oxypurinol (100 \(\mu\text{mol/L}, \text{Sigma-Aldrich}); (\pm)-\text{S-Nitroso-N-acetylpenicillamine (SNAP) (1 or 50} \(\mu\text{mol/L, Santa Cruz, Santa Cruz, CA, USA}); N\text{\textsuperscript{5}}-(1\text{-Imino-3-buteryl})\text{-L-Ornithine (L-VNIO) (100} \(\mu\text{mol/L, Enzo Life Sciences, Plymouth Meeting, PA, USA}); \text{Hydrogen peroxide (H}_2\text{O}_2\) (100 \(\mu\text{mol/L, Sigma-Aldrich}); (6R)-5,6,7,8\text{-Tetrahydrobiopterin dihydrochloride (BH}_4\) (300 \(\mu\text{mol/L, Sigma-Aldrich}). Experiments in control condition (no treatment) were run in parallel to each type of pharmacological intervention for each batch of cardiomyocytes.

**Detection of Reactive Oxygen Species (ROS)**

ROS were measured by using the sensitive probe 2’,7’-dichlorodihydrofluoresceine (H\textsubscript{2}DCF–DA, 10 \(\mu\text{M}; \text{Molecular Probes}) in two different ways. First, fresh isolated mouse cardiomyocytes were placed in the chamber of an IonOptix spectrofluorometer and the background fluorescence (\(F_0\)) was acquired with an excitation wavelength of 488 nm and emission fluorescence collected at 510 ± 15 nm. Then, cardiomyocytes were incubated during 30 min at 23°C or 37°C with H\textsubscript{2}DCF–DA and washed by superfusing fresh Tyrode (1.8 mM \(\text{CaCl}_2\)) solution for 15 minutes to allowed desterification of H\textsubscript{2}DCF. The initial fluorescence (\(F_0\)) and a second measure after 5 minutes (\(F\)) were acquired. Myocytes were stimulated at 1 Hz. ROS were expressed as:

\[
\text{ROS} = (F-F_0)-(F_0-F_a)
\]

Alternatively, control or 100 \(\mu\text{mol/L oxypurinol–treated cardiomyocytes were loaded with H}_2\text{DCF–DA for 15 minutes on polyllysine–coated microscope slides at 23°C or 37°C. After 10 minutes washing with Tyrode solution, cardiomyocytes were fixed with 2\% \text{p-formaldehyde in cold phosphate buffered saline (PBS) and then washed once with PBS. Mounting medium was applied and slides kept at 4°C until fluorescence was measured. Cardiomyocytes treated with 1 mmol/L \text{H}_2\text{O}_2 at either 23°C or 37°C, from each mouse model, were used as positive control for ROS and were used as maximal fluorescence signal for normalization of each group (F_{\text{max}}). Fluorescence (F) was captured by an inverted fluorescence microscope Olympus IX81 and a CDD camera Retiga 2000R (Q Imaging) with an excitation wavelength of 488 nm and 525 nm emission. Images were quantified by ImageJ (NIH) software and results were expressed as:}
\[ F_{DCF} = \frac{F - F_0}{F_{max} - F}, \]

where \( F_0 \) is background.

**Nitric Oxide Measurement**

Isolated WT mouse cardiomyocytes were loaded with the fluorescent NO-sensitive dye 4,5-diaminofluorescein diacetate (5 μmol/L DAF-2 DA; Calbiochem/EMD Biosciences, San Diego, CA, USA) for 20 minutes at room temperature and then washed for 20 more minutes to allow intracellular deesterification. Cells were set in the perfusion chamber of an IonOptix system and fluorescence (excited at 488 nm and emission collected at 510 ± 15 nm) was recorded three times during a stabilization period of 15 min at 23°C. Then, fluorescence was also acquired at 25°C, 30°C, 34°C and 37°C for each cell. DAF-2 fluorescence intensity (F) was expressed as F/F₀, where F₀ is the fluorescence intensity at 23°C after the stabilization time.

**NOS isoform expression**

Cardiomyocytes from 4 WT hearts were exposed to different temperature and then collected in RNA later lysis buffer (Qiagen, Valencia, CA). Total RNA was extracted from cells using Pure-Link Micro-to-Midi Total RNA Purification System (Qiagen) and reverse-transcribed using High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). All samples were treated with TurboTM DNase (Ambion, Austin, TX). Quantitative real-time PCR was performed in triplicate using a 20 μl reaction mixture containing 10 ng cDNA, TaqMan Universal PCR Master Mix (Roche, Branchburg, NJ) and primer/probe sets for nitric oxide synthase 1 (Nos1, Mm00435171_m1), Nos2 (Mm00440502_m1), and Nos 3 (Mm00435197_g1 ) (TaqMan Gene Expression Assay, Applied Biosystems, Foster City, CA). As an internal control glucoronidase beta (GUSB, Mm01197698_m1) was determined in each reaction. Reactions conditions were performed according to manufacturer instructions: 1 cycle of 50°C for 2 minutes, 1 cycle of 90°C for 10 minutes and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Software from iQ5 multicolor real-time PCR detection system (Bio-Rad, Hercules, CA) was used for PCR analyses. mRNA relative expression was calculated by 2ΔCt method. qPCR data reflects four different experiments each done at 23°C, 30°C, and 37°C.

**Statistical analysis**

Data are expressed as mean ± SEM. For Leak–Load relationship, an exponential growth fit, which compares independent fits with a global shared fit, was applied. Two or more groups of data were considered to fit different curves if \( p < 0.05 \). For comparisons of two groups, Student’s t test was used. For comparison of three or more groups, one or two way-ANOVA was performed. Two-way ANOVA was used when a second variable was involved. Newman-Keuls or Bonferroni’s post-hoc tests were used as appropriate by the GraphPad Prism version 4.02 (GraphPad Prism Software Corporation San Diego, CA USA). A \( p < 0.05 \) was considered significant.

**Reference List**


**Supplemental Figure legends**

**Online Figure I.** SR Ca2+ leak protocol. Protocol used to assess SR Ca2+ leak using tetracaine to block the RyR2 and caffeine to estimate SR Ca2+ load.

**Online Figure II.** Intermediate dose of SNAP does not change leak-temperature pattern in NOS1−/− cardiomyocytes. Dependence of SR Ca2+ leak on temperature in NOS1−/− cardiomyocytes in the absence or the presence of 50 µmol/L SNAP at matched SR Ca2+ load ≈60 µmol/L. Dependence of SR Ca2+ leak with temperature in WT cardiomyocytes is also displayed as reference.

**Online Figure III.** Ca2+ decay time constant. Ca2+ decay time constant (τ) in WT, NOS1−/− or GSNOR−/− cardiomyocytes, evaluated at 23°C or 37°C. Ca2+ decay is not different between WT and NOS1−/− at 23°C but it is slower in NOS1−/− cardiomyocytes at 37°C (*p* < 0.05, Student ‘s t-test). On the other hand, τ are similar at 37°C in WT and GSNOR−/− but smaller in GSNOR−/− at 37°C (*p* < 0.05 GSNOR−/− vs. WT, Student ‘s t-test).
Online Figure I

Stop pacing

+ Tetracaine

0Na+/0Ca²⁺

Leak

Caffeine
Online Figure II

SR Ca$^{2+}$ Leak (µM)

- WT
- NOS1$^{-/-}$
- NOS1$^{-/-}$ + 50 µM SNAP

Temperature (°C)
Online Figure III

![Bar chart showing Tau (τ) sec for different conditions: WT 23, NOS1⁺ 23, GSNOR⁺ 23, WT 37, NOS1⁻ 37, GSNOR⁺ 37. The bars are color-coded: black for WT and NOS1⁻, red for GSNOR⁺. The values are labeled on the bars: 21, 22, 15, 20, 21, 21. There are markings for NS and * indicating statistical significance.](image-url)