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>; 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-butadiene 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) solutions 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>] was calculated using the following equation, as described previously<sup>2</sup>:

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

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 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>], amplitude was considered as: peak [Ca<sup>2+</sup>] – resting [Ca<sup>2+</sup>].

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., a rapid switching to 0Na+/0Ca2+ solution containing 20 mM caffeine to assess SR Ca2+ content was applied. Following recovery of the cell, the same pacing protocol was assessed. After stop pacing, a switch to 0Na+/0Ca2+ 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 Ca2+ leak for a particular myocyte. After assessing Ca2+ leak, tetracaine was washed out by superfusing fresh 0Na+/0Ca2+ Tyrode solution and SR Ca2+ content was assessed by caffeine challenge. SR Ca2+ contents were calculated considering that SR represents 3.5% and cytosol 65% of the myocyte volume as previously described. The following equation from Shannon et al. was used:

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

[Ca2+]SR is the SR Ca2+ content, [Ca2+]caff is the SR Ca2+ released by caffeine, βmax-SR and Kd-SR are the usual Michaelis parameters for SR Ca2+ binding. 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 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 µmol/L, Calbiochem, Calbiochem/EMD Biosciences, San Diego, CA, USA); Oxypurinol (100 µmol/L, Sigma-Aldrich); (±)-S-Nitroso-N-acetylpenicillamine (SNAP) (1 or 50 µmol/L, Santa Cruz, Santa Cruz, CA, USA); N5-(1-Imino-3-butenyl)-L-Ornithine (L-VNIO) (100 µmol/L, Enzo Life Sciences, Plymouth Meeting, PA, USA); 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 (ROS)
ROS were measured by using the sensitive probe 2’,7’-dichlorodihydrofluoresceine (H2DCF–DA, 10 µM; Molecular Probes) in two different ways. First, fresh isolated mouse cardiomyocytes were placed in the chamber of an IonOptix spectrofluorometer and the background fluorescence (F0) 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 H2DCF–DA and washed by superfusing fresh Tyrode (1.8 mM CaCl2) solution for 15 minutes to allowed desterification of H2DCF. The initial fluorescence (F1) and a second measure after 5 minutes (F) were acquired. Myocytes were stimulated at 1 Hz. ROS were expressed as:

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

Alternatively, control or 100 µmol/L oxypurinol–treated cardiomyocytes were loaded with H2DCF–DA 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 (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 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 (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} = (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 desterification. 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 $F/F_0$, where $F_0$ 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 $2\Delta Ct$ 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

Ratio (F340/F380) vs. Time (sec)

- 0Na+/0Ca²⁺
- + Tetracaine
- Leak
- Stop pacing
- Caffeine
Online Figure II

- WT
- NOS1\(^{-/-}\)
- NOS1\(^{-/-}\) + 50 \(\mu\)M SNAP

SR \(\text{Ca}^{2+}\) Leak (\(\mu\)M)

Temperature (\(^\circ\)C)