Nitroxyl Improves Cellular Heart Function by Directly Enhancing Cardiac Sarcoplasmic Reticulum Ca\textsuperscript{2+} Cycling

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Abstract—Heart failure remains a leading cause of morbidity and mortality worldwide. Although depressed pump function is common, development of effective therapies to stimulate contraction has proven difficult. This is thought to be attributable to their frequent reliance on cAMP stimulation to increase activator Ca\textsuperscript{2+}. A potential alternative is nitroxyl (HNO), the 1-electron reduction product of nitric oxide (NO) that improves contraction and relaxation in normal and failing hearts in vivo. The mechanism for myocard effects remains unknown. Here, we show that this activity results from a direct interaction of HNO with the sarcoplasmic reticulum Ca\textsuperscript{2+} pump and the ryanodine receptor 2, leading to increased Ca\textsuperscript{2+} uptake and release from the sarcoplasmic reticulum. HNO increases the open probability of isolated ryanodine-sensitive Ca\textsuperscript{2+}-release channels and accelerates Ca\textsuperscript{2+} reuptake into isolated sarcoplasmic reticulum by stimulating ATP-dependent Ca\textsuperscript{2+} transport. Contraction improves with no net rise in diastolic calcium. These changes are not induced by NO, are fully reversible by addition of reducing agents (redox sensitive), and independent of both cAMP/protein kinase A and cGMP/protein kinase G signaling. Rather, the data support HNO/thiolate interactions that enhance the activity of intracellular Ca\textsuperscript{2+} cycling proteins. These findings suggest HNO donors are attractive candidates for the pharmacological treatment of heart failure. (Circ Res. 2007;100:96-104.)

Key Words: nitroxyl ■ contractility ■ ryanodine receptor ■ sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase ■ excitation/contraction coupling

Congestive heart failure affects an estimated 5 million people in the United States and has an annual mortality rate approaching 20%. More than half of the patients have depressed cardiac function, and, although improvement in function is clearly beneficial, as revealed by heart transplantation, development of effective pharmacological therapy to safely stimulate contraction has proven problematic.1 Most such agents rely on enhancing cAMP and protein kinase A (PKA) to stimulate activator Ca\textsuperscript{2+} and increase contractility. However, this approach is less effective in failing hearts, because of downregulation of the signaling,2 and is chronically linked to toxicity and increased mortality.

We recently reported that donors of nitroxy (HNO), the 1-electron reduction product of nitric oxide (NO),3 have novel cardiovascular effects quite different from NO. In intact in vivo hearts, the HNO donor Angeli’s salt (AS) enhances function independent of ß-adrenergic blockade or stimulation and unaccompanied by changes in cGMP.4,5 Unlike most prior positive inotropes, HNO donors are similarly effective in normal and failing hearts.5 Their combined ability to enhance heart function, while reducing venous pressures, has suggested potential utility as a heart failure treatment.

The mechanisms underlying cardiac action of HNO remain unknown. HNO can stimulate ion channels such as the N-methyl-D-aspartate receptor.6-7 Recent data suggest that it also activates the skeletal muscle ryanodine receptor (RyR).8 HNO is thought to react with targeted thiols9 and, more specifically, negatively charged thiols, or thiolates. These exist in several proteins involved in Ca\textsuperscript{2+} cycling, such as the sarcoplasmic reticular (SR) Ca\textsuperscript{2+} release channel,10 SR Ca\textsuperscript{2+}-ATPase, cyclic nucleotides and sarcoplasmic Ca\textsuperscript{2+}-mobilizing proteins.11-12

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pump (sarcoplasmic reticulum Ca^{2+}-ATPase [SERCA2a]), and possibly phospholamban.\textsuperscript{11} Hence, we hypothesized that HNO activity targets heart muscle cells and directly improves contraction and relaxation by enhancing Ca^{2+} cycling. Our results support improvement in SR Ca^{2+} uptake and release that is independent of cAMP/PKA or cGMP/PKG but, rather, related to thiol modification.

**Materials and Methods**

**Reagents**

AS (Na\(_2\)N\(_2\)O\(_3\)) was a generous gift of Dr Jon M. Fukuto and Matthew I. Jackson (University of California, Los Angeles). AS (100 mmol/L) stock solution was freshly prepared by dissolving AS in 10 mmol/L NaOH. Sodium-2-(\(N\),\(N\)-diethylamino)-diazenolate-2-oxide (DEA/NO) was purchased from Calbiochem (San Diego, Calif). Indo 1 acetylated ester (Indo 1-AM) was purchased from Molecular Probes/Invitrogen (Carlsbad, Calif). \(1H\)-[1,2,4]Oxadiazolo quinoxalin 1-one (ODQ) was obtained from Tocris (Ellisville, Mo). All other compounds were purchased from Sigma Chemical Co (St Louis, Mo; Milan, Italy).

**Contraction and Whole Ca\(^{2+}\) Transients in Mouse Ventricular Myocytes and Whole Ca\(^{2+}\) Transients and SR Ca\(^{2+}\) Load in Rat Ventricular Myocytes**

Wild-type 2- to 4-month-old mice were anesthetized with intraperitoneal pentobarbital sodium (100 mg/kg IP). Heart perfusion and isolation of rat ventricular myocytes were performed as described\textsuperscript{12} (see the online data supplement, available at http://circres.ahajournals.org). Functional measurements are described in the online data supplement. The protocols were all approved by the Animal Care and Use Committee of Johns Hopkins University.

**FRET Imaging**

Primary cultures of cardiac ventricular myocytes from 1- to 3-day-old Sprague–Dawley rats (Charles River Laboratories, Wilmington, Mass) were prepared according to Dostal et al.\textsuperscript{13} FRET analysis was performed as described\textsuperscript{14} (see online data supplement).

**Fluorescent Probes for Two-Photon Laser Scanning Microscopy and Image Acquisition**

The cationic potentiometric fluorescent dye tetracyanomethylrhodamine methyl ester (TMRM) was used to monitor changes in \(\Delta\psi_m\), as previously described.\textsuperscript{15} The production of the fluorescent glutathione adduct GSB from the reaction of cell permeant monochlorobimane (MCB) with reduced glutathione (GSH), catalyzed by glutathione S-transferase, was used to measure intracellular glutathione levels. Details of GSH measurements are provided in the online data supplement.

**Visualization of Spontaneous Ca\(^{2+}\) Sparks and Measurement of Spark Frequency**

Isolated mouse cardiac myocytes were loaded with the Ca\(^{2+}\) indicator fluo-4 acetylated ester (fluo-4/AM) (Molecular Probes, 20 \(\mu\)mol/L for 30 minutes). Confocal images were acquired using a confocal laser-scanning microscope (LSM510, Carl Zeiss) with a Zeiss Plan-Neofluor 40 oil immersion objective (NA = 1.3). Fluo-4/AM was excited by an argon laser (488 nm), and fluorescence was measured at 505 nm. Images were taken in the line-scan mode, with the scan line parallel to the long axis of the myocytes. Each image consisted of 512 line scans obtained at 1.92-ms intervals, each

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**Figure 1.** HNO increases contractility and relaxation in isolated ventricular myocytes. A, Effect of AS/HNO on sarcomere shortening in isolated mouse ventricular myocyte. B, Dose-response effect of AS/HNO and DEA/NO on cell shortening. \(\ast P<0.001\) vs control, \(\dagger P<0.01\) vs control, \(\ddagger P<0.0005\) vs control. C, AS/HNO effects on myocyte relaxation (time to 50% relengthening). \(\$ P<0.05\) vs control.
comprising 512 pixels at 0.10-μm separation. Digital image analysis used customer-designed programs coded in interactive data language and a modified spark detection algorithm.16

RyR2 Single-Channel Recordings in Planar Lipid Bilayers
Recording of single RyR2 in lipid bilayers was performed as described17 (see the online data supplement).

Measurements of ATP-Dependent Ca2+ Uptake by Murine Cardiac SR Vesicles
Crude cardiac microsomal vesicles containing fragmented SR were prepared as described18 (see also the online data supplement). SR membrane vesicles (0.4 mg/mL) suspended in a medium containing 100 mmol/L KCl, 1 mmol/L MgCl2, 50 μmol/L arsenazo III, 5 mmol/L sodium azide, and 20 mmol/L MOPS, pH 7.4, were mixed with an equal volume of an identical medium containing 1 mmol/L Na2ATP at 24°C in a manually operated stopped-flow apparatus (Applied Photophysics Ltd). The total [Ca2+]i in the uptake medium was 0.5 μmol/L, yielding a free [Ca2+]i in equilibrium with the Ca/arsenazo III complex of 0.2 μmol/L (Kd=3.3×10-11 mol/L). The change in [Ca2+]i was monitored at 0.1-second intervals using a single-beam UV-VIS spectrophotometer (AVIV, Model 14DS) with a monochromator setting of 650 nm. The signal change caused by vesicle light scattering was evaluated from separate measurements conducted under identical conditions at the isosbestic wavelength of 693 nm (red-shifted from 685 nm by the presence of protein). Addition of AS (250 μmol/L) to the incubation medium had no effect on the spectral characteristics of arsenazo III or its response to Ca2+. The kinetic and thermodynamic parameters for Ca2+ uptake were evaluated by fitting stopped-flow signals to 1- and 2-exponential decay functions plus a residual term using nonlinear regression. Residual plots of the difference between the fitted curve and data points were used to evaluate systematic errors in the fits and to calculate the sum-of-squares error used in selecting the best fit.

Results
To test whether HNO directly influences myocyte function, freshly isolated adult mouse myocytes (C57/Bl6) were exposed to AS (10−6 to 10−3 mol/L), matching concentrations relevant in vivo.4,5 Myocyte contractility rose in a dose-dependent manner (Figure 1A and 1B), peaking at 100% at 0.5 and 1 mmol/L (both P<0.00005). Myocyte relaxation rate also improved by 10% to 20% (Figure 1C; P<0.05). These changes plateaued after 10 to 15 minutes and were reversible (at ≤500 μmol/L) 15 minutes after stopping exposure to AS (Figure 1A). In contrast to HNO, the NO donor DEA/NO induced slight functional depression at low doses and minimal changes at higher doses (Figure 1B).

At physiological pH, AS decomposes into HNO and nitrite. We therefore tested whether nitrite might contribute to the observed response. AS decomposition in the identical medium and temperature as used in the myocyte studies yielded 25% nitrite generation after ≤5000 seconds (16 minutes). Identical results were obtained with 0.1 to 1 mmol/L AS. This

Figure 2. AS/HNO actions on myocyte function are cAMP and cGMP independent but modulated by the intracellular thiol content. A, left, Kinetics of cAMP-FRET recorded in a single living neonatal rat cardiomyocyte (inset) challenged with AS (1 mmol/L), followed by norepinephrine (NE) (10 μmol/L) and the broad-spectrum phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) (100 μmol/L). Graph depicts FRET average over the entire cell. Summary data are to the right. *P<10−5 vs control. B, PKA inhibition with 100 μmol/L Rp-CPT-cAMPS blunts ISO but not HNO inotropy. C, sGC (soluble guanylyl cyclase [ODQ]) or PKG (Rp-8Br-cGMPs) inhibition blunts NO but not HNO effects. D, NO has negative impact on concomitant β-adrenergic–stimulated contractility, whereas HNO effects are additive. E, Pretreatment with cell-permeable GSH reduces sarcomeere shortening enhancement by AS/HNO. †P<0.05 vs control.
meant that at the time of functional analysis, 25 to 250 μmol/L NO2− was expected. However, direct exposure to such levels of NO2− (and higher and lower doses) had no effect on sarcomere shortening.

Agents that concomitantly increase myocyte contraction and accelerate relaxation are often linked to a rise in intracellular cAMP and subsequent activation of PKA.10 To test whether this applied to AS/HNO, we performed real-time imaging of cAMP on neonatal rat cardiomyocytes transfected with a cAMP FRET probe.14 On exposure to 1 mmol/L AS, the FRET signal was unchanged (0.3%±0.1%; n=23; P=NS), whereas subsequent application of norepinephrine (10 μmol/L) or phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (100 μmol/L) both increased it by 12% (P<10−6) (Figure 2A). Pretreatment of adult mouse myocytes with the PKA inhibitor Rp-CPT-cAMPs (100 μmol/L; Figure 2B) did not alter HNO-enhanced sarcomere shortening.

AS/HNO-stimulated contractility was also independent of cGMP/PKG. Preincubation with the soluble guanylate cyclase inhibitor ODQ (10 μmol/L×30 minutes) prevented DEA/NO-induced negative inotropy but had no impact on AS/HNO inotropy. Pretreatment with a PKG inhibitor (Rp-8Br-cGMPs, 10 μmol/L) prevented DEA/NO negative inotropy, converting it to a modest positive response, yet had no impact on AS/HNO inotropy (Figure 2C).

NO donors exert a negative effect on β-adrenergic stimulation in vitro and in vivo; however, we previously found the opposite for HNO donors in intact hearts.5 We confirmed this in cardiomyocytes. Cells challenged with isoproterenol (ISO) (2.5 mmol/L) had a 100±27% increase in sarcomere shortening (P=0.002, n=30). This was markedly blunted by confusion of 0.25 mmol/L DEA/NO, whereas coapplication of 0.5 mmol/L AS/HNO doubled shortening above ISO alone (Figure 2D). Thus, AS/HNO acts in parallel with the β-adrenergic pathway.

HNO targets thiol groups on selective proteins.9 To test whether such interaction could underlie whole cell contractile effects, studies were performed in which myocyte thiol equivalents were first enhanced using a cell-permeable ester-derivative of GSH (GSH ethyl ester in Tyrode’s solution, 4 mmol/L for 3 hours). We hypothesized that by enriching the intracellular thiol content, the probability of trapping HNO before it targeted critical thiol residues related to excitation/contraction coupling would be enhanced. Pretreatment with GSH enhanced intracellular thiol equivalents (+6±1.5% in fluorescence arbitrary units versus controls, n=40, P<0.05) determined by fluorescence assay of GSH S-bimane production using 2-photon microscopy. Pretreated cells were then exposed to AS/HNO (0.5 mmol/L), and the contractility response was substantially blunted (+57±19%; P=0.02 versus base; P=0.05 versus AS alone) (Figure 2E). This supports the targeting of HNO on SH groups to exert its cardiotropic action.

Next, we examined Ca2+ cycling in adult mouse and rat cardiac myocytes. Cells were first exposed to AS/HNO for 5 to 10 minutes, then washed and loaded with Indo-1 or fluo-4 for 20 minutes. Pretreatment with AS was necessary because the drug reacted with the Ca2+ indicators (both fluo-4 and Indo-1) and altered their fluorescent properties. In mice, the Ca2+ transient amplitude assessed by confocal line-scan imaging increased by ≈40% over baseline with 0.5 mmol/L AS (n=27, P<0.001) (Figure 3A and 3B), and time to peak transient was prolonged (Figure 3C), whereas the decay time shortened (Figure 3D). Basal fluorescence (F0) was unchanged by AS pretreatment (Figure 3E). Similar results were obtained in rat myocytes (using Indo-1) for Ca2+ transient amplitude (Figure 4A and 4B) and decay time (Figure 4C). The increase in amplitude was not accompanied by an increase in diastolic Ca2+ level (ratio 405/485=0.239±0.006 [control] versus 0.243±0.008 [AS]; P=NS; see also Figure 3A and 3E and Figure 4A). Rapid sustained caffeine (10 mmol/L) application abruptly releases all SR Ca2+ and subsequent [Ca2+], decline is mediated mainly via Na/Ca exchange (NCX). The amplitude and tau of decline of the caffeine-induced Ca2+ transient indicates that HNO did not
alter SR Ca\(^{2+}\) content (Figure 4F) or NCX function (\(\tau=2.0\pm0.4 \) versus \(2.2\pm0.3 \) seconds; Figure 4E). These results indicate that the HNO-enhanced \([Ca^{2+}]_{cyt}\) decline was attributable to increased SERCA2a function, and the HNO-enhanced Ca\(^{2+}\) transient amplitude was caused by enhanced fractional SR Ca\(^{2+}\) release (Figure 4D) withunaltered SR Ca\(^{2+}\) content (Figure 4F).

Given evidence for enhanced SR Ca\(^{2+}\) reuptake and release, with no net gain in total SR Ca\(^{2+}\) content, we next examined direct effects of AS/HNO on the ryanodine-sensitive release channel (RyR2). In intact myocytes, AS enhanced RyR2 opening probability, as revealed by an increased frequency of Ca\(^{2+}\) sparks assessed by line-scan confocal microscopy (Figure 5A), in a dose-dependent manner (Figure 5B; 18-fold rise in spark frequency at 1 mmol/L AS, \(n=10\) to 24, \(P<0.001\)). Conversely, DEA/NO had no effect on spark generation (Figure 5C). Individual spark amplitude, rise time, and spatial width were unaltered by AS, indicating a primary effect on RyR2 activation. SR Ca\(^{2+}\) store depletion by thapsigargin (10 \(\mu\)mol/L, 30 minutes) or ryanodine exposure (10 \(\mu\)mol/L) abolished Ca\(^{2+}\) sparks in control and AS (0.5 mmol/L, data not shown). The influence of AS/HNO on Ca\(^{2+}\) sparks was thiol sensitive. Preincubating cells with reduced glutathione (4 mmol/L for 3 hours) before AS exposure prevented increased spark frequency (Figure 5D), indicating that increased intracellular thiol content effectively quenched HNO action.

To further test whether HNO directly interacted with RyR2 proteins to increase open probability, purified reconstituted RyR2 were expressed in planar lipid bilayers and steady-state activity recorded with or without AS/HNO. The cis (cytosolic) solution contained 10 \(\mu\)mol/L activating Ca\(^{2+}\), and recordings were made at positive 30-mV holding potential. AS (0.1 to 1 mmol/L) produced a dose-dependent rapid increase in frequency and the mean time of open events without altering unitary channel conductance (Figure 5E). The probability of the channel being open \(P_{o}\) increased from an average 0.16\pm0.03 without AS/HNO to 0.46\pm0.07 at 0.3 mmol/L AS added to the cytoplasmic side of the channel (\(n=4\)). This was reversible on addition of 2 mmol/L dithiothreitol (0.11\pm0.04). These findings support direct HNO/RyR2 interaction likely via a reversible reaction with thiol groups in the protein.

We investigated whether HNO directly enhances SR Ca\(^{2+}\) uptake by studying its effects on SR membrane vesicles isolated from pooled mouse hearts. Crude SR microsomal vesicles were incubated with 250 \(\mu\)mol/L AS before measuring ATP-dependent Ca\(^{2+}\) uptake by stopped-flow mixing at 24\(^\circ\)C. Arsenazo III was used to monitor Ca\(^{2+}\) removal from the extravesicular compartment and buffer the free \([Ca^{2+}]_{cyt}\) at a level producing half-saturation of the Ca\(^{2+}\) pump \((\approx0.2 \) mmol/L). The time course of Ca\(^{2+}\) accumulation monitored at 650 nm was biphasic (Figure 6A), likely reflecting different vesicle populations associated with the light and heavy fractions of SR.\(^{21}\) Incubation with 250 \(\mu\)mol/L AS for 15 minutes increased the activity of the fast (0.047 versus 0.64 sec\(^{-1}\); \(P<0.05\)) and the slow (0.069 versus 0.136 sec\(^{-1}\); \(P<0.0005\); \(n=6\)) uptake phases (Figure 6B; Table), without affecting total Ca\(^{2+}\) uptake (Table). Ca\(^{2+}\) uptake activity was abolished by preincubation with 10 \(\mu\)mol/L thapsigargin (not shown), whereas exposure to the Ca\(^{2+}\) ionophore A23187 (5 \(\mu\)g/mg SR protein) diminished total Ca\(^{2+}\) uptake by \(\approx50\%\) (Figure 6E). Stopped-flow signals acquired at the isosbestic wavelength of 693 nm were also biphasic (Figure 6C and 6D). The decrease in absorbance at 693 nm, representing scattered light associated with Ca\(^{2+}\) sequestration and osmotic vesicle swelling, was subtracted from the 650 nm signal before analysis. After subtraction, Ca\(^{2+}\) accumulation exhibited a monophasic time course with >90\% of uptake occurring within the initial 20 s (Figure 6F and 6G).

AS/HNO exposure increased the rate constant for Ca\(^{2+}\) uptake by 104\% based on exponential analysis of the 650 to 693 nm signal (0.1563 versus 0.3204 sec\(^{-1}\); \(P<0.0005\); \(n=6\)) (Figure 6H, left). The difference between total Ca\(^{2+}\) uptake at equilibrium and after exposure to AS/HNO was not
significant (Figure 6H, right; \(P=\text{NS; } n=6\)), indicating that activation by HNO increases the catalytic efficiency of the Ca\(^{2+}\) pump without changing its thermodynamic efficiency. The enhanced SERCA2a function and unaltered net SR Ca\(^{2+}\) uptake in these vesicle experiments are consistent with the acceleration of the decay of the [Ca\(^{2+}\)] transient by AS in intact cardiac myocytes (Figure 4C through 4F and Figure 5).

**Discussion**

In the physiological setting, cardiac contractile force and rate of force decay are enhanced via cAMP/PKA-coupled mechanisms that trigger activator Ca\(^{2+}\) to stimulate the myofilaments and SR uptake to hasten relaxation. Yet, altered cAMP/PKA signaling can contribute to chronic remodeling and failure. Therapies mimicking these pathways have generally proven ineffective for long-term treatment of cardiac failure. Here we reveal that HNO acts very differently on the heart muscle cell, augmenting contractility and accelerating relaxation, independent of cAMP/PKA, by enhancing the Ca\(^{2+}\) transient by increasing both SR Ca\(^{2+}\) uptake and release. These 2 counterbalancing effects likely explain why diastolic Ca\(^{2+}\) does not rise and total SR Ca\(^{2+}\) load remains unchanged. Moreover, this direct effect is redox sensitive and reversible and is very different to the effects produced by NO. Increased SR Ca\(^{2+}\) release with unaltered total SR Ca\(^{2+}\) content suggests HNO modifies RyR2 function rather than
induces a leak by increasing intra-SR Ca\(^{2+}\) stores. These effects are quite different from that exerted by NO donors, agonists, and caffeine. NO donors are reported to enhance or inhibit RyR2, but not alter basal Ca\(^{2+}\) spark frequency. Agonists stimulate RyR2 open probability via PKA-mediated phosphorylation, and Ca\(^{2+}\) spark frequency can increase by this mechanism and further by phosphorylation of phospholamban, which enhances SR Ca\(^{2+}\) load. In transgenic mice overexpressing human \(\beta_2\) receptors, Ca\(^{2+}\) sparks are larger and more frequent than in nontransgenic cells, despite having resting cytosolic Ca\(^{2+}\) and Ca\(^{2+}\) SR load similar to controls. This suggests that \(\beta\)-mediated cAMP-PKA activation alters not only RyR2 sensitivity to Ca\(^{2+}\) but also Ca\(^{2+}\) release-linked RyR2 inactivation, potentially changing SR stability. In contrast, HNO increases spark frequency without altering individual spark characteristics or adversely impacting Ca\(^{2+}\) stability. The action of HNO on RyR2 is also different from that of caffeine, which increases the frequency of spontaneous Ca\(^{2+}\)-release events (Ca\(^{2+}\) waves), an effect that persists even after discontinuing the drug, leading to a substantial decrease in SR Ca\(^{2+}\) content.

The unique action of HNO on RyR2 may relate to its thiophilic chemistry. HNO effects were rapidly reversed by reducing equivalents, suggesting real-time competition for HNO between free thiols and critical thiol residues on the RyR2. The data showing that a 6% increase in intracellular GSH blunts 57% of HNO effects on sarcomere shortening suggests HNO targets selective thiolate residues rather than having a generalized interaction. Identification of these specific targets awaits subproteome analysis of cysteine modification, with site mutagenesis, to confirm the functional importance of particular targets. Selective thiophilic action of HNO might suggest that it is an in vivo signaling molecule, although this remains speculative as methods to measure in vivo synthesis are currently unavailable.

To sustain cardiac inotropy in the presence of HNO-induced increase in the fractional release of Ca\(^{2+}\) from RyR2, the velocity of Ca\(^{2+}\) reuptake into the SR should increase.

![Graphs and Diagrams](http://circres.ahajournals.org/ supplemental/imageURL)
Effect of AS/HNO on Kinetic Parameters for Ca\textsuperscript{2+} Uptake by Cardiac SR Vesicles

<table>
<thead>
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<th>Absorbance</th>
<th>Without AS</th>
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<tr>
<td>$k_1$</td>
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<td>$k_2$</td>
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<td>&lt;0.0005 (n=6)</td>
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<td>0.005696±0.0006</td>
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<td>650–693 nm</td>
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<tr>
<td>$k$</td>
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<td>&lt;0.0005 (n=6)</td>
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<tr>
<td>$A$</td>
<td>0.00257±0.0003</td>
<td>0.00202±0.0004</td>
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$k_1$ indicates rate constant for fast phase of Ca\textsuperscript{2+} uptake; $k_2$, rate constant for slow phase of Ca\textsuperscript{2+} uptake; $A_1$, amplitude of fast phase of Ca\textsuperscript{2+} uptake; $A_2$, amplitude of slow phase of Ca\textsuperscript{2+} uptake; $k$ and $A$, rate constant and amplitude for Ca\textsuperscript{2+} uptake for 650–693 nm signal, respectively.

during relaxation.\textsuperscript{33} This latter process is slowed in the failing heart, and recent efforts to stimulate it by gene modulation (eg, manipulation of phospholamban\textsuperscript{34,35} or increased SERCA2a expression\textsuperscript{36}) highlight the therapeutic attractiveness of this target. AS/HNO stimulated Ca\textsuperscript{2+} uptake in both myocytes and isolated cardiac SR, supporting direct action on SERCA2a. The mechanism remains unknown but could involve direct targeting of SERCA2a by HNO, or releasing some of the inhibition of SERCA2a by phospholamban.\textsuperscript{37}

Although we did not assess whether HNO alters the phosphorylation of various EC coupling proteins (eg, RyR2, phospholamban) as a mechanism for inotropy, several lines of evidence suggests such changes are unlikely and/or separate from HNO modulation. First, both PKG and PKA blockade had no effect on HNO inotropy. Second, HNO did not alter cAMP. Third, HNO effects were rapidly reversible by adding thiol-reducing agents, which would not be observed if a primary phosphorylation mechanism was involved. Fourth, the RyR2 studies were performed in reconstituted membranes without kinases to stimulate phosphorylation, and the responses in this preparation were highly concordant with those observed by Ca\textsuperscript{2+} sparks in intact cells. Lastly, HNO inotropic response in myocytes was shown to be additive to \beta agonists, suggesting that HNO and \beta-adrenergic pathways act in parallel.

Our data provide important new insights into our prior intact animal studies\textsuperscript{4,5} that first revealed HNO donors improve function in the failing heart, independent of \beta-adrenergic blockade, and additive to \beta-adrenergic agonists. Initial studies had first suggested a possible role of HNO in stimulating calcitonin gene–related peptide (CGRP) release\textsuperscript{4}; however, subsequent studies confirmed this effect was sympathostimulatory, inhibited by \beta blockers, and not mediated by direct myocyte CGRP effects.\textsuperscript{38} The current data reveal a direct enhancement of myocyte Ca\textsuperscript{2+} cycling. However, changes in Ca\textsuperscript{2+} handling are not the sole mechanisms as other recent data from our laboratory have found AS/HNO also enhances maximal Ca\textsuperscript{2+}-activated force without altering diastolic Ca\textsuperscript{2+} levels in isolated rat trabeculae. Thus, HNO also acts as a myofilament Ca\textsuperscript{2+} sensitizer at systolic Ca\textsuperscript{2+} levels (T. Dai, Y. Tian, C. G. Tocchetti, T. Katori, D. A. Kass, N. Paolocci, W. Gao, manuscript submitted for publication). This factor would appear to work in concert with increased Ca\textsuperscript{2+} cycling revealed in the current study.

Several study limitations should be noted. First, cells from healthy hearts were studied, and the observed effects of HNO may not directly translate to myocytes from failing ventricles. However, in prior in vivo studies, we observed a similar efficacy of HNO on cardiac function in normal and failing hearts.\textsuperscript{5} Second, we did not examine the coupling between L-type calcium current and RyR2 activation (coupling gain), or determine whether the L-type current itself is altered by HNO. However, enhanced SR calcium uptake and release was demonstrated in isolated SR and reconstituted RyR2, where the gain interaction would not be relevant. Regarding the latter, the lack of change in Ca\textsuperscript{2+} extruded by the NCX and in total SR Ca\textsuperscript{2+} content suggests L-type Ca\textsuperscript{2+} current was unlikely to be altered.

The present data suggest an intriguing potential for the use of HNO donors to treat depressed heart function, particularly in light of prior work confirming efficacy in intact large animals with heart failure. Although an agent that increased SR Ca\textsuperscript{2+} release might raise concerns of proarrhythmia,\textsuperscript{39} the manner by which HNO achieves this effect is novel, and thus its consequences may be as well. Importantly, the current data show increased Ca\textsuperscript{2+} fractional release counterbalanced by improved uptake so that SR Ca\textsuperscript{2+} load and diastolic Ca\textsuperscript{2+} levels are unchanged.

Future studies examining HNO responses in myocytes from failing hearts, longer-term exposure studies, and, ultimately, clinical studies will be needed to prove HNO efficacy and safety for the treatment of decompensated hearts, but the present data provide a valuable starting point for such investigations.

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Disclosures

None.

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**Supplemental Material**

**Contraction and whole Ca\(^{2+}\) transients in isolated mouse ventricular myocytes.**

Wild type 2-4 month old mice were anesthetized with intraperitoneal pentobarbital sodium (100 mg/kg/ip). To assess for sarcomere shortening, cells were imaged using field stimulation (0.5 Hz) in an inverted fluorescence microscope (Diaphot 200; Nikon, Inc). Sarcomere length was measured by real-time Fourier transform (IonOptix MyoCam, CCCD100M). Twitch amplitude is expressed as a percentage of resting cell length. Twitch kinetics was quantified by measuring the time to peak shortening and the time from peak shortening to 50% relaxation. For whole Ca\(^{2+}\) transient measurements, myocytes were loaded with the Ca\(^{2+}\) indicator fluo-4/AM (Molecular Probes, 20 \(\mu\)M for 30 min) and Ca\(^{2+}\) transients were measured under field-stimulation (0.5 Hz) in perfusion solution by confocal laser scanning microscope (LSM510, Carl Zeiss). Digital image analysis used customer-designed programs coded in Interactive Data Language (IDL).

**Whole Ca\(^{2+}\) transients and SR Ca\(^{2+}\) load in isolated rat ventricular myocytes.**

Isolation of rat ventricular myocytes was carried as follows. The enzyme used for tissue dissociation was Liberase Blendzyme 3 or 4 (13-20 Wuensch Units/Heart) sometimes supplemented with 5-10 Units of Dispase II (both Roche Diagnostics, Indianapolis, IN). Ventricular myocytes were then plated onto superfusion chambers, with the glass bottoms treated with natural mouse laminin (Invitrogen, Carlsbad, CA). The standard Tyrode's solution used in all experiments contained (in mM): NaCl 140, KCl 4, MgCl\(_2\) 1, glucose 10, HEPES 5, and CaCl\(_2\) 1, pH 7.4. Myocytes were loaded with 6 \(\mu\)M Indo-1/AM for 25 min and subsequently perfused for at least 30 min to allow for deesterification of the dye. Some cells were pretreated with 0.5 mM AS (in some Caffeine experiments with 1 mM), washed and then loaded with Indo-1/AM. Concentration of the AS stock solution
was verified by absorbance at 250 nm. All experiments were done at room temperature (23–25°C) using field stimulation. Ca²⁺-transients were recorded with Clampex 8.0 and data analyzed with Clampfit.

**FRET analysis**

Cells were transfected with a FRET-based sensor for cAMP and imaged 48 hrs after transfection. Cells were continuously perfused with HEPES buffered Ringer’s modified saline (1 mmol CaCl₂/liter) at room temperature. Cells were imaged on an inverted Olympus IX50 microscope upon excitation at 430 nm. Images analysis was performed by using ImageJ (Rasband, W.S., ImageJ, NIH, Bethesda, Maryland, USA). FRET values were measured as the 480/535nm emission ratio intensity (R) and were normalized to the 480nm/535nm value at time 0s (R₀).

**Fluorescent probes for two-photon laser scanning microscopy and image acquisition.**

The cationic potentiometric fluorescent dye tetramethylrhodamine methyl ester (TMRM) was used to monitor changes in ∆Ψᵱ. The production of the fluorescent glutathione adduct GSB from the reaction of cell permeant monochlorobimane (MCB) with reduced glutathione (GSH), catalyzed by glutathione S-transferase, was used to measure intracellular glutathione levels. Details of GSH measurements are provided in the on-line supplement. The dish containing the cardiomyocytes was equilibrated at 37°C with unrestricted access to atmospheric O₂ on the stage of a Nikon E600FN upright microscope. Then, cells were loaded with 100 nM TMRM and 50 µM MCB for at least 20 min. Images were recorded using a two photon laser scanning microscope (Bio-Rad MRC-1024MP) with excitation at 740nm (Tsunami Ti:Sa laser, Spectra-Physics). The red emission of TMRE was collected at 605±25nm and the blue fluorescence of GSB
was collected at its maximal emission (480nm). Images were analyzed offline using ImageJ software (Wayne Rasband, NIH).

**RyR2 single channel recordings in planar lipid bilayers.**

Briefly, a phospholipid bilayer of PE:PS (1:1 dissolved in n-decane to 20 mg/ml) was formed across an aperture of ~300 µm diameter in a delrin cup. The cis chamber (900 µl) was the voltage control side connected to the head stage of a 200A Axopatch amplifier, while the trans chamber (800 µl) was held at virtual ground. Both chambers were initially filled with 50 mM cesium methanesulfonate and 10 mM Tris/Hepes pH 7.2. After bilayer formation, cesium methanesulfonate was raised to 300 mM in the cis side and 100 to 200 µg of mouse cardiac SR vesicles was added. After detection of channel openings, Cs⁺ in the trans chamber was raised to 300 mM to collapse the chemical gradient. Single channel data were collected at steady voltages (-30 mV) for 2-5 min. Channel activity was recorded with a 16-bit VCR-based acquisition and storage system at a 10 kHz sampling rate. Signals were analyzed after filtering with an 8-pole Bessel filter at a sampling frequency of 1.5-2 kHz. Data acquisition and analysis were done with Axon Instruments software and hardware (pClamp v8.0, Digidata 200 AD/DA interface).

**Preparation of crude SR vesicles from murine hearts.** Measurements of ATP-dependent Ca²⁺ uptake by murine cardiac SR vesicles.

Pooled hearts from C57 mice were placed in 0.9% saline on ice, trimmed of atrial and connective tissue, and weighed. The finely minced heart muscle was homogenized in 10 mM NaHCO₃ using a Polytron blender and the SR vesicles were separated from the myofilaments, mitochondria and nuclear membranes by differential centrifugation at 8,500 and 45,000 x g. SR vesicles suspended in 0.25 M sucrose + 10 mM MOPS, pH 7.0 were frozen and stored in liquid nitrogen prior to use. Twenty minutes prior to
measuring Ca\(^{2+}\) uptake, cardiac SR vesicles (1 mg/ml in storage buffer) were incubated with 250 \(\mu\)M AS. After dilution of the SR membranes in the Ca\(^{2+}\) uptake buffer, changes in kinetic behavior due to AS/HNO were seen after a delay of \(~15\) min and remained in effect for the duration of the experiment (45-60 min).