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

Nitric oxide (NO) exerts diverse regulation of cell signaling through a broad range of post-translational modifications, largely through S-nitrosylation of specific cysteine thiol moieties.\(^2\)-\(^4\) Despite the increased understanding of NO signaling and the identification of various NO synthase (NOS) isoforms in the cardiac myocyte, a consensus has not emerged on the mechanism underlying NO regulation of excitation–contraction coupling, and studies have reported diametrically opposite results. For example, NOS1–deficient mice-1 (NOS1\(^{-/-}\)) showed opposite behavior of cardiomyocytes in calcium (Ca\(^{2+}\)) handling and contractility\(^5\)-\(^7\) or sarcoplasmic reticulum (SR) Ca\(^{2+}\) leak\(^8\)-\(^9\) in comparison with wild-type (WT) cells. Similarly, studies showed opposite effects on β-adrenergic contractile responses.\(^1\)\(^-\)\(^7\) We hypothesized that other factor(s) can fundamentally change the direction of an NO-based physiological response. Here, we identify temperature as a key determinant of cardiomyocyte response to NO.

Temperature has a broad influence on Ca\(^{2+}\) signaling in cardiomyocytes\(^1\)\(^0\)-\(^1\)\(^1\) and there is a close relationship between body temperature and NO production in the pathogenesis...
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 Ca\(^{2+}\) leak. Post-translational modifications, such as S-nitrosylation, of RyR2 are influenced by cellular NO/redox state and can both positively and negatively affect SR Ca\(^{2+}\) leak.\(^{18,20}\) Signaling defects in Ca\(^{2+}\) handling, such as Ca\(^{2+}\) leak, contribute to impaired contractility in the failing heart, thus depleting SR Ca\(^{2+}\) 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 Ca\(^{2+}\) leak in isolated cardiomyocytes from WT mice or mouse models of aberrant S-nitrosylation, NOS1\(^{-/-}\) and S-nitrosogluthathione reductase–deficient (GSNOR\(^{-/-}\)) 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-Nos1\(^{tm1Plh}\); n=20; Jackson Laboratories, Bar Harbor, ME, stock number 002633); or with 5% CO\(_2\) and 95% O\(_2\) 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 Ca\(^{2+}\) was restored by sequential additions of CaCl\(_2\) in a Ca\(^{2+}\)-free Tyrode solution (see Online Data Supplement). Cardiomyocytes were resuspended in a 1.8 CaCl\(_2\) Tyrode solution at room temperature and then loaded with Fura-2. Myocytes were stimulated at 0.5 or 1 Hz.

**Intracellular Ca\(^{2+}\) Measurement**

Intracellular Ca\(^{2+}\) was measured using the Ca\(^{2+}\)-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 superfluensing a free Ca\(^{2+}\) and then a Ca\(^{2+}\) saturating (5 mmol/L) solutions both containing 10 μmol/L ionomycin (Sigma–Aldrich) until reaching a minimal (R\(_{\text{min}}\)) or a maximal (R\(_{\text{max}}\)) ratio values, respectively. [Ca\(^{2+}\)]\(_i\) was calculated as described previously.\(^3\)

**Measurement of SR Ca\(^{2+}\) Leak and SR Ca\(^{2+}\) Content**

SR Ca\(^{2+}\) leakage was assessed with 1 mmol/L tetracaine (Sigma–Aldrich) as described by Shannon et al.\(^2\) The observed decrease in the Fura-2 ratio in presence of tetracaine compared with the nontetra-caine-treated condition was considered the Ca\(^{2+}\) leak for a particular myocyte (Online Figure I). After assessing Ca\(^{2+}\) leak, tetracaine was washed out by superfluensing fresh 0Na\(^{+}/0Ca\(^{2+}\) Tyrode solution and SR Ca\(^{2+}\) content was assessed as described by Bassani et al.\(^3\) by a caffeine challenge. SR Ca\(^{2+}\) contents were calculated considering that SR represents 3.5% and cytosol 65% of the myocyte volume as previously described.\(^2\) SR leak–SR load pairs were grouped by similar SR 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 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); Oxyurinol (100 μmol/L; Sigma–Aldrich); (2)-S-Nitroso-N-acetylpenicillamine (SNAP; 1 or 50 μmol/L; Santa Cruz, Santa Cruz, CA); N\(_5\)-(1-imino-3-butenyl)-1-ornithine (1-VNIO; 100 μmol/L; Enzo Life Sciences, Plymouth Meeting, PA); Hydrogen peroxide (H\(_2\)O\(_2\); 100 μmol/L; Sigma–Aldrich) (6R)-5,6,7,8-Tetrahydrobiopterin dihydrochloride (BH\(_4\); 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′-dichlorodihydrofluorescein diacetate (10 μmol/L; Molecular Probes) in 2 different ways. First, fresh isolated mouse cardiomyocytes were placed in the chamber of an IonOptix spectrophotometer and the background fluorescence (F\(_0\)) 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 (F\(_i\)) and a second measure after 5 minutes (F\(_i\)) were acquired. Myocytes were stimulated at 1 Hz and ROS expressed as:

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

Alternatively, control or 100 μmol/L oxyurinol-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 H\(_2\)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 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:

\[
F_{\text{DCF}} = \frac{F - F_0}{F_{\text{max}} - F_0}
\]

where F\(_0\) 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 set in the perfusion chamber of an IonOptix system and fluorescence (excited at 488 nm and emission collected at 510±15 nm) was recorded during stabilization at 23°C. Then, fluorescence was also acquired at 25°C, 30°C, 34°C, and 37°C for each cell; 4,5-diaminofluorescein intensity was expressed as \( I_{F,0} \), where \( I_{F} \) 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 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 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 Bio systems, Foster City, CA). As an internal control glucuronidase, β-glucuronidase (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 iQ5 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, 34°C, and 37°C.

Statistical Analysis
Data were 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’s 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 Ca^{2+} 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 Ca^{2+} leak response to changes in temperature was different in these 2 models (Figure 1A). The pattern of SR Ca^{2+} leak–temperature, at matched SR Ca^{2+} 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. However, NOS1−/− cardiomyocytes exhibited a significant increase in leak when the temperature surpassed 30°C (Figure 1A) as we previously showed. This behavior is summarized in Figure 1B, which compares SR Ca^{2+} leak in NOS1−/− at 23°C with 37°C (Figure 1B; \( P=0.0054 \) and 2A).

Reactive Oxygen Species are Associated With the SR Ca^{2+} Leak
ROS signaling plays a role in the regulation of RyR channel gating by promoting redox post-translational modifications. In particular, oxidative stress induces RyR2 to leak Ca^{2+} from the SR. We found that SR Ca^{2+} 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 Ca^{2+} leak–temperature dependence, we first tested the effect of the superoxide scavenger Tempol on Ca^{2+} leak at 23°C, 30°C, and 37°C. Tempol eliminated the Ca^{2+} 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. Oxyipurinol 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 oxyipurinol between 23°C and 30°C ($K_{\text{Oxy}}=0.0089\pm0.0045$ versus $K_{\text{−Oxy}}=0.0048\pm0.0024$, $P=0.475$; Figure 3D), whereas at 34°C and 37°C oxyipurinol-treated NOS1−/− cardiomyocytes exhibited a leak–load curve that was less steep than NOS1−/− control ($K_{\text{Oxy}}=0.0030\pm0.0025$ versus $K_{\text{−Oxy}}=0.0117\pm0.0015$, $P=0.0481$ 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

Figure 2. Leak is associated to reactive oxygen species (ROS) in cardiomyocytes.

A, Sarcoplasmic reticulum (SR) Ca2+ leak in wild-type (WT) and neuronal nitric oxide synthase–deficient mice-1 (NOS1−/−) at 23°C or 37°C (at matched SR Ca2+ load ≈75 μmol/L; Student t test). B, ROS in WT and NOS1−/− at 23°C or 37°C (Student t test), as measured by epifluorescence of 2′,7′-dichlorodihydrofluorescein 5 minutes after the initial reading. C, Dependence of SR Ca2+ leak with temperature in WT or NOS1−/− cardiomyocytes in the absence or the presence of 1 mmol/L Tempol (at matched SR Ca2+ load ≈75 μmol/L; *$P<0.05$, **$P<0.01$ (−)Tempol vs +Tempol-treated WT; ††††$P<0.0001$ (−)Tempol vs +Tempol-treated NOS1−/− cardiomyocytes; 2-way ANOVA). D, SR Ca2+ load–leak relationship in WT or NOS1−/− cardiomyocytes, nontreated and Tempol-treated at 23°C (**$P<0.01$ WT (−)Tempol vs WT +Tempol; ††$P<0.01$ NOS1−/− (−)Tempol vs NOS1−/− +Tempol). E, At 37°C (**$P<0.01$ WT (−)Tempol vs WT +Tempol; †††$P<0.001$ NOS1−/− (−)Tempol vs NOS1−/− +Tempol; exponential growth fitting.
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 μmol/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 hyper S-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\(^{2+}\) content 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\(^2\)=0.9812; Figure 7B). This association suggests that temperature-dependent NOS activity plays an important role in SR Ca\(^{2+}\) leak regulation. We measured SR Ca\(^{2+}\) leak in WT cardiomyocytes at 23°C in the presence of tetrahydrobiopterin (BH\(_4\)), a NOS cofactor which also
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.$^{14}$

We previously showed that leak was increased in the absence of NOS1 at 37°C, whereas Wang et al$^8$ 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$^{27}$ 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,$^{18,27}$ the reduction in ROS by oxypurinol was associated with reduced SR Ca$^{2+}$ leak. In addition, the complete elimination of ROS by Tempol,
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 production 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 observed 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−/− cardiomyocytes, as seen by the chronic deletion of NOS1. The risk of using a higher dose of 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 cardiomyocytes. Moreover, this redox dysregulation would uncouple NOS1, further contributing to ROS production (see the proposed working model in Figure 8). Thus, recouping 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.

It has been demonstrated previously that NO-mediated S-nitrosylation of RyR2 enhances its activity. 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, suggesting 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.

Ca$^2+$ stores in NOS1$^{-/-}$ cardiomyocytes are highly dependent on the leak of Ca$^2+$ 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 Ca$^2+$ decay (Online Figure III) also contributes to the depletion of SR Ca$^2+$ stores at this temperature in NOS1$^{-/-}$. However, Ca$^2+$ decay is not different from WT at 23°C (Online Figure III), thus the decreased Ca$^2+$ leak would be enough to explain the augmented Ca$^2+$ content at low temperature. This dependence allows us to speculate that SR Ca$^2+$ stores in this model of NO/redox imbalance are poorly regulated. There was no obvious association between SR Ca$^2+$ content and leak within the range of temperatures studied in WT myocytes, suggesting that despite reduced Ca$^2+$ decay with decreasing temperature (Online Figure III), it may be enough to compensate for the increased leak in normal mice. Similarly, SR Ca$^2+$ 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 Ca$^2+$ 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.

**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/F$_0$) by normalization of the DAF-2 signal at each temperature (F) to the fluorescence at 23°C (F$_0$; n=4 mice; *P<0.05 vs 23°C; 1-way ANOVA). **B.** Linear correlation between sarcoplasmic reticulum (SR) Ca$^{2+}$ leak and NO production in WT cardiomyocytes at all studied temperatures. **C.** SR Ca$^{2+}$ leak (at matched SR Ca$^{2+}$ load $\approx$75 μmol/L) in WT cardiomyocytes in the absence (baseline [BL]) or the presence of 100 μmol/L tetrahydrobiopterin (BH$_4$) 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).

**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 Ca$^2+$ 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 Ca$^{2+}$/calmodulin-dependent protein kinase II, leading to Ca$^{2+}$ handling abnormalities, including SR Ca$^{2+}$ 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 Ca$^{2+}$ release into the cytosol, thereby improving cardioprotection and the
outcomes of this powerful intervention. Alternatively, according to our data, inhibition of GSNOR during hypothermia may also add substantial improvements to this protocol because Ca$_{\text{2+}}$ leak is kept low during cooling in the GSNOR-deficient model.

**Conclusion**

Here, we show that Ca$_{\text{2+}}$ leak increases when the temperature drops, coinciding with an XOR-mediated increase in ROS production and NO uncoupling. In contrast, the leak rises at temperatures >30°C in the absence of NO 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$_{\text{2+}}$ leak from the SR is regulated by a crucial interaction between temperature and NO/redox balance.

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**Disclosures**

None.

**References**


Temperature Influences NOS1 Signaling and Ca\(^{2+}\) Leak

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What Is Known?

- Nitric oxide (NO) signaling modulates cardiac function, including calcium cycling in cardiac myocytes.
- Abnormalities in calcium cycling, such as increased calcium leak from intracellular stores, severely affect cardiac performance.
- Changes in temperature affect the metabolism of NO and consequently NO signaling in the heart.

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 cardiac myocytes 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 postschisma or myocardial infarction, might be beneficial in preventing a temperature-dependent increase in calcium leak, thereby favoring cardioprotection and improving clinical outcomes.

References

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

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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<tm1Plh>J; n=20; Jackson Laboratories, Bar Harbor, ME); or S-nitrosoglutathione 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>:

\[
[Ca^{2+}]_i = K_d \times (Sf_2/Sb_2) \times (R - R_{min})/(R_{max} - R)
\]

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 Sf<sub>2</sub>=4608 and Sb<sub>2</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). ∆[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 0Na\textsuperscript{+}/0Ca\textsuperscript{2+} solution containing 20 mM caffeine to assess SR Ca\textsuperscript{2+} content was applied. Following recovery of the cell, the same pacing protocol was assessed. After stop pacing, a switch to 0Na\textsuperscript{+}/0Ca\textsuperscript{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 Ca\textsuperscript{2+} leak for a particular myocyte. After assessing Ca\textsuperscript{2+} leak, tetracaine was washed out by superfusing fresh 0Na\textsuperscript{+}/0Ca\textsuperscript{2+} Tyrode solution and SR Ca\textsuperscript{2+} content was assessed by caffeine challenge.\textsuperscript{5} SR Ca\textsuperscript{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 Ca\textsuperscript{2+} content, [\text{Ca}^{2+}]_{\text{caff}} is the SR Ca\textsuperscript{2+} released by caffeine, \(\beta_{\text{max-SR}}\) and \(K_d_{\text{SR}}\) are the usual Michaelis parameters for SR Ca\textsuperscript{2+} binding. SR leak-SR load pairs were grouped by similar SR Ca\textsuperscript{2+} load and expressed as a leak-load relationship fitted by an exponential growth function using the GraphPad 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 \(\mu\text{mol/L}\), Calbiochem, Calbiochem/EMD Biosciences, San Diego, CA, USA); Oxypurinol (100 \(\mu\text{mol/L}\), Sigma-Aldrich); (±)-S-Nitroso-N-acetylpenicillamine (SNAP) (1 or 50 \(\mu\text{mol/L}\), Santa Cruz, Santa Cruz, CA, USA); N\textsuperscript{5}-(1-Imino-3-butenyl)-L-Ornithine (L-VNIO) (100 \(\mu\text{mol/L}\), Enzo Life Sciences, Plymouth Meeting, PA, USA); Hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) (100 \(\mu\text{mol/L}\), 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 \(\mu\text{M}\); 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 CaCl\textsubscript{2}) solution for 15 minutes to allowed desterification of H\textsubscript{2}DCF. The initial fluorescence (\(F_i\)) and a second measure after 5 minutes (F) were acquired. ROS were expressed as:

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

Alternatively, control or 100 \(\mu\text{mol/L}\) oxypurinol–treated cardiomyocytes were loaded with H\textsubscript{2}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% 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 H\textsubscript{2}O\textsubscript{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:

**Supplemental Material- page 2**
\[ F_{DCF} = \frac{(F-F_0)}{(F_{max}-F)} \]

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 Bio systems, 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

![Graph showing effects of Tetracaine and Caffeine on ratio (F340/F380) over time.](image)

- **Ratio (F340/F380)**
- **Time (sec)**
- **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](chart.png)

- **Tau (τ) sec**
- **WT 23 C**: 21
- **NOS1 + 23 C**: 22
- **GSNOR + 23 C**: 15
- **WT 37 C**: 20
- **NOS1 + 37 C**: 21
- **GSNOR + 37 C**: 21

*NS* indicates a non-significant difference.

* indicates a significant difference.