Supporting Information

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SI Materials and Methods

Experimental Preparation. Experiments were performed in accordance with Public Health Service guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University. Male guinea pigs were anesthetized (3 cc nembutal, intraperitoneal), and their hearts were rapidly removed via medial thoracotomy and Langendorff perfused during the entire experiment with oxygenated (100% O_2) Tyrode's solution containing (in millimoles) 140 NaCl, 0.7 MgCl, 4.5 KCl, 5.5 dextrose, 5 Hepes, and 5.5 CaCl2 (pH 7.45, 34 °C). Perfusion pressure was maintained between 50 and 70 mmHg by regulating coronary flow using a pulsatile flow system. Endocardial cryoablations were performed in all preparations to eliminate the Purkinje fibers and deeper layers of myocardium (1). To measure intracellular Ca^{2+} transients, hearts were stained with the Ca^{2+}-sensitive indicator Indo-1 AM (Invitrogen) at a final concentration of 10 μM for 30 min at room temperature to minimize dye compartmentalization, followed by a 15-min washout period. In all experiments, 7 μM of cytochalasin-D (Sigma-Aldrich) was used to ensure that motion artifact did not influence our result.

The perfused hearts were placed in a Lexan chamber. The mapping field was positioned over the left anterior descending artery just below its bifurcation with the diagonal coronary artery. To avoid epicardial surface cooling and desiccation, the heart was immersed in the coronary effluent, which was maintained at a uniform temperature (34 °C) equal to the perfusion temperature with a heat exchanger located in the chamber. The volume-conducted electrocardiogram (ECG) was monitored by using three Ag-AgCl disk electrodes fixed to the chamber in positions roughly corresponding to ECG limb leads I, II, and III. ECG signals were filtered (0.3–300 Hz), amplified (1,000×), and displayed on an oscilloscope. A fine-gauge (0.003-inch diameter), polytetrafluoroethylene-coated silver bipolar electrode was inserted into the left ventricular anterior wall to stimulate the ventricular endocardial surface at twice diastolic threshold current. Physiological stability of the preparation was assured by monitoring the ECG, coronary pressure, coronary flow, and perfusion temperature continuously throughout each experiment. Preparations remain viable for 4–5 h, but the entire experimental protocol typically lasted 1–2 h.

Excitation light was obtained from a 365-nM, 500 mW light-emitting diode (LED) (Nichea) and directed through a flexible liquid light guide (Thermo Oriel) to the preparation. Fluorescent light from the preparation was collected with two high-numerical aperture complex Nikon photographic lenses placed facing each other. To measure high-fidelity Ca^{2+} signals, a 445-nm long-pass filter (Chroma Technology) was positioned to transmit the Indo-1 fluorescence to a 16 × 16 element photodiode array (Hamamatsu). For the present study, an optical magnification of 1.24× was used, resulting in a total mapping field of 14.2 × 14.2 mm, with 0.9-mm spatial resolution and 0.81-mm^2 pixel size. All recordings made after Indo-1 loading were AC coupled which eliminated background fluorescence originating from the dye and the tissue (background fluorescence) to maximize the dynamic range of the photodiode array to image very small changes in intracellular Ca^{2+}. To view the preparation, a mirror was positioned to reflect visible light to a charge-coupled device video camera (SciMedia; MiCam02-HR).

Whole Heart Studies. Intracellular Ca^{2+} was measured using high-resolution optical mapping from the anterior surface of Langendorff-perfused guinea pig heart in the following groups: (*i*) control, normal $[Ca^{2+}]_I$; (*ii*) NOS1 inhibition (SMTC), normal $[Ca^{2+}]_i$ (1.25 mM); (*iii*) control, elevated $[Ca^{2+}]_i$ (5.5 mM); (*iv*) NOS1 inhibition, elevated $[Ca^{2+}]_I$; (*v*) NOS1 inhibition + allopurinol (elevated $[Ca^{2+}]_i$); and (*vi*) H_2O_2 (elevated $[Ca^{2+}]_i$) and NOS1 inhibition + H_2O_2 (elevated $[Ca^{2+}]_i$). Constant pacing over a broad range of pacing cycle lengths for 1 min followed by a halt in pacing was used to elicit multicellular spontaneous Ca^{2+} release (mSCR) activity and arrhythmias as previously described (2).

S-methyl-L-thiocitrulline (SMTC; EMD Milipore) is a specific inhibitor of NOS1 and is reported to exhibit ~17-fold increased selectivity for rat NOS1 (IC_{50} = 300 nM) compared with NOS3 (IC_{50} = 5.4 μM). Also, in a separate study the selectivity of SMTC for NOS1 was shown based on K_i values of 1.2, 11, and 40 nM for human NOS1, NOS3, and NOS2, respectively (3).

The concentration of reactive oxygen species (ROS) in the intact heart was measured by perfusing for 15 min with the ROS-sensitive dye dihydrorhodamine 6G (Invitrogen). Fluorescence levels were measured (excitation/emission: ~530/566 nm) from the anterior surface during steady-state pacing under baseline treatment conditions (see text for details) and then repeated following H_2O_2 (0.2 mM) and H_2O_2 (10 mM) for calibration.

Isolated Myocytes. Myocytes were isolated enzymatically from guinea pig hearts using the enzymatic dispersion technique described previously (4). Sarcomere shortening was assessed using a video-based sarcomere length detection system (IonOptix). Intracellular Ca^{2+} transients were measured using Indo-1 AM and the L-type Ca^{2+} current was measured using standard patch-clamp methods. Cardiomyocytes where rapidly paced (4 Hz) for 60 s in the presence of normal tyrodes with and without NOS1 inhibition (S-methyl L-thiocitrulline, SMTC), SMTC + allopurinol and SMTC + S-nitrosoglutathione (GSNO). After a halt in pacing, intracellular Ca^{2+} and cell length were measured to assess for the presence of Ca^{2+} waves and aftercontractions.

Assessment of Ryanodine Receptor S-Nitrosylation and -Oxidation. ***Isolation of sarcoplasmic reticulum vesicles.*** Following experimental treatment, isolated guinea pig hearts were removed from the bioassay chamber and frozen rapidly. Sarcoplasmic Reticulum (SR) vesicles were prepared essentially as described (5). Briefly, whole hearts were homogenized in buffer containing 20 mM Hepes (pH 7.4), 0.5 mM EDTA, 0.3 M sucrose, and protease inhibitors (100 nM aprotinin, 20 μM leupeptin, 1 μM pepstatin, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine). Homogenates were then subjected to centrifugation at 9,000 × *g* for 20 min and the resultant supernatant was centrifuged at 100,000 × *g* for 1 h to generate the microsomal fraction. The microsomal pellet was resuspended and fractionated on a discontinuous sucrose gradient (20%-30%-40%) by centrifugation at 100,000 × *g* for 15 h, and the SR vesicle fraction was collected and stored under liquid nitrogen. Protein concentration was determined with a bicinchoninic acid-based assay.

Assessment of RyR2 S-nitrosylation by resin-assisted capture of S-nitrosothiols (SNO-RAC). SNO-RAC was performed essentially as described (6). Briefly, SR vesicles were solubilized in HEN buffer (50 mM Hepes, 1 mM EDTA, 0.1 mM neocuproine) containing 2% SDS, and free thiols were blocked by incubation with S-methylmethanethiosulfonate [MMTS; 0.1% (wt/vol)] at 50 °C for 20 min. After acetone precipitation, proteins were resuspended in HEN buffer containing 1% (wt/vol) SDS and incubated with thiopropyl sepharose (thiol-capture beads) in the presence of

ascorbate (50 mM) for 4 h. Bound proteins were eluted with 285 mM 2-mercaptoethanol and Western blotted with anti-RyR2 antibody.

Quantification of RyR2-free thiols. SR vesicles were incubated with the fluorescent indicator monobromobimane (MBB) (7) for 1 h at room temperature. Vesicles were then solubilized in 2% SDS and, after removal of unbound MBB by size exclusion chromatography (P6 filtration), proteins were separated by SDS/PAGE. Gels were stained and the band containing RyR2 was excised, followed by in-gel digestion with trypsin. Fluorescence intensity of digests was assessed with a fluorescence spectrophotometer and extracted peptides were quantified with a bicinchoninic acid-based assay. Free thiols were quantified with reference to MBB-labeled glutathione standards.

Cardiac Ryanodine Receptor Phosphorylation. Tissue was obtained from the left ventricular free wall from control and NOS1-inhibited hearts. Western blotting was performed to determine the relative expression levels of cardiac ryanodine receptor (RyR2) and RyR2-P_{S2814}. Cardiac homogenates were separated on SDS/PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Blots were probed with the following primary antibodies, respectively: mouse anti-RyR2 (Affinity Bioreagents) and polyclonal antirabbit RyR-P_{S2814} (X. H. T. Wehrens, Baylor College of Medicine, Houston, TX). They were then treated with horseradish peroxidase-conjugated antimouse antibodies (Amersham). Protein bands were quantitated using ImageQuant software.

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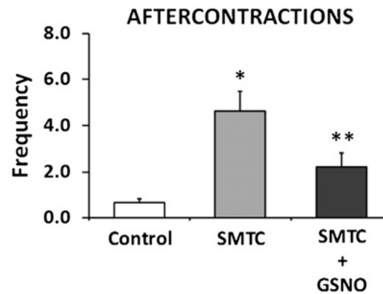

Fig. S1. S-nitrosoglutathione (GSNO) reverses the effects of NOS1 inhibition (SMTC) on arrhythmogenic aftercontractions in isolated myocytes. Treatment with GSNO ($n = 19$) during NOS1 inhibition suppressed aftercontractions compared with NOS1 inhibition alone ($n = 31$). *$P < 0.05$ compared with control. **$P < 0.05$ compared with SMTC.