NitroxyI(HNO)-Mediated Disulfide Bond Formation Between Cardiac Myofilament Cysteines Enhances Contractile Function

Wei Dong Gao1*, Christopher I. Murray2*, Ye Tian1,3, Xin Zhong1,3, Jenna F. DuMond4, Xiaoxu Shen1, Brian A. Stanley5, D. Brian Foster5, David A. Wink6, S. Bruce King4, Jennifer E. Van Eyk2,5, Nazareno Paolocci5,7.

1Department of Anesthesiology and Critical Care Medicine, 2Department of Biological Chemistry, Johns Hopkins University School of Medicine; Baltimore MD, 21205 USA; 3Department of Pathophysiology, Harbin Medical University, 150086, China; 4Department of Chemistry, Wake Forest University, Winston-Salem, NC, 27109, USA; 5Division of Cardiology, Department of Medicine, Johns Hopkins School of Medicine; Baltimore, MD, 21205, USA; 6Radiation Biology Branch, National Cancer Institute, NIH, Bethesda, MD, 20892, USA; 7Department of Clinical Medicine, University of Perugia, Perugia, 06126 Italy.

*WDG & CIM contributed equally to this work.

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Address correspondence to:
Dr. Wei Dong Gao
Department of Anesthesiology and Critical Care Medicine
The Johns Hopkins University School of Medicine
Zayed Tower 6208
1800 Orleans Street
Baltimore, MD 21205
Tel: 410-955-7519
Fax: 410-955-0994
e-mail: wgao3@jhmi.edu

Dr. Nazareno Paolocci
Division of Cardiology
Department of Medicine
The Johns Hopkins University School of Medicine
Traylor 911
720 Rutland Ave.
Baltimore, MD 21205
Tel: 410-502-5743
Fax: 410-502-2558
e-mail: npaoloc1@jhmi.edu

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**ABSTRACT**

**Rationale:** In the myocardium, redox/cysteine modification of proteins regulating Ca$^{2+}$ cycling can affect contraction and may have therapeutic value. Nitroxyl (HNO), the one electron reduced form of nitric oxide, enhances cardiac function in a manner that suggests reversible cysteine modifications of the contractile machinery.

**Objective:** To determine the effects of HNO modification in cardiac myofilament proteins.

**Methods and Results:** The HNO-donor, 1-nitrosocyclohexyl acetate (NCA), was found to act directly on the myofilament proteins increasing maximum force ($F_{\text{max}}$) and reducing the concentration of Ca$^{2+}$ for 50% activation ($Ca_{50}$) in intact and skinned cardiac muscles. The effects of NCA are reversible by reducing agents and distinct from those of another HNO-donor Angeli’s salt (AS), which was previously reported to increase $F_{\text{max}}$ without affecting $Ca_{50}$. Using a new mass spectrometry capture technique based on the biotin switch assay, we identified and characterized the formation by HNO of a disulfide linked actin-tropomyosin and myosin heavy chain (MHC)-myosin light chain 1 (MLC1). Comparison of the NCA and AS effects with the modifications induced by each donor indicated the actin-tropomyosin and MHC-MLC1 interactions independently correlated with increased Ca$^{2+}$ sensitivity and force generation, respectively.

**Conclusions:** HNO exerts a direct effect on cardiac myofilament proteins increasing myofilament Ca$^{2+}$ responsiveness by promoting disulfide bond formation between critical cysteine residues. These findings indicate a novel, redox-based modulation of the contractile apparatus which positively impacts myocardial function, providing further mechanistic insight for HNO as a therapeutic agent.

**Keywords:** contractility, nitroxyl, redox-switch, oxidation, calcium, oxidant signalling, redox

**Non-standard Abbreviations:**

- HF: heart failure
- PTM: post-translational modification
- redox: reduction/oxidation
- ROS: reactive oxygen species
- RNS: reactive nitrogen species
- HNO: nitroxyl
- DTT: dithiothreitol
- AS: Angeli’s salt
- NCA: 1-nitrosocyclohexyl acetate
- MS: mass spectrometry
- TM: tropomyosin
- MH: myosin heavy chain
- MLC1: myosin light chain 1
- NEM: N-ethylmaleimide
- biotin-HPDP: N-[6-(Biotinamido)hexyl]-3’-(2’-pyridyldithio) propionamide
- DIGE: Difference in-gel electrophoresis
- DTDP: 2,2’-dithiodipyridine.
INTRODUCTION

A hallmark of acute and chronic heart failure (HF) is the loss of contractile function. Contraction is stimulated by an influx of intracellular Ca$^{2+}$ and can be regulated by direct post-translational modification (PTM) of the myofilament proteins. The majority of therapies available (e.g. β agonists, phosphodiesterase inhibitors) increase intracellular Ca$^{2+}$ to strengthen contraction; however, they have also been associated with long-term mortality either by increasing diastolic force, worsening diastolic dysfunction or promoting cardiac arrhythmias. A promising alternative approach has been the development of inotropic agents that directly increase contraction by enhancing the response of the contractile myofilament proteins to the available Ca$^{2+}$. It has been hoped that this class of drugs would revolutionize the management of heart failure. However, recent clinical trials with Ca$^{2+}$ sensitizers such as levosimendan have met with limited success.

An area that has been overlooked in the heart until now is the possible manipulation of key reduction/oxidation (redox) sensitive sites or “switches”. Modification of these critical residues has been shown to impact processes involved in signal transduction, transcription and metabolism, leading to the assertion that similar switches may exist to regulate cardiac contraction. Cysteine (Cys), either individually or in pairs, forms the core of thiol-based switches that can sense the local redox-environment through the unique chemical properties of its thiol side chain. These sensors oscillate between a reduced and oxidized state, responding to reactive oxygen or nitrogen species (ROS/RNS) with a continuum of PTMs. Low fluxes of ROS/RNS can function as a signaling mechanism through the formation of reversibly oxidized Cys (e.g. disulfide or sulfenic acid-Cys). Higher fluxes can generate irreversibly modified Cys (e.g. sulfinic or sulfonic acid-Cys) which may lead to cellular apoptosis and eventually necrosis. Regulatory redox-switches have been found in components of excitation-contraction coupling where they contribute to modulate cardiac contraction, either reversibly, as seen with S-nitrosylation during signaling events, or more permanent oxidative modifications of the ryanodine receptor which can lead to dysfunction.

The characterization of a similar Cys-based regulation in the myofilaments remains elusive. Skinned cardiac muscle fibers, comprising only the contractile machinery, displayed reduced force generation when treated with oxidizing agents such as H$_2$O$_2$ or O$_2^-$ likely in the presence of transition metals. Conversely, cardiac muscles superfused with donors of the thiophylic RNS agent, nitroxyl (HNO), increased force production. This increase in function is notably larger than can be accounted for by the associated rise in Ca$^{2+}$ transient, suggesting HNO acts directly on the myofilaments, sensitizing them to Ca$^{2+}$. HNO reacts with the thiol on Cys forming either a sulfaminamide or, in the presence of an additional free thiol, a disulfide bond (Figure 1). The effects of HNO on myofilaments have been found to be readily reversible by the reducing agent dithiothreitol (DTT), suggesting that its action involves the formation of intra- or inter-protein disulfide bonds. Yet, nature and sites of HNO-induced modifications in myofilaments are currently unknown.

Our hypothesis is that HNO induces the formation of intra- or inter-protein disulfide bonds between critical Cys in the myofilament proteins to enhance contractility. We investigated the effects of two chemically unrelated HNO donors (Angeli’s salt (AS)) and 1-nitrosocyclohexyl acetate (NCA) on cardiac contraction in intact and skinned muscles and then identified and characterized HNO modified residues utilizing a new mass spectrometry (MS) site mapping technique.
METHODS

**Determination of force, \([\text{Ca}^{2+}]_i\), and steady-state force-[\text{Ca}^{2+}]_i** relationships in intact cardiac muscle. See supplemental material.

**Steady-state force-[\text{Ca}^{2+}]** relationships in skinned cardiac and skeletal muscles. Details for muscle skinning are in the online supplement. After skinning, the muscles were activated with solutions of varied \([\text{Ca}^{2+}]\) while diastolic sarcomere length was kept constant. The force-[\text{Ca}^{2+}] relationships were generated before and after treatment with NCA (2.5 µmol/L) in the same preparations. After obtaining the force-[\text{Ca}^{2+}] relation for the control phase, the muscle was exposed to NCA in relaxing solution for 10 min. There was no increase in resting force during the treatment. Afterwards, the muscle was activated with activating solutions of varied \([\text{Ca}^{2+}]\) containing NCA. These activations in the presence of NCA were repeated once to ensure similar force-[\text{Ca}^{2+}] relations were obtained.

**Detection of HNO modifications by biotin switch assay.** HNO modified thiols were detected using a variation of the standard biotin switch protocol. In brief, 100 µg of isolated rat myofibrils/treatment were diluted to 0.5 µg/µL in HEN (250 mmol/L HEPES pH 7.4, 1 mmol/L EDTA and 0.1 mmol/L neocuproine) including 0.1% (w/v) SDS, treated for 10 min at 37°C and removed by acetone precipitation. Treatments included: NCA, 2.5 µmol/L; AS, 500 µmol/L; and their decomposed, inactive equivalents (reagent at rt for >96 h) in the absence of Ca²⁺. Additional treatments were DTT, 5 mmol/L and DEA/NO, 125 mmol/L. Remaining free thiols were blocked with 20 mmol/L N-ethylmaleimide (NEM). HNO and/or NO modified thiols were reduced using 5 mmol/L DTT or 1 mmol/L ascorbate and biotinylated with 0.8 mmol/L N-[6-(Biotinamido)hexyl]-3´-(2´-pyridylidithio) propionamide (biotin-HPDP). Excess biotin-HPDP was removed by acetone precipitation (2 volumes) and resultant pellets were washed with additional acetone. Biotinylated proteins were captured with Ultralink Immobilized Streptavidin, separated by SDS PAGE and silver stained. For MS studies, 200 µg of starting material was used and after labeling, biotinylated proteins were digested overnight with trypsin or chymotrypsin prior to capture with streptavidin (see supplemental material).

**Gel shift assay.** 10 µg of rat myofibrils (0.5 µg/µL) were exposed to treatment or control conditions for 20 min at 37°C. Samples were diluted to 0.1 µg/µL in LSD sample buffer, treated with 0 or 5 mmol/L DTT and separated by SDS PAGE. Proteins were visualized by silver stain or western blot and identified by MS as needed (see supplemental material).

**Difference in-gel electrophoresis (DIGE) analysis.** NCA/AS treated purified tropomyosin (TM) or myofibril samples were labelled using the fluorescent CyDyes 2, 3, 5 according to the manufacturer’s recommended protocol and analysed by SDS PAGE (see supplemental material).

**Statistical analysis.** Student’s t-test and one-way analysis of variance (ANOVA) was used for statistical analysis of the data (Systat 10.2). A value of P<0.05 was considered to indicate significant differences between groups. Unless otherwise indicated, pooled data were expressed as mean ± standard error of the mean (S.E.M.).
RESULTS

HNO donors augment force generation in isolated cardiac muscle.

In isolated intact cardiac muscle, the HNO-donor, NCA, increased force development in a dose dependent manner from 0.2 to 20 µmol/L with no significant changes in resting force at 0.5 mmol/L [Ca\(^{2+}\)]\(_o\) (Figure 2A and 2B). NCA (10 µmol/L) augmented force up to 37.7±3.8 mN/mm\(^2\) (p<0.001 vs. 8.8±1.0 mN/mm\(^2\) in control muscles). The rise in Ca\(^{2+}\) transient was not significantly different (0.43±0.11 vs. 0.27±0.06 µmol/L control, p=0.23) and diastolic Ca\(^{2+}\) only rose significantly at high doses. In the presence of NCA, twitch force remained higher at any given external Ca\(^{2+}\) concentration. However, the amplitude of intracellular Ca\(^{2+}\) transients was not different from control (Figure 2C). The force-frequency relationship was also enhanced by NCA without increasing intracellular Ca\(^{2+}\) concentration (Figure 2D).

The disproportionate increase in force compared to changes in Ca\(^{2+}\) transients may be the result of increased myofilament Ca\(^{2+}\) responsiveness with NCA. To test this directly, steady-state force-[Ca\(^{2+}\)]\(_i\) relations were obtained by tetanizing the muscles in the presence of ryanodine. Steady-state force-[Ca\(^{2+}\)]\(_i\) relations in control and muscles exposed to NCA (2.5 µmol/L) are shown in Figure 3A. Both maximal Ca\(^{2+}\)-activated force (F\(_{max}\)) and [Ca\(^{2+}\)]\(_i\) required for 50% of activation (Ca\(_{50}\)) increased significantly in muscles exposed to NCA (Table 1). Furthermore, the increased Ca\(^{2+}\) responsiveness persisted after removing membrane-delineated organelles with detergent (skinning), indicating that NCA acts directly on the myofilament proteins (Figure 3B and Table 1). The increases in myofilament Ca\(^{2+}\) sensitivity caused by HNO were completely abolished by 5 mmol/L DTT (Figure 3C), confirming that the effects of HNO are sensitive to reducing equivalents. Although HNO is the primary nitrogenous hydrolysis product of NCA (>50%), other products include acetic acid/sodium acetate and cyclohexanone. These compounds did not produce any measurable effects in the muscles (data not shown). Furthermore, 1-nitrosocyclohexyl pivalate (NCP), a compound of similar chemical structure that does not release HNO had no effects (Figure 3D). Together, these data suggest the positive inotropic effect of NCA is specific to HNO.

HNO targets specific cysteine residues in the myofilament proteins.

The nature of the HNO induced Cys modifications was investigated using a new variation of the biotin switch assay (Figure 4A). Isolated rat cardiac myofibrils were used in place of skinned fibres due to ease in preparing sufficient quantities. Mg-ATPase measurements confirmed the integrity of the myofibrils and NCA treatment resulted in a similar decrease in Ca\(_{50}\) observed in intact and skinned muscle fibres (Online Figure I). A change in the maximal myofibril ATPase rate was not anticipated as we have reported previously. For the biotin switch experiments, 5 mmol/L DTT was used to target the same HNO induced modifications which were reversed in the physiological studies. To compare HNO modification to those induced by NO, 1 mmol/L ascorbate was used to reduce S-nitrosylation groups. The streptavidin capture of intact biotinylated proteins revealed that HNO modified proteins could be specifically isolated and that they were distinct from those induced by treatment with the NO donor, DEA/NO (Figure 4B). HNO modifications were resistant to reduction with ascorbate while DEA/NO modifications were reversed by both reducing agents.

To map and evaluate the effects of HNO modification on individual Cys, a comparison was done between the changes induced by NCA to those of the traditional HNO donor, AS. AS co-releases HNO and nitrite while NCA decomposes to HNO, acetate and cyclohexanone. We have previously reported that AS increased F\(_{max}\) but did not affect Ca\(^{2+}\) sensitivity (Ca\(_{50}\)) in cardiac muscle. Using the modified biotin switch technique with different donors, a comparative proteomic strategy was devised to parse the effects of the different HNO-donors; Cys modifications common to NCA and AS treatments were attributed to the increase in F\(_{max}\) while sites unique to NCA were considered candidates for the decrease in
A total of 12 HNO-modified Cys were identified on 8 proteins between the two treatments (Figure 4C and Table 2). Of those, 4 sites (TM Cys190, actin Cys257 and MHC Cys947 and Cys1750) were unique to NCA treatment (Online Table I).

**HNO induces dimeric forms of MHC-MLC1 and actin-TM.**

To investigate the HNO-induced modifications, non-reducing western blot analysis was used to observe the presence of higher molecular weight species. Higher molecular weight forms of actin, TM, MHC and MLC1 were found which were reversed in the presence of 5 mmol/L DTT, indicating HNO induced inter-protein disulfide bonds (Figure 5A). MHC and MLC1 were found to be modified in a similar manner with both NCA and AS treatment as indicated by the MS analysis. The two higher molecular weight species observed were consistent with the formation of an MLC1 homodimer (25 kDa + 25 kDa = ~50 kDa) and an MHC–MLC1 heterodimer (25 kDa + 212 kDa = ~240 kDa). The formation of a higher molecular weight TM species only occurred with NCA treatment, consistent with MS analysis. A higher molecular weight form of actin, approximately 80 kDa, was observed in both NCA and AS treatment. Additionally, NCA treatment produced a loss of antibody binding for the monomeric form of actin (42 kDa). MS analysis of silver stained gel bands revealed that the actin monomer displays increased gel mobility with NCA. Analysis of other bands revealed that a similar, but less abundant, shift for actin also occurred in AS treated samples (Figure 5B and Online Table II). No molecular weight shifts were observed for any of the candidate proteins following treatment with the NO-donor.

The higher molecular weight actin and TM bands were examined by SDS-PAGE with better resolution in that region. Comparison of purified TM and isolated myofibrils revealed a higher migration in the NCA-treated myofilament preparations (Figure 5C, left). This suggests that in myofilaments, NCA does not induce the formation of a TM homodimer but forms another disulfide linked interaction. Analysis of the higher forms of actin revealed a difference between the NCA and AS treated samples. NCA treatment of myofibrils produced a specific band at a slightly lower molecular weight than the bands observed for AS (Figure 5C, right). Comparison of the TM and actin NCA-bands suggests a linked, co-migrating species. Difference in-gel electrophoresis (DIGE) analysis revealed a distinct myofibril NCA-specific band (Cy3 - green) higher than the purified TM homodimer (Cy2 - blue) and below a series of AS-specific bands (Cy5 – red) (Figure 5D). MS analysis of the molecular weight region identified both actin and TM at that location which were lost under reducing conditions (Figure 5E and Online Table II). These findings, in combination with the site mapping results, suggest the presence of an NCA-specific heterodimer linked by the formation of a disulfide bridge between TM (Cys190) and actin (Cys257).

Other sites of modification identified in this study were considered as possible candidates for the NCA specific increase in Ca^{2+} sensitivity. The two sites identified on MHC (Cys947, Cys1750) with NCA treatment were ruled out. Western blot analysis of MHC revealed no NCA-specific higher molecular weight species; only a small shift in common between the two donor treatments, determined to be an interaction with MLC1, was observed. It is possible that an intramolecular disulfide bond was formed between the residues, but considering both modifications were mapped to the linear tail region in areas not known to closely associate, it is unlikely these candidates represent meaningful sites of modification. Additional analysis into the different action of the two HNO donors revealed that the co-release of nitrite from AS had no role in inhibiting the formation of the actin-TM heterodimer. Co-incubation of NCA and nitrite did not affect heterodimer formation (Online Figure II).

**NCA increases Ca^{2+} sensitivity but not maximum force production in skeletal muscle due to the lack of dimeric MLC1 formation.**

To determine if MLC1 Cys81 is involved in the increased maximum force production, the effect of HNO donors was investigated in skeletal muscle preparations. Skeletal muscle isoforms of
myofilament proteins contain all potential target Cys except for MLC1, which lacks the candidate site Cys81, providing a natural mutant sequence (Figure 6A). Steady-state force-[Ca\textsuperscript{2+}], relations of skeletal muscle before and after exposure to NCA (2 µmol/L) are presented in Figure 6B. C\textsubscript{a50} decreased significantly in the presence of NCA while F\textsubscript{max} remained unchanged (F\textsubscript{max} 33±3.8 vs. 31.7±3.7 mN/mm\textsuperscript{2}, p=NS; C\textsubscript{a50} 0.8±0.1 vs. 1.07±0.05 µmol/L, p<0.05; Hill, 4.36±0.81 vs. 3.47±0.82, p=NS). The same insensitivity of F\textsubscript{max} to AS was also observed in skinned skeletal muscles (data not shown). Diagonal gel western blots revealed an absence of dimeric forms of MLC1 in NCA-treated skeletal samples but confirmed the presence of a higher molecular weight form of TM (Figure 6C and Online Figure III).

These results indicate that MLC1 Cys81 is a critical residue and redox-switch for the HNO induced increase in cardiac force production.

DISCUSSION

The redox-switch is emerging as a diverse signalling system for detecting and reacting to changes in the oxidative environment. Disulfide bonds have been the least appreciated candidate for these regulatory modifications.\textsuperscript{22} Here we show that two chemically unrelated HNO-donors act directly on specific myofilament proteins to form disulfide bonds between redox-switches which increase Ca\textsuperscript{2+} sensitivity and/or force. HNO-treatment resulted in the formation of an actin-TM heterodimer which correlates with the increase in Ca\textsuperscript{2+} sensitivity, and dimeric forms of MHC and MLC1 which are associated with increased force generation. These effects are reversed by treatment with reducing agents and distinct from those induced by NO.

**HNO action on the cardiac myofilaments.**

The formation of regulatory disulfide bonds in the myofilaments that enhance contraction is a novel finding. Our data demonstrate that NCA/HNO can specifically enhance myofilament response to Ca\textsuperscript{2+} which correlates with the *in vitro* formation of an actin-TM heterodimer. In muscle, TM provides a physical barrier, reducing the probability of myosin S1 heads binding to the actin filament during relaxation.\textsuperscript{2} When Ca\textsuperscript{2+} binds to the troponin complex TM moves from its inactivated (*blocked, B-*) state over actin subdomains 1 and 2 to its Ca\textsuperscript{2+}-activated (*closed, C-*) state over actin subdomains 3 and 4.\textsuperscript{23} This shift in position increases the probability of myosin binding to the sites on actin, further shifting TM’s position to the fully open (*M-*) state (Online Figure IV).\textsuperscript{23-25} Our findings indicate that HNO treatment results in the formation of a disulfide bond between the Cys residues in actin’s subdomain 4 (Cys257) and TM (Cys190),\textsuperscript{26,27} given their availability and proximity to each other during activation.\textsuperscript{28-30}

The average position and movement of TM over the actin filament is a reflection of equilibrium distribution between the B-, C- and M-states.\textsuperscript{2} In the absence of Ca\textsuperscript{2+}, the distribution is ≈70/25/5%, respectively,\textsuperscript{31,32} which changes in response to Ca\textsuperscript{2+} or the presence of myosin heads.\textsuperscript{33} It is tempting to speculate that the formation of a disulfide bond between Cys190 of TM and Cys257 of actin, would tether a subpopulation of TMs in a position over actin’s subdomains 3 and 4 similar to the natural C- or M-state. This would disturb the local positional distribution of TM and may bias its local equilibrium position toward a state that is more permissive to myosin-binding; effectively priming the myofilaments for Ca\textsuperscript{2+}-activation. HNO treatments were preformed in the absence of Ca\textsuperscript{2+} indicating that myofilament activation is not required for dimer formation. Only low-stoichiometry cross-linking could have a disproportionately large inotropic effect, since the effect on one TM is communicated to neighboring TMs by end-to-end interactions along the actin filament. Such local effects on TM by low-stoichiometry cross-linking do not appear to impinge on its ability to adopt the proper B-state globally, since NCA-treated muscles show normal relaxation kinetics. Increased resting force was observed for intact muscle, but not skinned preparations, and only high HNO concentrations which correlated with an increase in diastolic calcium,
consistent with previous findings. Alterations of TM positional equilibrium by PTMs have been demonstrated by several recent studies using C-terminus truncated troponin I.

An outstanding question in the analysis is a definitive demonstration for the requirement of an actin-TM heterodimer to influence contraction. While no other potential interactions were identified, conformation of the role of an actin-TM disulfide bond will require additional investigation including: site-directed mutagenesis via an in vivo gene-delivery approach or extraction/reconstitution of the thin filament with gelsolin, FRET techniques and 3D cryo-reconstruction of NCA treated thin filaments. These investigations will be the subject of future studies.

The lack of HNO-dependent increase in skeletal muscle Fmax is due to the absence of the dimeric forms of MLC1 and MHC. Both MHC and MLC1 are essential components of the cross-bridges which form during steady-state activations. Fmax is dependent on cross-bridge turnover kinetics, the number of cross-bridges, and the force per cross-bridge. Therefore, alterations in the interaction between MHC and MLC1 (specifically Cys81 of MLC1) likely impacts force production. The regulatory MLC1 is positioned like a collar just below the head region on the MHC (a region called the lever arm). It has been proposed that the swing of this lever arm produces contraction which leads to force development. MLC1 plays an essential role in stabilizing the arm. The orientation of these structures brings the identified residue, Cys81, in close proximity with its pair and Cys37 in MHC’s enzymatic head region (Figure S4). We therefore propose that the formation of a covalent interaction in this region, via the HNO induced disulfide bond, provides additional rigidity of the lever arm, facilitating a stronger swing of the arm, ultimately producing more force. Whether MHC-MLC1 heterodimer or MLC1-MLC1 homodimer are responsible is not clear as neither occurred in skeletal muscle treatments.

The slope of force-[Ca2+] relation (Hill coefficient) was not altered by NCA treatment suggesting no change in the cooperative activation in of force/cross-bridges. Cooperative activation occurs through the transmission of Ca2+ binding to TnC across neighbouring functional units (FUs) resulting in greater cross bridge formation. The results indicate that NCA has little effect on Ca2+ binding to TnC, but instead suggest that it may have a greater influence on the force production of individual cross-bridges rather than the number formed. However, an NCA effect on cooperativity cannot be ruled out since not all aspects of cooperative activation are represented by changes in Hill coefficient. Further investigation will be required to address this possibility.

**HNO donors, redox chemistry and disulfide bond formation in the heart.**

The formation of disulfide bonds is not specific to HNO; however, the broader circumstances of HNO derived disulfide formation appear to be unique among ROS and RNS. HNO is capable of forming disulfide bonds and has been shown to enhance in vivo cardiac inotropy, while the levels of ROS or NO necessary to induce disulfide bonds have been associated with wider cellular dysfunction and, in the case of myocardium, diminished contractility. It is likely the distinct chemistries of these oxidative species are the source of the difference. ROS can lead to rapid and transient disulfide formation via sulfenyl (thiyl) radical or sulfenic acid reactions. Both modifications can then react with nearby available thiols to form disulfides, but under conditions of persistently high oxidative stress, over-oxidation generates sulfenic and sulfonic acids which prevent this reaction. In contrast, HNO modification results in only one of two possible products, a sulfinamide or disulfide. The positive inotropic effects suggest the targeted chemistry of HNO is not predisposed to more harmful and permanent oxidative modifications.

A recent example of the detrimental effects of oxidative stress was presented by Canton and co-workers who reported that left ventricular specimens obtained from explanted failing human hearts displayed greater levels of actin and TM carbonylation that correlated with contractile impairment. Carbonylation is an irreversible PTM likely formed by the high oxidative stress experienced over the
course of heart failure. In addition to carbonylation, they also found that TM participated in a disulfide cross-linked complex. The composition of this complex remains undetermined although actin was ruled out as a component. These findings highlight that differences in the levels of oxidative stress and the oxidizing agent can determine the type of PTM formed and dictate the functional or pathological outcome. Our study suggests HNO induces mildly oxidizing conditions which are manifestly different from those experienced by failing hearts contributing to contractile dysfunction.

One key observation in the current study was that treatment with HNO donors NCA and AS had distinct effects on the myofilaments. While both NCA and AS release HNO, they do so by different mechanisms and rates, yielding different by-products. AS co-releases HNO and an equal amount of nitrite upon protonation with a half life of 2.1 min at 37°C at pH 7.4.21 Hydrolysis of NCA releases HNO, acetate and cyclohexanone with a half life that depends on the reaction conditions (t₁/₂ = 0.8 min in 0.1 N NaOH/MeOH and t₁/₂ = >2 h in pH 7.4 phosphate buffer/MeOH)18 (Online Figure V). The effect of nitrite or a possible reaction between thiol and the NCA compound have been ruled out experimentally (Online Figure II). The unique physiological effects of AS and NCA are more likely the result of their different release kinetics. The rapid release of HNO by AS is more likely to flood an area resulting in the simultaneous conversion of opposing thiols to N-hydroxysulfenamides, preventing the formation of a potential disulfide. The much slower HNO release from NCA produces a lower, more constant, concentration of HNO in the system. Low levels of HNO would favor the generation of the N-hydroxysulfenamide intermediate at only one in a pair of proximal thiols. The other thiol would remain un-modified permitting rapid reaction and disulfide formation before any rearrangement to a sulfinamide (Figure 1).

Translational implications and conclusions.

Since the pharmacological management of acute HF has changed little over the past 15 years, our findings may also be of clinical interest. Current available therapies (e.g. β-agonists, phosphodiesterase inhibitors) increase intracellular [Ca²⁺] with negative long-term effects. HNO targets specific redox-switches to enhance the myofilament response to Ca²⁺, without affecting diastolic Ca²⁺ levels and its inotropic action is preserved in failing hearts.51 Treatment with HNO potentially offers an interesting opportunity to improve both systolic and diastolic function in HF free from the negative consequences of sustained Ca²⁺ manipulation.4,13,14 Clinical trials are ongoing to test this possibility in acutely decompensate HF patients.52

Oxidative modifications in the myocardium have primarily been associated with negative functional consequences,53 but evidence suggests this may not be uniformly true. Application of the HNO donor, NCA, reproduced and exceeded the performance of previously characterized HNO-donors in cardiac tissue.17 Here we established that HNO acts directly and reversibly on the myofilament proteins to modify specific cysteine-thiols, increasing contractility. Characterization of the covalent interactions between key contractile proteins via the formation of disulfide bonds provides insight into the mechanism of this novel redox-based modulation of cardiac function. These findings provide further support for HNO-donors as a potential new class of therapeutic for the management of HF.

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DISCLOSURES
Nazareno Paolocci is a founder and stock holder of Cardioxyl Pharmaceuticals Inc.

REFERENCES


45. Razumova MV, Bukatina AE, Campbell KB. Different myofilament nearest-neighbor interactions have distinctive effects on contractile behavior. Biophys J. 2000;78:3120-3137.


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FIGURE LEGENDS

Figure 1. HNO reacts with free thiols via a reactive intermediate. Following the formation of N-hydroxysulfinamide, the reaction proceeds by one of two pathways; A, isomerization to form a sulfanamide group or B, in the presence of an additional thiol formation of a disulfide bond and hydroxylamine.16

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TABLES

Table 1. Effect of NCA on parameters of steady-state force-[Ca2+]i relationships in intact and skinned cardiac muscles.

<table>
<thead>
<tr>
<th></th>
<th>Fmax (mN/mm²)</th>
<th>Ca₅₀ (µmol/L)</th>
<th>Hill Coefficient (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intact muscles (n=5)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before NCA</td>
<td>96±5</td>
<td>0.57±0.03</td>
<td>3.94±0.18</td>
</tr>
<tr>
<td>after NCA</td>
<td>123±18*</td>
<td>0.42±0.01*</td>
<td>4.92±0.84</td>
</tr>
<tr>
<td><strong>Skinned muscles (n=6)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>before NCA</td>
<td>82±4</td>
<td>1.35±0.36</td>
<td>3.21±1.18</td>
</tr>
<tr>
<td>after NCA</td>
<td>94±2*</td>
<td>0.30±0.13*</td>
<td>2.39±1.02</td>
</tr>
</tbody>
</table>

* p<0.05 vs. before NCA in intact muscles; # p<0.05 vs. before NCA in skinned muscles.

Table 2. Sites of HNO modification determined by biotin switch assay.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Position of modified Cys</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NCA</strong></td>
<td></td>
</tr>
<tr>
<td>α-troponemin</td>
<td>190</td>
</tr>
<tr>
<td>Actin</td>
<td>257</td>
</tr>
<tr>
<td>myosin heavy chain</td>
<td>947(β)*</td>
</tr>
<tr>
<td></td>
<td>1750(α/β)</td>
</tr>
<tr>
<td><strong>NCA / AS</strong></td>
<td></td>
</tr>
<tr>
<td>myosin heavy chain</td>
<td>37(α)</td>
</tr>
<tr>
<td>myosin light chain 1</td>
<td>81</td>
</tr>
<tr>
<td>Actin</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>219</td>
</tr>
<tr>
<td>α-actinin</td>
<td>889</td>
</tr>
<tr>
<td>myosin binding protein C</td>
<td>475</td>
</tr>
<tr>
<td>troponin C</td>
<td>35</td>
</tr>
<tr>
<td><strong>AS</strong></td>
<td></td>
</tr>
<tr>
<td>myosin heavy chain</td>
<td>907(α/β)</td>
</tr>
</tbody>
</table>

(*) denotes sites present in different isoforms of myosin heavy chain sequence.
Novelty and Significance

What is Known?

- Perturbed tissue redox conditions contribute to cardiac structural disarray and mechanical dysfunction, particularly under conditions of stress.
- High fluxes of oxidizing agents could irreversibly alter myofilaments and their regulatory proteins, reducing cardiac force generation.
- Nitroxyl (HNO), a reactive nitrogen species (RNS) related to nitric oxide (NO), increases cardiac inotropy/lusitropy in a reversible redox-dependent manner.

What New Information Does This Article Contribute?

- HNO donors act directly on specific myofilament proteins to form disulfide bonds between cysteine-based redox-switches.
- HNO treatment forms actin-tropomyosin heterodimers and dimeric forms of myosin heavy chain (MHC) and myosin light chain 1 (MLC1) account for increased myofilament responsiveness to Ca\textsuperscript{2+}.
- HNO-induced modifications can be detected using a dithiothreitol-based biotin switch capture technique combined with mass spectrometry (MS).

Redox-switches modulate the function of channels/pumps that control cardiac Ca\textsuperscript{2+} cycling. However, evidence supporting the role of cysteine-based sensors in myofilaments is still scant, as redox-changes have been exclusively associated with reduced force generation. Here we provide evidence that HNO augments force development via reversible disulfide bond formation between specific cysteine residues in myofilaments. HNO increases Ca\textsuperscript{2+} sensitivity but not maximum force production in skeletal muscle, which lacks some sites as revealed by a new biotin-switch/MS approach. The novel idea is that redox-switches in myofilaments are dynamic in nature and undergo a continuum of redox-modifications where the functional or detrimental outcome is likely dictated by the oxidant’s nature and the redox-milieu of the myofilament compartment. The pharmacological treatment of heart failure (HF) has changed little over the past 15 years. β-agonists and phosphodiesterase inhibitors increase intracellular [Ca\textsuperscript{2+}] but can have negative long-term effects. Conversely, HNO enhances myofilament Ca\textsuperscript{2+} responsiveness without affecting diastolic Ca\textsuperscript{2+} levels, and its action is preserved in failing hearts despite highly oxidizing conditions. Thus, HNO donors may improve systolic/diastolic function in HF patients, avoiding the negative consequences of sustained Ca\textsuperscript{2+} manipulation.
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Nitroxyl(HNO)-Mediated Disulfide Bond Formation Between Cardiac Myofilament Cysteines Enhances Contractile Function

Wei Dong Gao, Christopher I. Murray, Ye Tian, Xin Zhong, Jenna F. DuMond, Xiaoxu Shen, Brian A. Stanley, D. Brian Foster, David A. Wink, S. Bruce King, Jennifer E. Van Eyk and Nazareno Paolocci

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Supplemental Methods

Force and [Ca\textsuperscript{2+}], measurements in intact cardiac muscle

Rat hearts were exposed via mid-sternotomy after the animals were anesthetized with intraperitoneal injection of pentobarbital (100 mg/kg), and were then rapidly excised and aorta cannulated. The hearts were perfused retrogradely (~15 mL/min) with Krebs-Henseleit (K-H) solution containing 2.3-butanedione monoxime (BDM, 20 mmol/L), equilibrated with 95% O\textsubscript{2} and 5% CO\textsubscript{2}. The K-H solution is composed of (in mmol/L): NaCl 120, NaHCO\textsubscript{3} 20, KCl 5, MgCl\textsubscript{2} 1.2, glucose 10, and CaCl\textsubscript{2} 1.0, pH 7.35-7.45. Trabeculae were quickly dissected from the right ventricles of the hearts and mounted between a force transducer and a motor arm. The muscles were superfused with K-H solution at a rate of ~10 mL/min and stimulated at 0.5 Hz. Force was measured using a force transducer system (Si, Germany) and expressed in mN/mm. Sarcomere length was measured by laser diffraction.\textsuperscript{1,2} Fura-2 potassium salt was microinjected iontophoretically into one cell and allowed to spread throughout the whole muscle (via gap junctions). The epifluorescence of fura-2 was measured by exciting at 380 and 340 nm. The fluorescent light was collected at 510 nm by a photomultiplier tube (R1527, Hamamatsu). [Ca\textsuperscript{2+}] was given by (after subtraction of the autofluorescence): 

\[ [Ca^{2+}] = K_d' \frac{(R-R_{\text{min}})}{(R_{\text{max}}-R)} \]

where R is the observed ratio of fluorescence (340/380), \( K_d' \) is the apparent dissociation constant, \( R_{\text{max}} \) is the ratio of 340 nm/380 nm at saturating [Ca\textsuperscript{2+}], and \( R_{\text{min}} \) is the ratio of 340 nm/380 nm at zero [Ca\textsuperscript{2+}]. The values of \( K_d' \), \( R_{\text{max}} \), and \( R_{\text{min}} \) were determined by in vivo calibrations.\textsuperscript{1,2} Tetanization of the trabecula was achieved by addition of ryanodine (1.0 µmol/L) and by increasing the stimulus rate to 10 Hz briefly (~3 sec) (isometric at sarcomere length of 2.2-2.3 µm) to obtain steady-state force-[Ca\textsuperscript{2+}] relations. Different levels of tetanized force were obtained by increasing [Ca\textsuperscript{2+}] in the perfusate (up to 20-25 mmol/L). The data was fit to the Hill equation:
\[
F = F_{\text{max}} \frac{[\text{Ca}^{2+}]}{K_{1/2}^n + [\text{Ca}^{2+}]}^n,
\]
where \(F_{\text{max}}\) is the maximal force, \(K_{1/2}\) is \([\text{Ca}^{2+}]\) at half \(F_{\text{max}}\), and \(n\) is the Hill coefficient.

**Steady-state force-Ca\(^{2+}\) relationships in skinned cardiac and skeletal muscles**

Trabeculae were skinned by \(\sim 15\) min exposure to \(1.0\%\) Triton in relaxation solution containing (mmol/L) KCl 80, HEPES 25, \(K_2\text{EGTA}\) 10, creatine phosphates sodium salt (Na\(_2\text{CrP}\)) 15, Na\(_2\text{ATP}\) 5, MgCl\(_2\) 5.15, and leupeptin 0.5, pH 7.2. After skinning, the muscles were activated with solutions (mmol/L: Ca\(^{2+}\)-EGTA 10, KCl 80, HEPES 25, Na\(_2\text{CrP}\) 15, Na\(_2\text{ATP}\) 5, MgCl\(_2\) 4.75, and leupeptin 0.5, pH 7.2) of varied \([\text{Ca}^{2+}]\) while diastolic sarcomere length was kept constant. The force-pCa relationships were generated before and after treatment with NCA (2.5 umol/L) in the same preparations. After obtaining the force-pCa\(^{2+}\) relation for the control phase, the muscle was exposed to NCA in relaxing solution for \(10\) min. There was no increase in resting force during the treatment. Afterwards, the muscle was activated with activating solutions of varied \([\text{Ca}^{2+}]\) containing NCA. These activations in the presence of NCA were repeated once to make sure similar force-pCa relations were obtained.

For skeletal muscle preparations, incisions were made to the legs of the rats and gastrocnemius muscle was dissected and placed in the dissection dish superfused with K-H solution containing 2,3-butanedione monoxime (BDM, 20 mmol/L), equilibrated with 95% O\(_2\) and 5% CO\(_2\). Under the microscope, muscle strips (130-150 µm thick, 200-250 µm wide, 2-3 mm long) were further dissected and mounted between a force transducer and a motor arm. The preparation was stabilized in KH (without BDM) with Ca\(^{2+}\) of 0.5 mM for \(\sim 10\) min. The muscle was then skinned in relaxation solution containing \(1.0\%\) Triton X-100 for 1-2 hrs. Force-Ca\(^{2+}\) relations were obtained in the same fashion as in skinned cardiac muscle preparation. The stability of the preparation was tested at the end of each experimental run at maximal activation, and the data were discarded if
maximal $Ca^{2+}$-activated force decreased over 20% at the end of the experiment. The experiments were performed at room temperature.

**Isolation of Myofibrils**

Rat myofibrillar preparations were obtained from frozen ventricles (Pel Freez Biologicals, www.pelfreez-bio.com) minced in 20 volumes/tissue weight of 4°C relax buffer (standard rigor buffer, SRB (mmol/L: 75 KCL, 10 imidazole pH 7.4, 2 MgCl$_2$) plus 4 phosphocreatine, 1 ATP, 50 BDM, 1 benzamidine-HCL, 0.1 PMSF, 1 μg/mL leupeptin, 1 μg/mL pepstatin, 1.0% (v/v) Trixon X-100) and adjusted to 10 mmol/L EDTA, as previously described.$^3$ Minced preparations were centrifuged for 8 min at 3000xg and the supernant was decanted. Resulting pellets were resuspended in 10 volumes of SRB plus 1.0% Triton X-100 and subjected to 6 strokes in a Duall tissue homogenizer and centrifuged as above. Pellets were gently resuspended and centrifuged as above twice more in SRB including 1.0% (v/v) Triton X-100, twice in SRB lacking Triton X-100 and once in K-60 buffer (mmol/L: 60 KCL, 20 MOPS, 2 MgCl$_2$ pH 7.4) before being resuspended in 5 volumes of K-60.

**Myofibril ATPase Assay**

Isolated myofibrils were diluted to 0.5 μg/ul in K-60 buffer and treated with 2.5 μmol/L NCA for 10 min at room temperature. After 10 min 5 mmol/L DTT was added to some samples. Following treatment, myofibrils were centrifuged for 5 min at 200xg and resuspended in fresh K-60 buffer. For ATPase activity, 15 μg of myofibrils were combined with various [Ca$^{2+}$] (pCa 8 – 5.125) and 2 mmol/L ATP in a 70 μl reaction volume and incubated for exactly 10 min at 31 °C. Reactions were halted by the addition of 100 μl of Stop solution (3.6% (w/v) Ascorbate, 0.6 mol/L HCl, 0.3% (w/v) ammonium molybdate and 7.2% (w/v) SDS). After allowing the signal to develop for 10 min at room
temperature, 100 µl of stabilizing solution (2% (w/v) Sodium Citrate, 2% (w/v) Sodium m-Arsinite, 2% (w/v) Acetic Acid) was added. Signal was determined by measuring absorbance at 595 nm. The amount of inorganic phosphate released during the reaction was determined by comparison to a 5 point ammonium phosphate standard curve.

Detection of HNO modifications by biotin switch assay

HNO modified thiols were detected using a modification to the standard biotin switch protocol.\(^4\) In brief, 100 µg of rat myofibrils/treatment were diluted to 0.5 µg/µL in HEN (mmol/L: 250 HEPES pH 7.4, 1 EDTA and 0.1 neocuproine) including 0.1% (w/v) SDS and exposed to a treatment for 10 min at 37°C which was subsequently removed by acetone precipitation. Treatments included: NCA, 2.5 µmol/L; AS, 500 µmol/L; and their decomposed, inactive equivalents which were solutions prepared and left at room temperature for greater than 96 hours. Additional treatments were DTT, 5 mmol/L and DEA/NO, 125 mmol/L. Remaining free thiols were blocked by resuspension in 300 µL of HEN including 2.5% (w/v) SDS and 20 mmol/L N-ethylmaleimide (NEM), incubated for 20 min at 50°C. Excess NEM was removed by acetone precipitation. HNO and/or NO modified thiols were reduced using 5 mmol/L DTT or 1 mmol/L ascorbate in 150 µL of HEN including 1% (w/v) SDS and biotinylated with 0.8 mmol/L Biotin-HPDP (Thermo Fisher Scientific, www.thermofisher.com) for one hour at room temperature. Excess biotin-HPDP was removed by acetone precipitation (2 volumes) and resultant pellets were carefully washed with an additional volume of acetone. Biotinylated proteins were resuspended in 1 mL of HEN including 0.1% (w/v) SDS and captured by incubation with 15 µL of washed, packed Ultralink Immobilized Streptavidin (Thermo Fisher Scientific) for one hour at room temperature. Beads were washed four times in 50 bead volumes of HEN (twice including 0.1% (w/v) SDS, twice including 600 mmol/L NaCl) and twice with Elution Buffer (mmol/L: 20 HEPES pH 7.4, 100 NaCl, 1 EDTA). Captured proteins
were eluted with 40 μL of EB containing 100 mmol/L DTT, mixed with 15 μL of 4X LDS sample buffer, boiled, separated by SDS PAGE and silver stained.\textsuperscript{5} For MS studies, 200 μg of starting material was used and all subsequent volumes were doubled accordingly. Biotinylated proteins were digested overnight with trypsin (Promega, www.promega.com) or chymotrypsin (Roche, www.roche.com) prior to capture and washed ten additional times with 5 mmol/L ammonium bicarbonate/20% acetonitrile before being eluted in 100 μL of wash buffer including 100 mmol/L DTT.\textsuperscript{6}

**HNO donor preparation**

Stock solutions of Angeli’s salt (50 mmol/L each) was prepared by dissolving powder in 10 mM NaOH and stored at -80°C for up to 2 months.\textsuperscript{7} Immediately before use, a 10x stock was prepared in HEN buffer and applied to sample. NCA was prepared by first diluting 1:5 in DMSO immediately before use. From that, a 10x stock solution was made by diluting the DMSO solution 1:5000 in HEN buffer and applied to the sample.

**LC/MS/MS analysis**

Peptide identification by liquid chromatography/tandem mass spectrometry (LCMS/MS) analysis was performed using an LTQ ion trap MS (Thermo Fisher Scientific, www.thermofisher.com) interfaced with a 2D nanoLC system (Eksigent, www.eksigent.com). Peptides were desalted on a C18 trap (75 μm x 30 mm, 5-10 μm, 120Å, YMC Gel) at 8 μL/min for 5 min with Buffer A (0.1% formic acid). After desalting, peptides were separated on a C18 column (75 μm x 100 mm, 5 μm, 120Å, YMC ODS-AQ, Waters, www.waters.com) with an 8 μm emitter tip (New Objective, www.newobjective.com) using 5-60% B (90% acetonitrile in 0.1% formic acid) gradient over 60 min at 300 nL/min. Acquired spectra were searched against the IPI rat (v3.53) primary sequence database using Sequest (version v.27, rev. 11, Thermo Fisher
Scientific) utilizing the Sorcerer platform (Sage-N Research Inc., www.sagenresearch.com) and analyzed using Scaffold (Proteome Software Inc., www.proteomesoftware.com). Search parameters included peptides cleaved by trypsin or chymotrypsin with up to 1 missed cleavage. Sequest was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 1.2 Da. NEM modification of cysteine was specified in Sequest as a variable modification. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm⁸ and met with a visual inspection. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm.⁹ Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. A determination of a site of HNO was made when a cysteine containing peptide was reliably identified in HNO treated samples and absent from control treated samples and its position was mapped against the protein full sequence.

**Gel shift assay**

10 μg of rat myofibrils per treatment at 0.5 μg/μL in HEN were exposed to a treatment or control conditions for 20 min at 37°C. Samples were diluted to 0.1 μg/μL in 1x LSD sample buffer (Invitrogen, www.invitrogen.com), treated with 0 or 5 mmol/L DTT and separated on a Bis-Tris 4-12% acrylamide 12 well, 1mm gels (Invitrogen) either using MES or MOPS running buffer. Proteins were silver stained or transferred to nitrocellulose and immunoblotted with primary antibodies for tropomyosin (CH1, sigma, www.sigmaaldrich.com), actin (AC-40, sigma), myosin light chain 1 (MLM527, Abcam, www.abcam.com) or myosin heavy chain α/β (3-48, abcam). For some silver stained gel bands of interest, in-gel digestion was done following the established protocol.⁵
**Diagonal gel shift assay**

10 μg of rat cardiac or skeletal myofibrils per treatment at 0.5 μg/μL in HEN were exposed to NCA treatment for 20 min at 37°C. Samples were diluted to 0.4 μg/μL in 1x LSD sample buffer (Invitrogen) and separated on a Bis-Tris 4-12% acrylamide 12 well, 1 mm gels (Invitrogen) either using MOPS running buffer. After separation gel lanes were excised using a razor blade and incubated for 20 min at 37 °C in 4 mL of 1x LDS sample buffer contain in 100 mmol/L DTT. Gel lanes were loaded onto Bis-Tris 4-12% acrylamide IPG well, 1.5 mm gels (Invitrogen) sealed with agarose and proteins were separated using MOPS running buffer. Proteins were silver stained or transferred to nitrocellulose and immunoblotted as described above.

**Purification of tropomyosin**

TM was purified as previously described.¹⁰

**In-gel digestion**

Gel slices of interest were excised from the gels cut into 1 mm³ pieces. Silver stained gel pieces were destained in 1:1(v/v) 30 mM potassium ferricyanide and 100 mmol/L sodium thiosulfate and washed three times with ddH₂O. Gel pieces were dehydrated in 100% acetonitrile and reswelled in 10 mmol/L DTT and incubated at 55 °C for 1 hour. After the DTT solution was removed, a solution of 55 mmol/L iodoacetaimide was added and gel slices were incubated at room temperature protected from light. Gel slices were then washed 3 times with 50% (v/v) ACN, 25 mmol/L (NH₄)HCO₃ and then fully dehydrated in 100% ACN and dried in a speed vac. Gel pieces were reswelled in a 12.5 ng/μL trypsin (Promega) solution containing 25 mmol/L (NH₄)HCO₃ and incubated at 37°C for >16 hours. Digested peptides were extracted by addition of 5% (v/v) formic acid and
incubation for 15 min followed by the addition of an equal volume of 100% ACN and 15 min incubation, this step was repeated and the extracts were combined. Proteins were identified using an Orbitrap LTQ tandem mass spectrometer (Thermo Fisher) and analyzed as described above.5

**Difference in-gel electrophoresis (DIGE) analysis**

Fluorescent labeling using the CyDyes (GE Healthcare, www.gehealthcare.com) was performed according to the manufacturer's recommended protocol.11 Treated samples were labeled as follows, 0.1 µg of purified tropomyosin (Cy2), 2 µg of NCA treated cardiac myofibrils (Cy3), 2 µg of AS treated cardiac myofibrils (Cy5). Labeling mixtures were incubated on ice in the dark for 30 min. The reactions were terminated by adding 0.8 µL of 10 mmol/L lysine (Sigma-Aldrich), vortexed and incubated on ice for 10 min. Samples were then combined and mixed with LDS sample buffer and separated by 1 dimensional Bis-Tris 4-12% acrylamide 12 well, 1 mm gel (Invitrogen) using MOPS running buffer under non-reducing conditions. After SDS–PAGE, CyDye-labeled proteins were visualized using a TyphoonTM 9410 imager (GE Healthcare) using the excitation/emission wavelength Cy2-488/520 nm, Cy3-532/580 nm, Cy5-633/670 nm. Gels were scanned at 100 µm (pixel size) resolution and photomultiplier tube (PMT) was set to 550 V using normal sensitivity. Scanned images were overlayed and intensities were adjusted to provide optimal resolution of the bands.

**References**


Online Figure I. NCA treatment of isolated myofibrils reduces pCa_{50} but does not affect max ATPase rate. ATPase activity was measured in isolated rat cardiac myofibrils over a range of [Ca^{2+}] pretreated with 2.5 μmol/L NCA for 10 min or treated with NCA and then reduced with 5 mmol/L DTT (n=8). NCA treated myofibrils displayed a left shift in Ca_{50} compared to DTT treated (0.17±0.03 vs. 0.33±0.04 μmol/L, p=0.006). No change was observed in maximal myofibrillar ATPase rate (59.1±2.1 vs. 56.1±2.6 nmoles P/min/mg protein, p=0.385) or Hill coefficient (2.18±0.61 vs. 1.4±0.24, p=0.2).
Online Figure II. Nitrite is not implicated in the differential action of the HNO donors, NCA and AS. Gel shift assay of 1 µg of rat cardiac myofibrils treated and separated by reduced or non-reduced western blot (n=3). TM forms a higher molecular weight species (determined to be an actin-TM heterodimer) in the presence of NCA but not AS. The co-release of nitrite from AS does not inhibit this reaction as co-incubation of NCA and nitrite does not effect dimer formation.
Online Figure III. Diagonal gel of cardiac myofibrils treated with NCA. Example of a diagonal silver stained gel where 10 µg of cardiac myofibrils were treated with 2.5 µmol/L NCA and separated first under non-reducing conditions (horizontal) and then under reducing conditions (100 mmol/L DTT) (vertical). The higher molecular weight forms of actin and TM are visible in this gel (upper and lower arrow heads, respectively). The dimeric forms of MLC1 could not be visualized by silver stain at this protein load; at greater protein loads, the higher molecular weight forms of MLC1 were difficult to resolve due to smearing in the non-reducing direction.
Online Figure IV. Proposed mechanisms of NCA induced decrease in Ca$_{50}$ and in maximum force generation. **A**, On the left is the thin filament with the relative positions of TM in the inactivated B- (black) and Ca$^{2+}$-activated C-state (blue) (the M-state was omitted for clarity). Subdomains of actin (1-4) relative to TM position are indicated in the blow-up (light grey) along with the approximate location of the weak (green) and strong (red) myosin binding sites. NCA induced cross-linking of a small proportion of TM (Cys190) to the inner domain of actin (subdomain 4, Cys 257) imposes restraints on TM’s movement biasing its equilibrium position toward a Ca$^{2+}$-activated state (right). This effect primes the thin filament conferring the enhanced myofilament response to Ca$^{2+}$. **B**, Proposed mechanism for increase in maximum force generation. Depicted are the thin and thick filament including position of MLC1 relative to head region of heavy chain indicating the effect of a cross link between two proteins stabilizing the position of the lever arm enhancing force production at fully activating Ca$^{2+}$ concentrations.
Online Figure V. Chemistry of NCA HNO release or reaction with thiols. A) Hydrolysis of NCA (1) results in its decomposition (2) to HNO, acetate and cyclohexane. HNO can then react with free thiols to form disulfide bonds. B) Alternatively, NCA may react with a thiol to form an organic N-hydroxysulfenamide adduct (3). Reaction of this intermediate with an additional free thiol would yield a protein disulfide releasing oxime (4) and acetate without the HNO intermediate.