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Nitroxyl enhances myocyte Ca^{2+} transients by exclusively targeting SR Ca^{2+}-cycling

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1. ABSTRACT

Nitroxyl (HNO), the 1-electron reduction product of nitric oxide, improves myocardial contraction in normal and failing hearts. Here we test whether the HNO donor Angeli's salt (AS) will change myocyte action potential (AP) waveform by altering the L-type Ca^{2+} current (ICa) and contrast the contractile effects of HNO with that of the hydroxyl radical (.OH) and nitrite (NO2-), two potential breakdown products of AS. We confirmed the positive effect of AS/HNO on basal cardiomyocyte function, as opposed to the detrimental effect of .OH and the negligible effect of NO2-. Upon examination of the myocyte AP, we observed no change in resting membrane potential or AP duration to 20% repolarization with AS/HNO, whereas AP duration to 90% repolarization was slightly prolonged. However, perfusion with AS/HNO did not elicit a change in basal ICa, but did hasten ICa inactivation. Upon further examination of the SR, the AS/HNO-induced increase in cardiomyocyte Ca^{2+} transients was abolished with inhibition of SR Ca^{2+}-cycling. Therefore, the HNO-induced increase in Ca^{2+} transients results exclusively from changes in SR Ca^{2+}-cycling, and not from ICa.

2. INTRODUCTION

The process of excitation-contraction coupling underlies cardiomyocyte contraction. In this process, an influx of Ca^{2+} through the L-type Ca^{2+} current (I_{Ca}) provides the trigger for the release of additional Ca^{2+} from the sarcoplasmic reticulum (SR) via the ryanodine receptors (RyR), thus inducing myocyte contraction (1). I_{Ca} can also serve to load the SR with Ca^{2+} and directly activate myofilament contraction. In order for cardiomyocyte relaxation to occur, the Ca^{2+} available for contraction must either be re-sequestered into the SR via the SR Ca^{2+} ATPase (SERCA2a) or extruded out of the myocyte through the Na^{+}/Ca^{2+} exchanger.

We recently reported that nitroxyl (HNO), the one-electron reduction product of nitric oxide (NO), improves myocardial contraction in both normal (2) and failing hearts (3). This functional improvement was elicited upon administration of the HNO donor Angeli's salt (AS), and was demonstrated to occur independent of {beta}-

adrenergic receptor ({beta}-AR) stimulation, and distinct from the effects NO and cGMP (3-5). The effects of HNO are due, in part, to the enhancement of SR Ca^{2+}-cycling (4), and likely occur through the targeting of critical thiol groups (6, 7). More specifically, HNO increases ATP-dependent SR Ca^{2+} uptake (4), and the activity of RyR (4, 8). HNO also works to enhance myocardial contraction by increasing the sensitivity of the myofilaments to Ca^{2+}, thus allowing for increased force development without a concomitant increase in actomyosin ATPase activity (5). The net result of HNO administration is an improvement in myocardial contraction, resulting from an increase in systolic Ca^{2+} transients, which serve to drive a more Ca^{2+}-sensitive contractile apparatus. However, previous reports have not examined the role of extracellular Ca^{2+} in the positive inotropic action of HNO. Further, under certain extreme biochemical conditions, the breakdown of AS may lead to the formation the hydroxyl radical (˙OH) (9, 10), which is a well-known negative modulator of myocardial function (11-13). Another potential breakdown product of AS includes nitrite (NO_2^-). Therefore, it is important to establish that the effect of AS on myocardial contraction occurs through the formation of HNO, and not via hydroxyl radical or nitrite production.

Here we investigate the role of extracellular Ca^{2+} in the positive inotropic action of HNO by examining action potential waveform and I_{Ca}. We also contrast the contractile effects of HNO with that of the hydroxyl radical, a possible breakdown product of AS, and nitrite, a reactive nitrogen species co-released during the decomposition of AS. Additionally, we provide further evidence that the effects of HNO are independent from {beta}-AR stimulation and are distinct from those effects typically observed with NO, cGMP, and other reactive oxygen and nitrogen species (14-17).

3. MATERIALS AND METHODS

3.1. Cardiomyocyte Isolation

Ventricular cardiomyocytes were isolated from mouse (C57BL/6, male) and rat (LBN-F1, male) hearts, as previously described (16). Briefly, hearts were excised from animals anesthetized via intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1}). Using a Langendorff apparatus, hearts were perfused with nominally Ca^{2+}-free Joklik Modified MEM (Sigma, St. Louis, MO) for 4 minutes at 37°C. Perfusion was then switched to the same solution, but now containing Liberase Blendzyme 4 (Roche Diagnostics, Indianapolis, IN). Hearts were digested until the drip rate reached one per second. Following digestion, the heart was taken down and the tissue minced, triturated, and filtered. The cell suspension was then rinsed and stored in Joklik Modified MEM containing 200 {micro}mol/L Ca^{2+}. Cells were used within 6 hours of isolation. This investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University.

3.2. Simultaneous Measurement of Systolic Ca^{2+} Transients and Shortening

Systolic Ca^{2+} transients and shortening were measured in isolated myocytes as previously described (16). Briefly, isolated myocytes were loaded at 22°C with 10 {micro}mol/L Fluo-4 AM (Molecular Probes, Eugene, OR) for 30 minutes. Excess dye was removed by washout with 200 {micro}mol/L Ca^{2+} normal Tyrode solution. Myocytes were then de-esterfied for an additional 30 minutes. Following loading, cells were stimulated at 1 Hz via platinum electrodes connected to a Grass Telefactor S48 stimulator (West Warwick, RI). Fluo-4 was excited with 480±20 nm light, and the fluorescent emission of a single cell was collected at 530±25 nm using an epifluorescence system (Cairn Research Limited, Faversham, UK). The illumination field was restricted to collect the emission of a single cell. Data were expressed as {delta}F/F_0, where F was the fluorescence intensity and F_0 was the intensity at rest. For experiments utilizing sodium nitrite, 10 {micro}mol/L Indo-1 AM (Molecular Probes) was utilized. Indo-1 was excited with 365±10 nm light, and the fluorescent emission of a single cell was collected at 405±30 nm and 485±25 nm. Data were expressed as {delta}$Ratio_{405/485}$. Simultaneous measurement of shortening was performed using an edge detection system (Crescent Electronics, Sandy, UT). Cardiomyocyte shortening amplitude was normalized to resting cell length (%RCL). For experiments utilizing sodium nitrite, sarcomere shortening was measured using the IonOptix MyoCam (Milton, MA). Sarcomere shortening amplitude was expressed as the percent of fractional shortening (%FS). All measurements were recorded at room temperature (22°C) except where noted.

3.3. Hydroxyl Radical Generation

Hydroxyl radicals were generated via Fenton chemistry using the H_2O_2+Fe^{2+}-nitrilotriaceticacetate (Fe^{2+}-NTA) system, as previously described (13). In this system, the concentration of Fe^{2+}-NTA within the perfusion solution was 10 {micro}mol/L; H_2O_2 was infused into the perfusion solution through a separate line to a final concentration of 3.75 {micro}mol/L. This allows hydroxyl radical formation to occur as closely to the preparation as possible. With the use of this system, the concentration of hydroxyl radicals generated in the perfusion solution is approximately 2 {micro}mol/L (12, 13). Systolic Ca^{2+} transients and shortening were simultaneously recorded as described above at a frequency of 1 Hz, with the exception that cells were loaded with 10 {micro}mol/L Indo-1 AM (Molecular Probes) instead of Fluo-4 AM, as hydroxyl radical exposure is known to induce bleaching of the Ca^{2+} indicator. Therefore, the ratiometric properties of Indo-1 AM will serve to counteract the effect of hydroxyl radical exposure on the Ca^{2+} indicator. Indo-1 was excited with 365±10 nm light, and the fluorescent emission of a single cell was collected at 405±30 nm and 485±25 nm. Data were expressed as $Ratio_{405/485}$ and {delta}$Ratio_{405/485}$. All measurements were recorded at room temperature (22°C).

3.4. Action Potential Measurement

Action potentials were recorded using the whole cell ruptured patch current clamp technique and an

Axopatch-200B amplifier with pCLAMP 9.0 software (Axon Instruments), as previously described (15). Electrodes (borosilicate glass tubing) with a resistance of 8-12 M{ohm} were filled with (in mmol/L): K-aspartate (130), KCl (10), NaCl (8), HEPES (5), and MgATP (5); pH 7.2 adjusted with KOH. All measurements were recorded at room temperature (22°C).

3.5. L-Type Ca^{2+} Current Measurement

L-type Ca^{2+} current was measured using the whole cell ruptured patch voltage clamp technique and an Axopatch-200B amplifier with pCLAMP 9.0 software (Axon Instruments), as previously described (15). Electrodes (borosilicate glass tubing) with a resistance of 1.5-3 M{ohm}, were filled with (in mmol/L): CsCl (120), $MgCl_2$ (6), EGTA (10), HEPES (10), and MgATP (2); pH 7.2 adjusted with CsOH. The bath solution consisted of (in mmol/L): NaCl (120), CsCl (4), $MgCl_2$ (1), $CaCl_2$ (1), glucose (10), HEPES (5), L-arginine (1); pH 7.4 adjusted with CsOH or HCl. L-type Ca^{2+} current was elicited by 200 ms pulses to 0 mV from a holding potential of -80 mV (following a pre-pulse to -40 mV) at a frequency of 0.2 Hz. This procedure isolates I_{Ca} by inactivation of the Na^+ current with the pre-pulse; replacement of K^+ with Cs^+ eliminates the K^+ current. I_{Ca} inactivation (tau, time constant for I_{Ca} decline) was determined using a single exponential fitted to the decay phase of the current. All measurements were recorded at room temperature (22°C).

3.6. SR Inhibition

For SR inhibition, cardiomyocytes were pretreated with 1 {micro}mol/L thapsigargin for 15 minutes in order to completely block SR function. SR inhibition was verified by the absence of 10 mmol/L caffeine-induced Ca^{2+} transients. To enhance the Ca^{2+} influx-induced Ca^{2+} transients, the $[Ca^{2+}]$ in the perfusion solution was increased to 20 mmol/L (18). Systolic Ca^{2+} transients were recorded as described above at frequency of 0.5 Hz. All measurements were recorded at room temperature (22°C).

3.7. Solutions and Drugs

Normal Tyrode control solution consisted of (in mmol/L): NaCl (140), KCl (4), $MgCl_2$ (1), $CaCl_2$ (1), Glucose (10), and HEPES (5); pH = 7.4 adjusted with NaOH/HCl. Angeli's salt (AS; Calbiochem, La Jolla, Ca) was dissolved in 10 mmol/L NaOH and used as an HNO donor. Sodium nitrite ($NaNO_2$, Sigma, St. Louis, MO) was used as a source of nitrite (NO_2^-). Isoproterenol (ISO; Sigma) was used as a non-selective {beta}-AR agonist. Thapsigargin (Sigma) was dissolved in dimethyl sulfoxide (DMSO, Sigma) and used as a specific inhibitor of SERCA activity. All solutions were made fresh on the day of experimentation.

3.8. Statistics

Data are presented as the mean±S.E.M. Statistical significance ($p<0.05$) was determined between groups using an ANOVA (followed by Newman-Keuls test) for multiple groups or a paired Student's *t*-test for two groups.

4. RESULTS

4.1. Effect of AS on cardiomyocyte function

We first confirmed the positive effect of Angeli's salt (AS, HNO donor) on basal function in isolated murine cardiomyocytes. AS (500 {micro}mol/L) significantly increased basal systolic Ca^{2+} transients and myocyte shortening (Systolic Ca^{2+} Transient: 0.7±0.1 vs. 1.1±0.1 {delta}F/F_0, Shortening: 4.0±0.5% vs. 10.5±2.5% RCL, $p<0.05$ vs. Control). This effect is shown in the representative traces (Figure 1A) and in the summary data (Figure 1B). AS/HNO also increased the time to peak of the systolic Ca^{2+} transient, while accelerating the decay of the systolic Ca^{2+} transient (data not shown). Similar effects were observed with AS/HNO upon repetition of the same experimental protocol at physiological temperature (37°C) (Systolic Ca^{2+} Transient: 47±10% vs. 55±10% change from Control, Shortening: 219±72% vs. 162±47% change from Control, p = NS). This effect is shown in the summary data expressed as a percent of control (Figure 1C). In a previous publication, we demonstrated that AS did not alter diastolic Ca^{2+} or diastolic cell length (4). Thus, AS/HNO induces positive inotropic effects in isolated cardiomyocytes.

4.2. Effect of hydroxyl radical and nitrite exposure on cardiomyocyte function

The possibility exists for AS to generate the hydroxyl radical (·OH) under certain extreme biochemical conditions (9, 10). Therefore, we examined the effect of acute hydroxyl radical exposure on isolated rat cardiomyocyte function. Acute hydroxyl radical exposure slightly increased systolic Ca^{2+} transients (data not shown), and induced a significant increase in diastolic Ca^{2+} that was accompanied by a significant decrease in diastolic cell length and myocyte shortening (Diastolic Ca^{2+}: 2.4±0.6 vs. 2.8±0.7 $Ratio_{405/485}$, Diastolic cell length: 110±4.9 vs. 105±5.2 {micro}m, Shortening: 7.2±0.9% vs. 5.0±0.6% RCL, $p<0.05$ vs. Control). This effect is shown in the summary data expressed as a percent of control (Figure 2).

Additionally, nitrite (NO_2^-) is another breakdown product of AS. In our previous publication, we determined that AS yielded approximately 25% nitrite after 15 minutes of continuous infusion (4). Therefore, we examined the effect of 125 {micro}mol/L $NaNO_2$ on isolated murine cardiomyocyte function. Exposure to nitrite, however, yielded no appreciable effect on basal systolic Ca^{2+} transients or sarcomere shortening (Systolic Ca^{2+}: 0.7±0.1 vs. 0.6±0.1 {delta}$Ratio_{405/485}$, Shortening: 2.6±0.3% vs. 2.8±0.4% FS, p = NS). This effect is shown in the summary data (Figure 3). Therefore, the acute effects of hydroxyl radical and nitrite exposure are distinct from those effects observed with AS/HNO.

4.3. AS and action potential waveform

We next examined the effect of AS/HNO on murine myocyte AP waveform. AS (500 {micro}mol/L) did not alter resting membrane potential (RMP: -79±3 vs. -78±4 mV) or the AP duration (APD) to 20% repolarization (APD_{20}: 1.0±0.2 vs. 1.3±0.3 ms), but did induce a slight prolongation of the APD to 90% repolarization (APD_{90}: 59±8 vs. 77±13 ms, $p<0.05$ vs. Control). This effect is

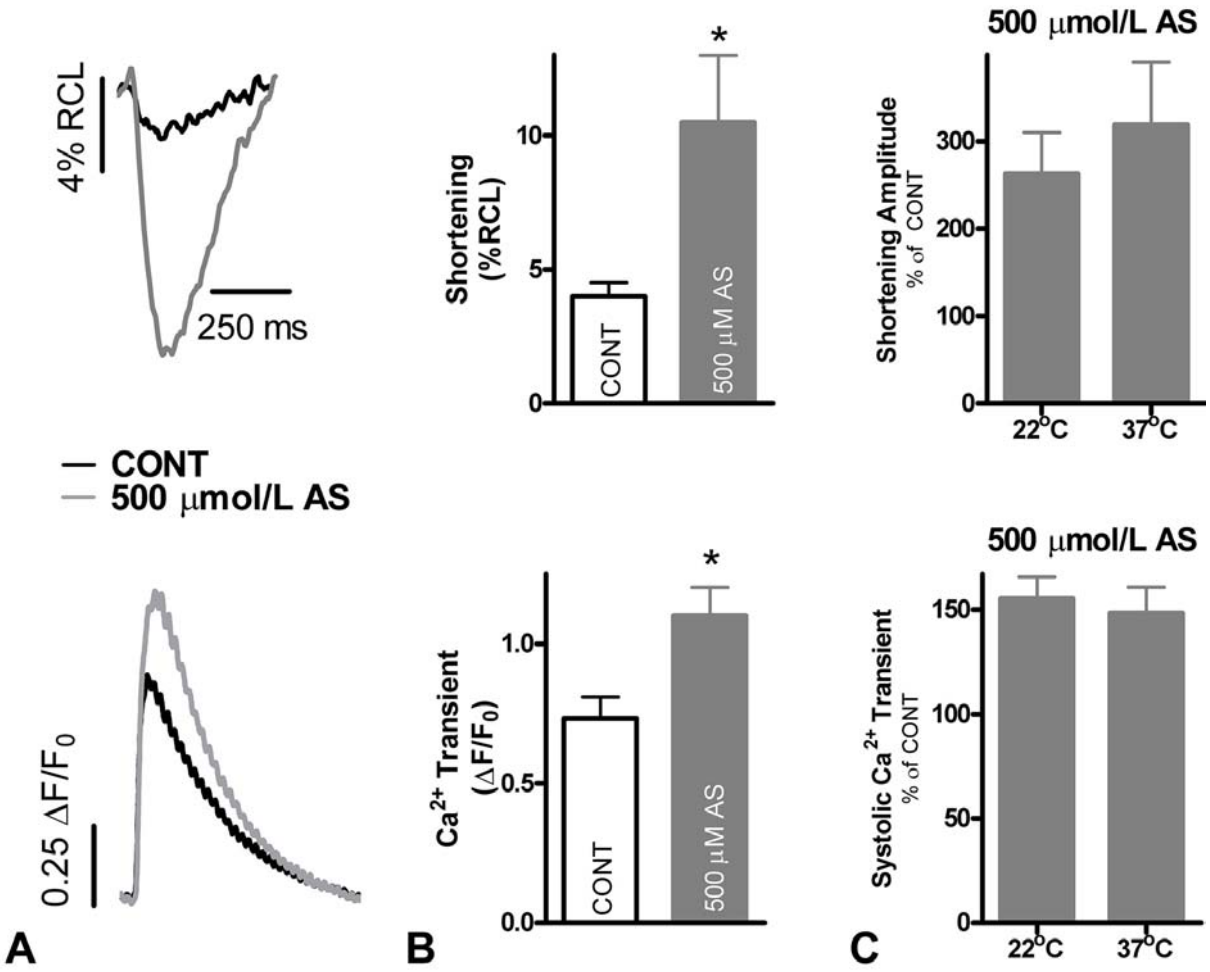

Figure 1. AS enhances systolic Ca^{2+} transients and cell shortening in cardiomyocytes. A) Individual, steady-state cell shortening (top) and systolic Ca^{2+} transient (bottom) traces representing the effect of control (normal Tyrode) and 500 {micro}mol/L AS in isolated murine cardiomyocytes. NOTE: the timescale found in the upper panel of Figure 1A applies to both the upper and lower panels of Figure 1A. B) Pooled data (mean±S.E.M.) demonstrating the effect of control (normal Tyrode) and 500 {micro}mol/L AS on cell shortening (top) and systolic Ca^{2+} transients (bottom) in cardiomyocytes (n = 14 myocytes/5 hearts). *p<0.05 vs. Control. C) Pooled data (mean±S.E.M.) demonstrating the effect of 500 {micro}mol/L AS on cell shortening (top) and systolic Ca^{2+} transients (bottom) in cardiomyocytes displayed as a % of control at room temperature (22°C) and physiological temperature (37°C).

shown in the representative traces (Figure 4A) and in the summary data (Figure 4B-C). Additionally, we did not observe any delayed afterdepolarizations (DADs) with the prolongation of the APD_{90} (data not shown).

4.4. AS does not alter I_{Ca}

We subsequently investigated the effect of AS/HNO on murine myocyte I_{Ca}. Surprisingly, we observed no change in basal I_{Ca} with either 100 {micro}mol/L or 500 {micro}mol/L AS (100 {micro}mol/L: 2.8±0.5 vs. 2.7±0.5 -pA/pF; 500 {micro}mol/L: 2.5±0.5 vs. 2.5±0.5 -pA/pF). This lack of effect is shown in the representative traces (Figure 5A), the representative time plot (Figure 5B), and in the summary data (Figure 5C). Further, AS/HNO had no effect on the current-voltage relationship for I_{Ca} (Figure 5D). However, AS/HNO did induce significantly faster inactivation of I_{Ca}, measured as the time constant for I_{Ca} decline (Control: 42±7 vs. HNO: 36±6 ms, p<0.05 vs. Control). This effect can be seen in the normalized I_{Ca} traces (Figure 5E) and in the summary data (Figure 5F).

AS/HNO also failed to elicit a change in I_{Ca} following pre-stimulation with 0.01 {micro}mol/L ISO (Control: 2.0±0.8 vs. ISO: 4.2±0.9* vs. ISO+AS: 3.9±0.5* -pA/pF, *p<0.05 vs. Control). This lack of effect can be seen in the representative traces (Figure 6A) and in the summary data (Figure 6B).

4.5. AS has no effect during SR inhibition

Since we observed no change in I_{Ca}, we further investigated the role of SR Ca^{2+}-cycling in the effects of AS/HNO. Upon complete inhibition of SR Ca^{2+}-cycling, systolic Ca^{2+} transients should be derived entirely from

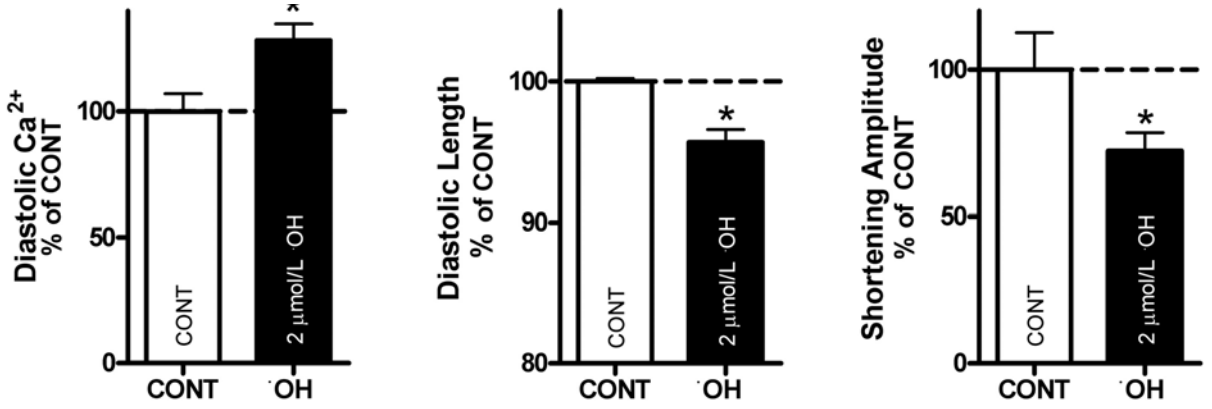

Figure 2. Hydroxyl radical exposure decreases cardiomyocyte contraction. Pooled data (mean±S.E.M.) demonstrating the effect of control (normal Tyrode) and hydroxyl radical exposure (2 {micro}mol/L ·OH) on diastolic Ca^{2+} (left), diastolic cell length (center), and shortening amplitude (right) in rat cardiomyocytes (n = 13 cardiomyocytes/5 hearts). *p<0.05 vs. Control.

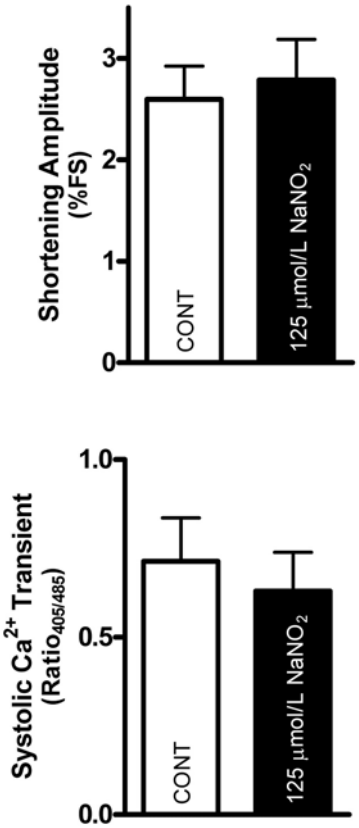

Figure 3. Nitrite does not alter cardiomyocyte contraction. Pooled data (mean±S.E.M.) demonstrating the effect of control (normal Tyrode) and 125 {micro}mol/L $NaNO_2$ on shortening (top) and systolic Ca^{2+} transients (bottom) in murine cardiomyocytes (n = 13 myocytes/2 hearts).

extracellular Ca^{2+} influx, mainly via I_{Ca}. Therefore, to further examine the role of SR Ca^{2+}-cycling, we examined the effect of AS on systolic Ca^{2+} transients in murine cardiomyocytes during inhibition of SR Ca^{2+}-cycling with thapsigargin. SR inhibition was verified by the absence of 10 mmol/L caffeine-induced systolic Ca^{2+} transients (data not shown). Further evidence of SR inhibition can be seen in the small size of the systolic Ca^{2+} transient, the slowed decline of the systolic Ca^{2+} transient, and the inability of ISO to hasten the systolic Ca^{2+} transient decline (Figure 7A). To enhance the Ca^{2+} influx-induced Ca^{2+} transients, the $[Ca^{2+}]$ in the perfusion solution was increased to 20 mmol/L (18). SR inhibition completely abolished the positive effect of 500 {micro}mol/L AS on systolic Ca^{2+}

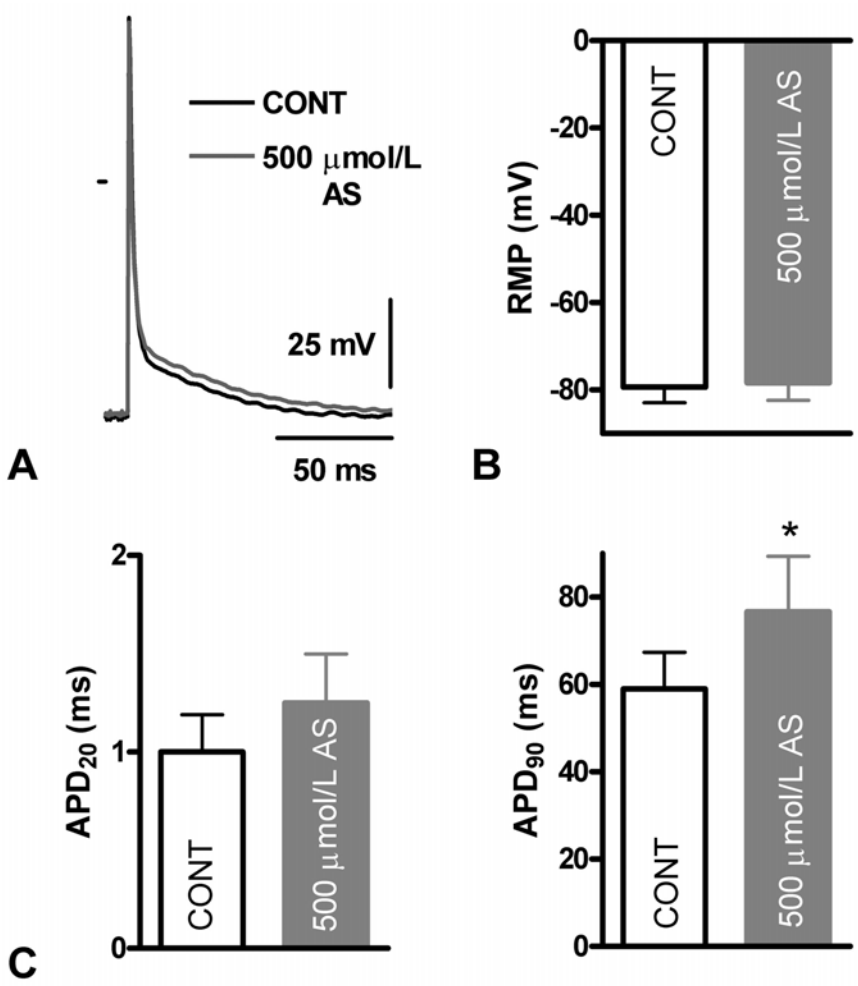

Figure 4. AS induces a slight change in AP waveform. A) Individual, steady-state AP traces representing the effect of control (normal Tyrode) and 500 {micro}mol/L AS in isolated murine cardiomyocytes. B) Pooled data (mean±S.E.M.) demonstrating the effect of control (normal Tyrode) and 500 {micro}mol/L AS on resting membrane potential (RMP). C) Pooled data (mean±S.E.M.) demonstrating the effect of control (normal Tyrode) and 500 {micro}mol/L AS on AP duration to 20% repolarization (APD_{20}, left) and to 90% repolarization (APD_{90}, right) (n = 8 cardiomyocytes/3 hearts). *p<0.05 vs. Control.

transient amplitude (0±4% change from control), as seen in the representative traces (Figure 7A) and in the summary data (Figure 7B). Conversely, 0.01 {micro}mol/L ISO still induced a significant increase in systolic Ca^{2+} transient amplitude even with SR inhibition (46±16% change from control, p<0.05 vs. Control), as seen in the representative traces (Figure 7A) and in the summary data expressed as a percent of control (Figure 7B). Vehicle treatment alone (DMSO) did not alter basal myocyte contraction or the response to 0.01 {micro}mol/L ISO (data not shown). Thus, HNO works exclusively at the level of the SR to increase systolic Ca^{2+} transients in isolated cardiomyocytes (Figure 7C), and not from the recruitment of extracellular Ca^{2+}.

5. DISCUSSION

Nitroxyl (HNO) was previously demonstrated to enhance myocardial contraction partly through effects on SR Ca^{2+}-cycling (2-4). However, our previous studies did not examine the role of extracellular Ca^{2+} in the positive inotropic action of HNO. In our current study, we demonstrate for the first time that HNO induces a slight change in AP waveform, but does not recruit additional extracellular Ca^{2+}, namely I_{Ca}, in order to increase systolic Ca^{2+}. Additionally, we did not observe DADs with the HNO-induced prolongation of the APD. Thus, sarcolemmal Ca^{2+} does not contribute to the HNO-induced increase in systolic Ca^{2+} transients. However, SR inhibition completely abolished the positive effect of HNO on systolic Ca^{2+} transients. Moreover, the current study is the first to contrast the positive inotropic action of HNO with that of the hydroxyl radical and nitrite. The generation of the former occurs from the breakdown of AS during extreme biochemical conditions, whereas the latter is normally co-released with HNO by AS. Therefore, the AS-induced increase in cardiomyocyte systolic Ca^{2+} occurs from the direct enhancement of SR Ca^{2+}-cycling by HNO.

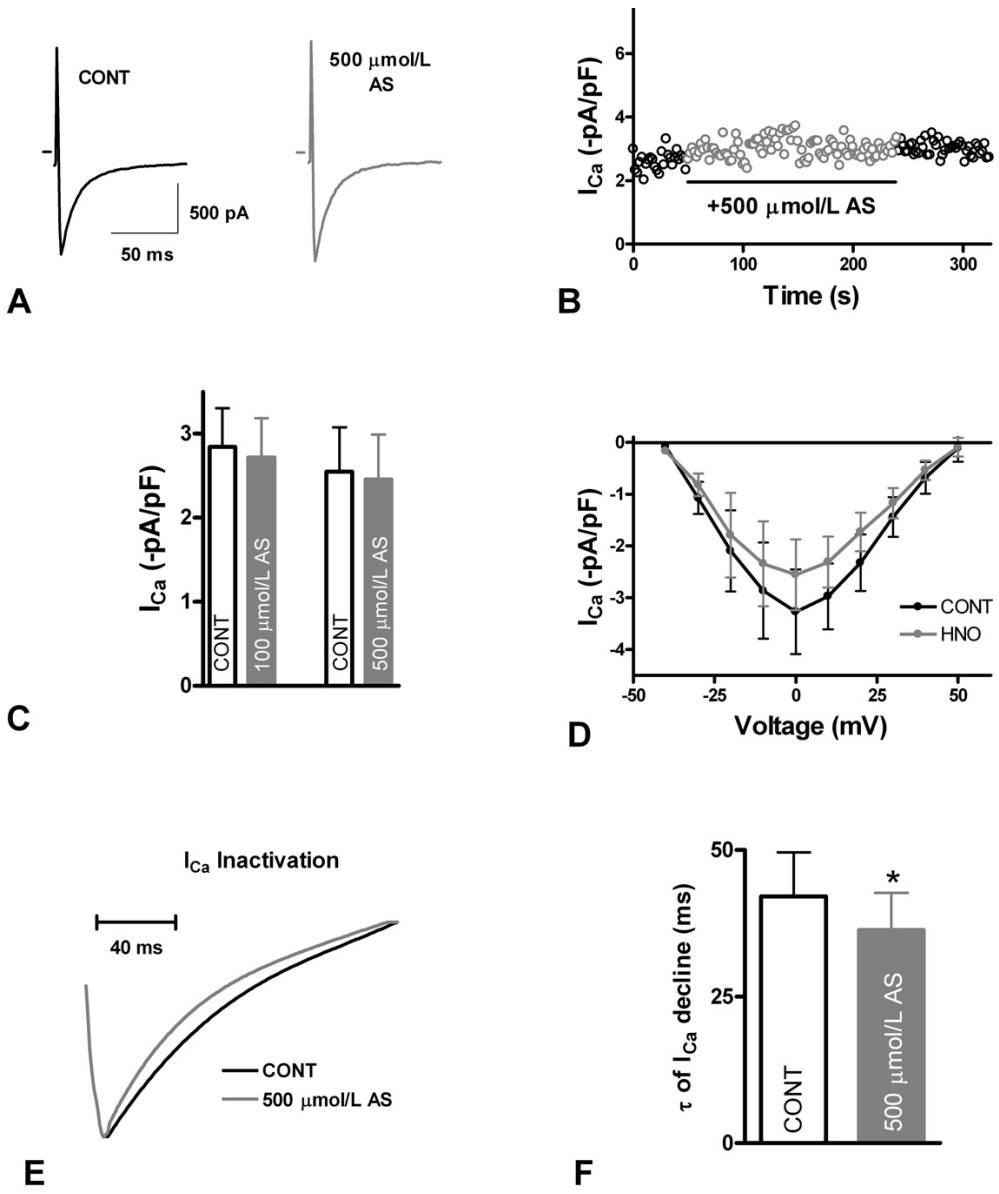

Figure 5. AS does not alter basal I_{Ca}, but does hasten the rate of I_{Ca} inactivation. A) Individual, steady-state I_{Ca} traces representing the effect of control (normal Tyrode) and 500 {micro}mol/L AS in isolated murine cardiomyocytes. B) Representative I_{Ca} time plot demonstrating the effect of control (normal Tyrode) and 500 {micro}mol/L AS. C) Pooled data (mean±S.E.M.) demonstrating the effect of control (normal Tyrode) and AS (100 & 500 {micro}mol/L) on cardiomyocyte I_{Ca}. D) Pooled data (mean±S.E.M.) demonstrating the effect of control (normal Tyrode) and 500 {micro}mol/L AS on the current-voltage relationship for I_{Ca}. E) Normalized I_{Ca} traces representing the effect of control (normal Tyrode) and 500 mmol/L AS on I_{Ca} inactivation in isolated murine cardiomyocytes. F) Pooled data (mean±S.E.M.) demonstrating the effect of control (normal Tyrode) and AS (500 {micro}mol/L) on cardiomyocyte I_{Ca} inactivation (tau, time constant for I_{Ca} decline) (n = 8-11 myocytes/4 hearts). *p<0.05 vs. Control.

5.1. HNO enhances cardiomyocyte contraction

We confirmed the positive effect of HNO in murine cardiomyocytes, and noted a significant increase in systolic Ca^{2+} transients and cell shortening (Figure 1). HNO also increased the time to peak of the systolic Ca^{2+} transient, while accelerating the decay of the systolic Ca^{2+} transient. These results are consistent with our previous study (4), which also demonstrated that HNO was without effect on diastolic Ca^{2+} or diastolic cell length. Further, the positive inotropic action of HNO was unaffected by temperature, as we observed similar increases in systolic Ca^{2+} transients and cell shortening at physiological temperature (37°C), compared to the effects observed at room temperature (22°C) (Figure 1). This result is consistent with a previous publication, where we examined the effect of HNO *in vivo* and observed a large increase in

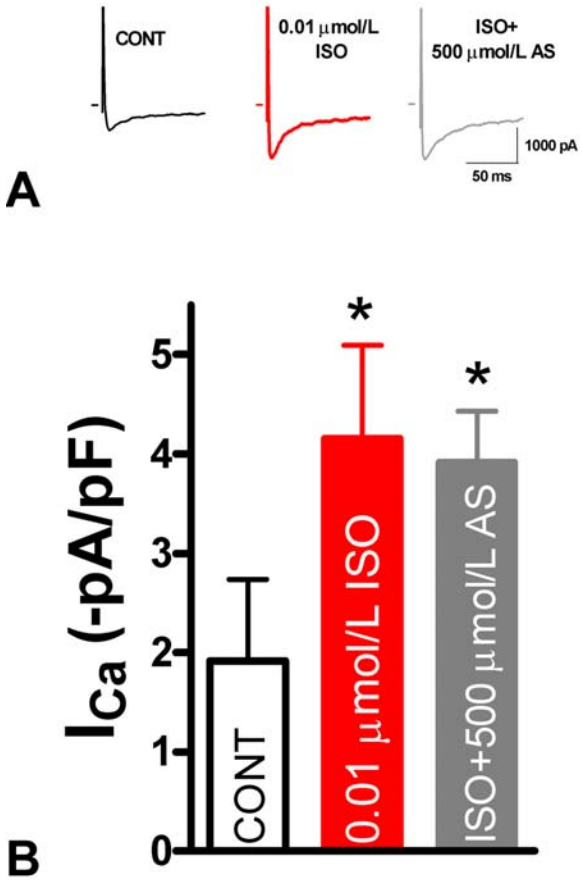

Figure 6. AS does not alter {beta}-AR-stimulated I_{Ca}. A) Individual, steady-state I_{Ca} traces representing the effect of control (normal Tyrode), 0.01 {micro}mol/L ISO and 500 {micro}mol/L AS in isolated murine cardiomyocytes. B) Pooled data (mean±S.E.M.) demonstrating the effect of control (normal Tyrode), ISO (0.01 {micro}mol/L) and AS (500 {micro}mol/L) on cardiomyocyte I_{Ca} (n = 6 cardiomyocytes/3 hearts). *p<0.05 vs. Control.

myocardial contractility, as well as enhanced myocardial relaxation (3).

5.2. Hydroxyl radical exposure is detrimental to cardiomyocyte function

AS is considered to be an HNO donor, but the chemistry of AS is rather complex (19). Studies have demonstrated hydroxyl radical production using very high concentrations of AS (>1 mmol/L) under conditions of very low pH (pH 4-6) (9, 10). Although hydroxyl radical production is minimal under our experimental conditions at pH 7.4, hydroxyl radical production may occur in certain intracellular compartments of low pH (i.e., mitochondria). Therefore, we conducted additional experiments in order to demonstrate that the contractile effects induced by AS were distinct from those observed with hydroxyl radical exposure. Hydroxyl radical exposure proved to be extremely detrimental to cardiomyocyte function by increasing diastolic Ca^{2+}, and decreasing diastolic cell length and myocyte shortening (Figure 2). These results are consistent with previous findings (11-13), and are in contrast to the effects of AS. Treatment with AS caused a large increase in systolic Ca^{2+} transients and myocyte shortening (Figure 1), without a change in diastolic Ca^{2+} or diastolic cell length (4). The effects of hydroxyl radical exposure are indicative of Ca^{2+}-overload, as evidenced by the changes in diastolic Ca^{2+} and length. However, AS does not appear to lead to Ca^{2+}-overload, as we observed no change in diastolic Ca^{2+} or length. This indicates that the effects of AS are distinct from those observed with the hydroxyl radical and likely other reactive oxygen species, and do not result from a generalized thiol oxidation. Thus, hydroxyl radical generation via AS is not likely to be a confounding factor in our experimental design.

5.3. Nitrite does not alter cardiomyocyte function

Nitrite is co-released with HNO during the decomposition of AS at physiological pH and temperature. However, nitrite had no effect on systolic Ca^{2+} transients or sarcomere shortening (Figure 3). These effects are consistent with our previous study (4), and are in direct contrast to those effects observed with HNO (Figure 1). Therefore, nitrite production via AS is not likely to underlie the positive inotropic action of AS/HNO.

5.4. HNO alters action potential waveform

Although HNO enhanced cardiomyocyte contraction, HNO did not induce a change in RMP or the

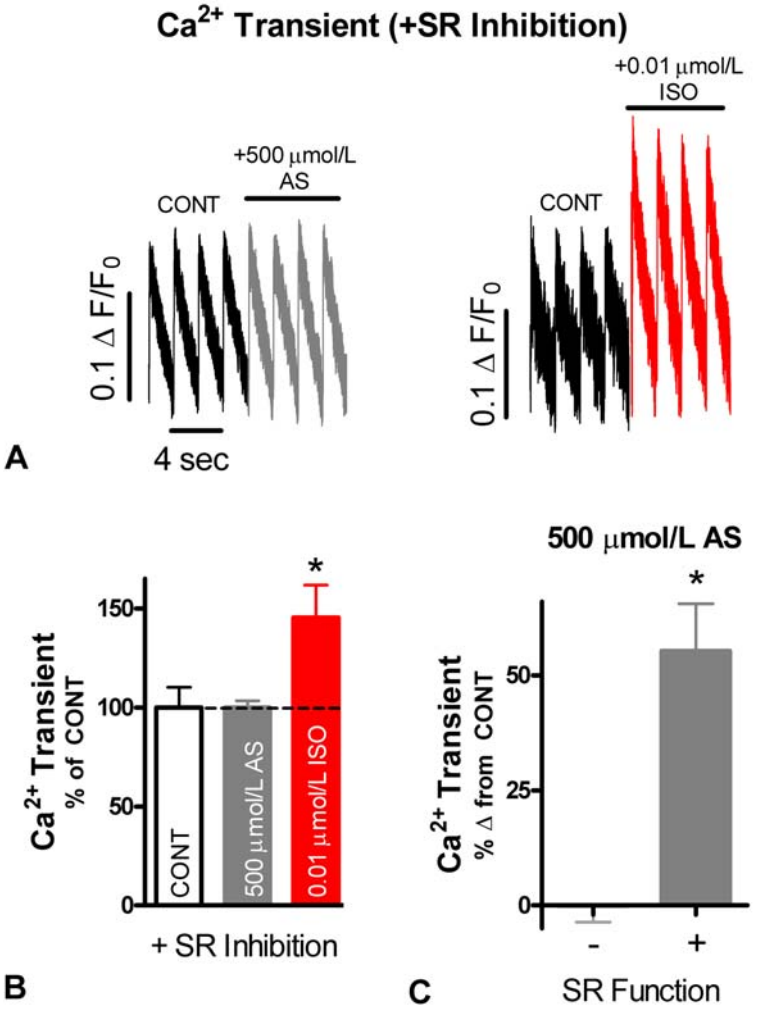

Figure 7. SR inhibition attenuates effect of AS. A) Individual, steady-state systolic Ca^{2+} transient (bottom) traces representing the effect of control (normal Tyrode) and 500 {micro}mol/L AS (left) or 0.01 {micro}mol/L ISO (right) during SR inhibition in isolated murine cardiomyocytes. NOTE: the black traces prior to the addition of AS (left) and ISO (right), represent basal systolic Ca^{2+} transients with SR inhibition under control conditions (normal Tyrode). B) Pooled data (mean±S.E.M.) demonstrating the effect of control (normal Tyrode), AS (500 {micro}mol/L), and ISO (0.01 {micro}mol/L) on cardiomyocyte systolic Ca^{2+} transient amplitude during SR inhibition (n = 8-9 cardiomyocytes/3 hearts). *p<0.05 vs. Control and AS. C) Pooled data (mean±S.E.M.) demonstrating the effect of AS on cardiomyocyte systolic Ca^{2+} transient amplitude with and without SR function. *p<0.05 vs. without SR Function.

APD_{20}, but did slightly prolong the APD_{90} (Figure 4). Importantly, we did not observe DADs with the HNO-induced prolongation of the APD_{90}. These effects on AP waveform are distinct from the effects of NO signaling, which has been shown to reduce the APD (15). Studies have shown that prolongation of the APD_{90} can result from changes in I_{Ca} (15, 20).

5.5. HNO does not alter I_{Ca}

Despite the HNO-induced increase in the APD_{90}, HNO had no effect on basal I_{Ca} (Figure 5). This lack of effect on I_{Ca} is surprising given that HNO greatly enhanced systolic Ca^{2+} transients and prolonged the APD_{90}. HNO also had no effect on the current-voltage relationship for I_{Ca} (Figure 5). However, faster inactivation of I_{Ca} was observed with HNO (Figure 5). These data are consistent with the HNO-induced increase in SR Ca^{2+}-cycling, which may accelerate the Ca^{2+}-dependent inactivation of I_{Ca} (21, 22). Since peak I_{Ca} was not altered by HNO, the prolongation of the action potential duration by HNO likely results from the targeting of repolarizing K^+ channels, namely $I_{K,slow1}$, $I_{K,slow2}$, and I_{ss} (23, 24), and warrants further study.

Additionally, HNO was without effect on {beta}-AR-stimulated I_{Ca} (Figure 6). Thus, the effects of HNO on I_{Ca} appear to be very different from the effects of exogenous and endogenous NO signaling, which has been shown to decrease {beta}-AR-stimulated I_{Ca} (15, 25). Further, the effects of {beta}-AR signaling also differ from HNO, as {beta}-AR stimulation has been demonstrated to

either increase or decrease I_{Ca} depending on which {beta}-AR subtype (i.e., $\{beta\}_1$-AR, $\{beta\}_2$-AR, $\{beta\}_3$-AR) is activated (15, 26, 27).

5.6. SR inhibition abolishes the effects of HNO

Inhibition of SR function completely abolished the positive effects of HNO on systolic Ca^{2+} transients (Figure 7), and indicates that SR Ca^{2+}-cycling is the sole source for the HNO-induced enhancement of systolic Ca^{2+} transients. Since {beta}-AR stimulation increases I_{Ca} (Figure 6), ISO was used as a positive control in order to verify that myocytes with complete SR inhibition could still exhibit an increase in systolic Ca^{2+} transients (Figure 7). Additionally, these results provide further verification that I_{Ca} and other extracellular Ca^{2+} influx do not play a role in the effects of HNO. This is particularly important given the pathologic nature of enhanced extracellular Ca^{2+} influx (28). The enhanced inactivation of I_{Ca} with HNO is also consistent with an increase in SR Ca^{2+}-cycling.

5.7. Limitations

A potential limitation of the current study involves the use of rodent cardiomyocytes. More specifically, the rodent AP tends to be more triangular compared to larger mammals (rabbit, human, etc.), and has a very short plateau phase (29). This brief plateau phase can be attributed to the presence of repolarizing currents that are markedly different from larger mammals. Rodent cardiomyocytes are also less reliant upon extracellular Ca^{2+} influx via I_{Ca} during the process of excitation-contraction coupling, but are instead more reliant upon Ca^{2+} derived from the SR. Therefore, future studies will address the effects of HNO on isolated cardiomyocyte function in larger mammal species.

Another potential limitation of the current study results from indicator loss. Angeli's salt slightly decreased the fluorescent emission of Fluo-4 in cuvette studies conducted over the same time course as our functional experiments (<10%). The possibility also exists for hydroxyl radical exposure to decrease the fluorescent emission of Indo-1. Indeed, a previous study found that hydroxyl radical exposure reduced the fluorescent emission of Indo-1 at both wavelengths (405, 485 nm), without altering the Ca^{2+} sensitivity of the indicator (11). Further, these changes were not wavelength dependent. Therefore, the ratiometric properties of Indo-1 should overcome the loss in fluorescence intensity due to hydroxyl radical exposure. In addition, our cell shortening measurements during hydroxyl radical exposure were consistent with the changes in Ca^{2+} observed with Indo-1 (i.e., increased diastolic Ca^{2+}, decreased diastolic cell length). We also observed an increase in diastolic force in trabecular preparations following hydroxyl radical exposure in a prior study (13). This increase in diastolic force is consistent with an increase in diastolic Ca^{2+}.

5.8. Conclusions

In heart failure the process of excitation-contraction coupling becomes dysfunctional due to a reduction in SR Ca^{2+}-cycling (30, 31). Since SR Ca^{2+}-cycling is diminished, cardiomyocyte contraction is also reduced. Classical pharmacological agents used in the treatment of heart failure ({beta}-AR agonists, phosphodiesterase inhibitors, etc.) have been shown to be detrimental over the long term due to adverse remodeling, increased arrhythmogenesis and increased apoptosis (32-35). These effects are likely due, in part, to the detrimental effects of enhanced extracellular Ca^{2+} influx via I_{Ca}. Prolonged activation of I_{Ca} results in adverse remodeling and has been shown to lead to pathological cardiac hypertrophy through activation of the calcineurin/NFAT signaling pathway (36, 37). Additionally, I_{Ca} can increase the generation of arrhythmias and has been demonstrated to increase the incidence of both early afterdepolarizations (EADs) and DADs (15, 38-40). We did not observe DADs at the myocyte level with AS/HNO, and HNO administration did not trigger arrhythmias *in vivo*, even with concomitant {beta}-AR stimulation (3). Further, activation of I_{Ca} can induce apoptotic cell death in the myocardium (28). However, the pool of Ca^{2+} that induces cardiomyocyte contraction, such as that enhanced by HNO, appears distinct from the Ca^{2+} pool that contributes to pathological signaling (41). The pool of Ca^{2+} which contributes to pathological signaling seems to be composed mainly of Ca^{2+} derived from enhanced extracellular influx. The differential regulation of contraction and pathological signaling by Ca^{2+} is likely due to the presence of specialized subcellular Ca^{2+} signaling domains in the cardiomyocyte. Thus, these data support the potential use of HNO donors as therapeutics for heart failure, as HNO works independent of I_{Ca}.

In conclusion, the HNO-induced enhancement of systolic Ca^{2+} transients in cardiomyocytes is independent and distinct from the non-specific effects of the hydroxyl radical and nitrite, and stems exclusively from an increase in SR Ca^{2+} release and re-uptake without the recruitment of extracellular Ca^{2+} via I_{Ca}. Thus, the positive inotropic action of HNO results from the enhancement of systolic Ca^{2+}, exclusive to SR Ca^{2+}-cycling, and increased myofilament Ca^{2+} sensitization. Interestingly, these changes are likely mediated through the targeting of specific cysteine residues of critical excitation-contraction coupling proteins. More specifically, we previously demonstrated that the effects of nitroxyl were due, in part, to the formation of a disulfide bond between two cysteine residues of phospholamban (6). This modification served to alter the confirmation of phospholamban, thus relieving sarcoplasmic reticulum Ca^{2+}-ATPase inhibition. Another study demonstrated that nitroxyl increased ryanodine receptor activity via disulfide bond formation (8), while a third report demonstrated that nitroxyl increased sarcoplasmic reticulum Ca^{2+}-ATPase activity through the direct glutathiolation of cysteine 674 (7). Although it is possible for HNO to increase cardiomyocyte contraction by targeting cysteine residues found in other excitation-contraction coupling proteins, the present study provides definitive evidence that extracellular Ca^{2+} is not required for the positive inotropic action of HNO.

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7. REFERENCES

1. Bers, DM: Cardiac excitation-contraction coupling. *Nature*, 415, 198-205 (2002)

2. Paolocci, N, WF Saavedra, KM Miranda, C Martignani, T Isoda, JM Hare, MG Espey, JM Fukuto, M Feelisch, DA Wink & DA Kass: Nitroxyl anion exerts redox-sensitive positive cardiac inotropy in vivo by calcitonin gene-related peptide signaling. *Proc Natl Acad Sci U S A*, 98, 10463-8 (2001)

3. Paolocci, N, T Katori, HC Champion, ME St John, KM Miranda, JM Fukuto, DA Wink & DA Kass: Positive inotropic and lusitropic effects of HNO/NO- in failing hearts: independence from beta-adrenergic signaling. *Proc Natl Acad Sci U S A*, 100, 5537-42 (2003)

4. Tocchetti, CG, W Wang, JP Froehlich, S Huke, MA Aon, GM Wilson, G Di Benedetto, B O'Rourke, WD Gao, DA Wink, JP Toscano, M Zaccolo, DM Bers, HH Valdivia, H Cheng, DA Kass & N Paolocci: Nitroxyl improves cellular heart function by directly enhancing cardiac sarcoplasmic reticulum Ca2+ cycling. *Circ Res*, 100, 96-104 (2007)

5. Dai, T, Y Tian, CG Tocchetti, T Katori, AM Murphy, DA Kass, N Paolocci & WD Gao: Nitroxyl increases force development in rat cardiac muscle. *J Physiol*, 580, 951-60 (2007)

6. Froehlich, JP, JE Mahaney, G Keceli, CM Pavlos, R Goldstein, AJ Redwood, C Sumbilla, DI Lee, CG Tocchetti, DA Kass, N Paolocci & JP Toscano: Phospholamban Thiols Play a Central Role in Activation of the Cardiac Muscle Sarcoplasmic Reticulum Calcium Pump by Nitroxyl. *Biochemistry*, 47, 13150-13152 (2008)

7. Lancel, S, J Zhang, A Evangelista, MP Trucillo, X Tong, DA Siwik, RA Cohen & WS Colucci: Nitroxyl Activates SERCA in Cardiac Myocytes via Glutathiolation of Cysteine 674. *Circ Res*, 104, 720-723 (2009)

8. Cheong, E, V Tumbev, J Abramson, G Salama & DA Stoyanovsky: Nitroxyl triggers Ca2+ release from skeletal and cardiac sarcoplasmic reticulum by oxidizing ryanodine receptors. *Cell Calcium*, 37, 87-96 (2005)

9. Stoyanovsky, DA, NF Schor, KD Nylander & G Salama: Effects of pH on the cytotoxicity of sodium trioxodinitrate (Angeli's salt). *J Med Chem*, 47, 210-7 (2004)

10. Ivanova, J, G Salama, RM Clancy, NF Schor, KD Nylander & DA Stoyanovsky: Formation of nitroxyl and hydroxyl radical in solutions of sodium trioxodinitrate: effects of pH and cytotoxicity. *J Biol Chem*, 278, 42761-8 (2003)

11. Josephson, RA, HS Silverman, EG Lakatta, MD Stern & JL Zweier: Study of the mechanisms of hydrogen peroxide and hydroxyl free radical-induced cellular injury and calcium overload in cardiac myocytes. *J Biol Chem*, 266, 2354-61 (1991)

12. Zeitz, O, AE Maass, P Van Nguyen, G Hensmann, H Kogler, K Moller, G Hasenfuss & PM Janssen: Hydroxyl radical-induced acute diastolic dysfunction is due to calcium overload via reverse-mode Na(+)-Ca(2+) exchange. *Circ Res*, 90, 988-95 (2002)

13. Hiranandani, N, T Bupha-Intr & PM Janssen: SERCA overexpression reduces hydroxyl radical injury in murine myocardium. *Am J Physiol Heart Circ Physiol*, 291, H3130-5 (2006)

14. Ziolo, MT, MJ Kohr & H Wang: Nitric oxide signaling and the regulation of myocardial function. *J Mol Cell Cardiol*, 45, 625-632 (2008)

15. Wang, H, MJ Kohr, DG Wheeler & MT Ziolo: Endothelial nitric oxide synthase decreases {beta}-adrenergic responsiveness via inhibition of the L-type Ca2+ current. *Am J Physiol Heart Circ Physiol*, 294, H1473-80 (2008)

16. Kohr, MJ, H Wang, DG Wheeler, M Velayutham, JL Zweier & MT Ziolo: Targeting of phospholamban by peroxynitrite decreases {beta}-adrenergic stimulation in cardiomyocytes. *Cardiovasc Res*, 77, 353-61 (2008)

17. Katori, T, S Donzelli, CG Tocchetti, KM Miranda, G Cormaci, DD Thomas, EA Ketner, MJ Lee, D Mancardi, DA Wink, DA Kass & N Paolocci: Peroxynitrite and myocardial contractility: in vivo versus in vitro effects. *Free Radic Biol Med*, 41, 1606-18 (2006)

18. Sun, J, E Picht, KS Ginsburg, DM Bers, C Steenbergen & E Murphy: Hypercontractile female hearts exhibit increased S-nitrosylation of the L-type Ca2+ channel alpha1 subunit and reduced ischemia/reperfusion injury. *Circ Res*, 98, 403-11 (2006)

19. Fukuto, JM, MI Jackson, N Kaludercic & N Paolocci: Examining nitroxyl in biological systems. *Methods Enzymol*, 440, 411-31 (2008)

20. Janczewski, AM, HA Spurgeon & EG Lakatta: Action potential prolongation in cardiac myocytes of old rats is an adaptation to sustain youthful intracellular Ca2+ regulation. *J Mol Cell Cardiol*, 34, 641-8 (2002)

21. Adachi-Akahane, S, L Cleemann & M Morad: Cross-signaling between L-type Ca2+ channels and

ryanodine receptors in rat ventricular myocytes. *J Gen Physiol*, 108, 435-54 (1996)

22. Sham, JS: Ca2+ release-induced inactivation of Ca2+ current in rat ventricular myocytes: evidence for local Ca2+ signalling. *J Physiol*, 500, 285-95 (1997)
23. Xu, H, W Guo & JM Nerbonne: Four kinetically distinct depolarization-activated K+ currents in adult mouse ventricular myocytes. *J Gen Physiol*, 113, 661-78 (1999)

24. Zhou, J, S Kodirov, M Murata, PD Buckett, JM Nerbonne & G Koren: Regional upregulation of Kv2.1-encoded current, IK,slow2, in Kv1DN mice is abolished by crossbreeding with Kv2DN mice. *Am J Physiol Heart Circ Physiol*, 284, H491-500 (2003)

25. Wahler, GM & SJ Dollinger: Nitric oxide donor SIN-1 inhibits mammalian cardiac calcium current through cGMP-dependent protein kinase. *Am J Physiol*, 268, C45-54 (1995)

26. Xiao, RP & EG Lakatta: Beta 1-adrenoceptor stimulation and beta 2-adrenoceptor stimulation differ in their effects on contraction, cytosolic Ca2+, and Ca2+ current in single rat ventricular cells. *Circ Res*, 73, 286-300 (1993)

27. Zhang, ZS, HJ Cheng, K Onishi, N Ohte, T Wannenburg & CP Cheng: Enhanced inhibition of L-type Ca2+ current by beta3-adrenergic stimulation in failing rat heart. *J Pharmacol Exp Ther*, 315, 1203-11 (2005)

28. Chen, X, X Zhang, H Kubo, DM Harris, GD Mills, J Moyer, R Berretta, ST Potts, JD Marsh & SR Houser: Ca2+ influx-induced sarcoplasmic reticulum Ca2+ overload causes mitochondrial-dependent apoptosis in ventricular myocytes. *Circ Res*, 97, 1009-17 (2005)

29. Nerbonne, JM: Studying cardiac arrhythmias in the mouse--a reasonable model for probing mechanisms? *Trends Cardiovasc Med*, 14, 83-93 (2004)

30. Houser, SR & KB Margulies: Is depressed myocyte contractility centrally involved in heart failure? *Circ Res*, 92, 350-8 (2003)

31. Bers, DM, DA Eisner & HH Valdivia: Sarcoplasmic reticulum Ca2+ and heart failure: roles of diastolic leak and Ca2+ transport. *Circ Res*, 93, 487-90 (2003)

32. Burger, AJ, U Elkayam, MT Neibaur, H Haught, J Ghali, DP Horton & D Aronson: Comparison of the occurrence of ventricular arrhythmias in patients with acutely decompensated congestive heart failure receiving dobutamine versus nesiritide therapy. *Am J Cardiol*, 88, 35-9 (2001)

33. Cuffe, MS, RM Califf, KF Adams, Jr., R Benza, R Bourge, WS Colucci, BM Massie, CM O'Connor, I Pina, R Quigg, MA Silver & M Gheorghiade: Short-term intravenous milrinone for acute exacerbation of chronic heart failure: a randomized controlled trial. *Jama*, 287, 1541-7 (2002)
34. Adamopoulos, S, JT Parissis, EK Iliodromitis, I Paraskevaidis, D Tsiapras, D Farmakis, D Karatzas, M Gheorghiade, GS Filippatos & DT Kremastinos: Effects of levosimendan versus dobutamine on inflammatory and apoptotic pathways in acutely decompensated chronic heart failure. *Am J Cardiol*, 98, 102-6 (2006)

35. Mann, DL & MR Bristow: Mechanisms and models in heart failure: the biomechanical model and beyond. *Circulation*, 111, 2837-49 (2005)

36. Wilkins, BJ, YS Dai, OF Bueno, SA Parsons, J Xu, DM Plank, F Jones, TR Kimball & JD Molkentin: Calcineurin/NFAT coupling participates in pathological, but not physiological, cardiac hypertrophy. *Circ Res*, 94, 110-8 (2004)

37. Fiedler, B, SM Lohmann, A Smolenski, S Linnemuller, B Pieske, F Schroder, JD Molkentin, H Drexler & KC Wollert: Inhibition of calcineurin-NFAT hypertrophy signaling by cGMP-dependent protein kinase type I in cardiac myocytes. *Proc Natl Acad Sci U S A*, 99, 11363-8 (2002)

38. January, CT, JM Riddle & JJ Salata: A model for early afterdepolarizations: induction with the Ca2+ channel agonist Bay K 8644. *Circ Res*, 62, 563-71 (1988)

39. January, CT & JM Riddle: Early afterdepolarizations: mechanism of induction and block. A role for L-type Ca2+ current. *Circ Res*, 64, 977-90 (1989)

40. Tweedie, D, SE Harding & KT MacLeod: Sarcoplasmic reticulum Ca content, sarcolemmal Ca influx and the genesis of arrhythmias in isolated guinea-pig cardiomyocytes. *J Mol Cell Cardiol*, 32, 261-72 (2000)

41. Houser, SR & JD Molkentin: Does contractile Ca2+ control calcineurin-NFAT signaling and pathological hypertrophy in cardiac myocytes? *Sci Signal*, 1, pe31 (2008)

Abbreviations: AP, action potential; AS, Angeli's salt; {beta}-AR, {beta}-adrenergic receptor; FS, fractional shortening; HNO, nitroxyl; ISO, isoproterenol; I_{Ca}, L-type Ca^{2+} current; NO, nitric oxide; NO_2^-, nitrite; $^{\cdot}OH$, hydroxyl radical; PLB, phospholamban; RCL, resting cell length; RyR, ryanodine receptor; SERCA, sarco-endoplasmic reticulum Ca^{2+}-ATPase; SR, sarcoplasmic reticulum

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