Nitroxyl Activates SERCA in Cardiac Myocytes via Glutathiolation of Cysteine 674

Steve Lancel, Jingmei Zhang, Alicia Evangelista, Mario P. Trucillo, XiaoYong Tong, Deborah A. Siwik, Richard A. Cohen, Wilson S. Colucci

Nitroxyl (HNO) exerts inotropic and lusitropic effects in myocardium, in part via activation of SERCA (sarcoplasmic reticulum calcium ATPase). To elucidate the molecular mechanism, adult rat ventricular myocytes were exposed to HNO derived from Angeli’s salt. HNO increased the maximal rate of thapsigargin-sensitive Ca^{2+} uptake mediated by SERCA in sarcoplasmic vesicles and caused reversible oxidative modification of SERCA thiols. HNO increased the S-glutathiolation of SERCA, and adenoviral overexpression of glutaredoxin-1 prevented both the HNO-stimulated oxidative modification of SERCA and its activation, as did overexpression of a mutated SERCA in which cysteine 674 was replaced with serine. Thus, HNO increases the maximal activation of SERCA via S-glutathiolation at cysteine 674.

Nitroxyl (HNO), the 1-electron reduced and protonated form of nitric oxide (NO), exerts a bioactivity profile that differs markedly from NO and other reactive nitrogen species such as peroxynitrite. In the cardiovascular system, HNO derived from Angeli’s salt (AS) exerts inotropic and lusitropic effects in the myocardium and causes relaxation of vascular smooth muscle. These observations have raised the possibility that HNO is involved in cardiovascular regulation and/or may have therapeutic potential.

In cardiac myocytes, HNO increases calcium cycling in association with increasing the activities of SERCA (sarcoplasmic reticulum ATPase) and the calcium release channel (CRC). In vascular smooth muscle cells SERCA activity can be increased by NO-induced S-glutathiolation. Accordingly, we hypothesized that in cardiac myocytes HNO can activate SERCA via S-glutathiolation.

Materials and Methods

In all experiments, adult rat ventricular myocytes (ARVMs) were exposed for 15 minutes to 500 μmol/L AS dissolved in 10 mmol/L NaOH. Detailed methods are provided in the online supplement at http://circres.ahajournals.org.

Results and Discussion

HNO Activation of SERCA Involves Reversible, Oxidative Thiol Modification

AS increased myocyte shortening (∼2-fold) and accelerated relaxation (Figure I in the online data supplement), confirming the findings of Tocchetti et al. In the absence of dithiothreitol (DTT), AS (500 μmol/L; 15 minutes) increased maximal SERCA activity ∼3-fold (Figure 1A). In the presence of DTT (2 mmol/L), HNO had no effect on SERCA activity (+3±11%; P=NS; n=3). Iodoacetamide binds preferentially to reactive thiolate anions at pH 6.5 and to cysteine 674 of SERCA, in particular. Oxidative modification of SERCA thiols was assessed using biotinylated iodoacetamide (BIAM). HNO (500 μmol/L; 15 minutes) decreased BIAM binding to SERCA thiols by 27±3% (Figure 1B; P<0.0001; n=14). In some experiments, ARVMs exposed to AS for 15 minutes were washed for an additional 15 minutes. After washout, the amount of BIAM-labeled SERCA was similar in HNO-treated and control cells (Figure 1C and 1D; P<0.01; n=4), indicating that the HNO-mediated modification is reversible. This finding is consistent with our observation that HNO-stimulated SERCA activation is prevented by DTT and the prior observation by Tocchetti et al that the effects of HNO on cardiac myocyte function are reversed by DTT or removal of AS from the perfusion buffer.

HNO Activates SERCA via S-Glutathiolation

We have shown that oxidative activation of SERCA and p21 Ras is mediated via the formation of mixed disulfides leading to protein S-glutathiolation. S-Glutathiolation of SERCA was assessed by immunoprecipitation of SERCA followed by immunoblotting with an antibody directed against glutathione. HNO caused an 18±4% increase in SERCA S-glutathiolation (P<0.05; n=5), which was abolished by DTT (Figure 2A and 2B). To further examine the role of S-glutathiolation, glutaredoxin-1 (GRX) was overexpressed (∼10-fold) via adenoviral infection (supplemental Figure II). In control ARVMs expressing β-galactosidase, HNO decreased BIAM-labeled SERCA by 43±13% (P<0.05; n=4), whereas in GRX-expressing cells, the effect of HNO was abolished (Figure 2C and 2D; P=NS; n=4). In β-galactosidase expressing cells, AS increased maximal SERCA activity by 42±5%, whereas in GRX-expressing cells HNO had no effect (Figure 2E; P=NS; n=4). Together with the demonstration that HNO increases S-glutathiolation of SERCA, the ability of GRX to prevent SERCA thiol oxidative modification and SERCA activation supports the conclusion that the major HNO-induced oxidative modification of SERCA is S-glutathiolation and is consistent with our prior demonstration that SERCA S-glutathiolation stimulates maximal enzyme activity in vascular smooth muscle cells and heart or purified SERCA in phospholipid vesicles.
Cysteine 674 Modulates HNO-Stimulated SERCA Activity

Of the 14 surface thiols of SERCA, iodoacetamide preferentially binds to cysteine 674.10,11 To test the role of cysteine 674 in mediating the effect of HNO in ARVMs, we overexpressed (5-fold; supplemental Figure III) wild-type SERCA or a mutated SERCA (C674S) in which cysteine 674 was replaced by serine. Of note, the amount of accessible SERCA thiols labeled by BIAM was reduced by 52±7% in cells expressing C674S (Figure 3A and 3B; \( P<0.001 \), \( n=4 \)), indicating that cysteine 674 accounts for approximately half of the labeling of wild-type SERCA. In cells expressing wild-type SERCA, HNO decreased BIAM labeling by 65±8% (\( P<0.01 \), \( n=3 \)) but, in contrast, caused no further decrease in the BIAM labeling of SERCA in cells expressing the C674S mutant (Figure 3A and 3B). In cells expressing wild-type SERCA, HNO stimulated SERCA activity by 59±12% but caused no stimulation in cells expressing the C674S mutant (Figure 3C). Likewise, HNO had no effect on myocyte contraction or relaxation in cells expressing the C674S mutant (supplemental Figure I). Thus, the cysteine 674 thiol is the molecular target that accounts for the HNO-mediated oxidative modification and activation of SERCA. Although it is possible that HNO causes oxidative modifications of other SERCA reactive cysteines14 and/or other amino acids, our data indicate that in cardiac myocytes S-glutathiolation of cysteine 674 is the most abundantly modified thiol and plays an essential role in HNO-mediated activation of SERCA.

Mechanism of Oxidative Modification

It remains to be determined how HNO causes S-glutathiolation of SERCA. It is possible that HNO leads to generation of NO and/or peroxynitrite. We previously found that peroxynitrite formed from NO can cause S-glutathiolation of SERCA in vascular smooth muscle cells7 and that this effect can be prevented by the peroxynitrite scavenger uric acid.7 Likewise, in ARVMs, we found that peroxynitrite decreases SERCA BIAM labeling and that this decrease is prevented by uric acid (100 \( \mu \)mol/L; data not shown). However, uric acid had no effect on the decrease in SERCA BIAM labeling or SERCA activation caused by AS (supplemental Figure IV). This finding suggests that peroxynitrite does not mediate HNO-stimulated S-glutathiolation of SERCA. Although further studies will be required to determine the chemical mechanism, our data are consistent with a direct effect of HNO.15 In this regard, it has been proposed that HNO can mediate S-glutathiolation via the formation of a protein thiol N-hydroxysulfenamide intermediate followed by a reaction with GSH.15 Alternatively, HNO might cause GSH depletion, resulting in an oxidative environment that promotes protein S-glutathiolation.16

Role of Phospholamban

Recently, Froehlich et al17 showed that SERCA was not activated by HNO in the absence of phospholamban or with mutation of critical phospholamban cysteines, thus implicating oxidative cysteine modifications of phospholamban. Thus, the regulation of SERCA by HNO may involve oxidative modifications of both SERCA and phospholamban.17 For example, SERCA activation via oxidative modification of cysteine 674 may require and/or synergize with a conformational effect mediated via oxidative thiol modification of phospholamban.

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Figure 2. HNO activates SERCA via S-glutathiolation. A, AS (500 μmol/L, 15 minutes) increases S-glutathiolation of SERCA (*P<0.05 vs control; n=5). B, Representative Western blot showing increased S-glutathiolation by addition of AS, and reversal by the addition of DTT (10 mmol/L) before BIAM labeling. C, GRX prevents the decrease in BIAM labeling caused by AS (*P<0.05 vs control; n=4). D, Representative Western blot. E, GRX prevents the AS-stimulated increase in maximal SERCA activity (*P<0.05 vs control; n=4). Data in A and C were normalized to the means of the control values.

Figure 3. Cysteine 674 modulates HNO-stimulated SERCA activation. A, AS decreases SERCA BIAM labeling in myocytes expressing wild-type SERCA (WT). In contrast, in myocytes expressing the C674S SERCA mutant, BIAM labeling is decreased as compared to wild type but is not decreased further by addition of AS (†P<0.01 vs control vehicle; n=3). B, Representative Western blot showing effect of C674S mutant on BIAM labeling. C, AS does not increase SERCA activity in myocytes overexpressing the C674S SERCA mutant (*P<0.05 vs AS in wild type; n=3). Data in A were normalized to the mean of the wild-type control value.
Disclosures

None.

References


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Nitroxyl Activates SERCA in Cardiac Myocytes via Glutathiolation of Cysteine 674: Correction

The authors wish to report an error they discovered in one of the adenoviral vectors used in the studies published on page 720 of the March 27, 2009 issue.\textsuperscript{1} We found that the adenoviral vector intended to express human wild type SERCA2b in the experiments shown in Figure 3 of this paper actually encoded the SERCA2b C674S mutant and in addition was contaminated with E1A adenovirus. The incorrect adenoviral vector was identified by PCR of the full-length SERCA present in the viral DNA within infected cells. We have reported details of the methods used elsewhere by which nested primer pairs were used to cover the immediate upstream adenoviral sequence and the full-length sequence of the virally expressed SERCA.\textsuperscript{2} Our finding of the error in Lancel et al\textsuperscript{1} came when checking the SERCA vectors after discovering contamination of another pair of viral vectors being amplified in our laboratory as reported in Haeussler et al.\textsuperscript{2} We then sought an explanation of the different effects of overexpression of two SERCA C674S mutants that we realized had occurred in the experiments shown in Figure 3 of Lancel et al.\textsuperscript{1} As indicated in Figure 3 of Haeussler et al,\textsuperscript{2} we checked for and found that the vector intended to express only the wild type SERCA was also contaminated by the adenoviral E1A gene.\textsuperscript{3} Contamination by E1A and its unintended functional effects has been noted by others to occur inadvertently by homologous recombination of the E1A gene that is present within the HEK 293 cell line and which enables amplification of the adenoviral vector which lacks this gene.\textsuperscript{3} We have also suggested remedies to prevent E1A contamination during amplification of replication-deficient adenoviral vectors.\textsuperscript{2} Furthermore, as discussed by Tong et al in their correction notice,\textsuperscript{4} we have discovered that infection of rat aortic smooth muscle cells with E1A stimulates endogenous SERCA expression, which may explain why in our prior experiments the cells infected with the contaminated SERCA mutant retained nitroxyl-stimulated SERCA activity that mimicked that observed in LacZ controls.

Because the contaminated vector was used in Figure 3 of Lancel et al,\textsuperscript{1} we produced a new wild type SERCA 2b adenoviral vector, verified both its sequence and that it and other vectors used lacked E1A contamination, and now provide a revised Figure 3. Methods are provided in full in Lancel et al\textsuperscript{1} and Haeussler et al.\textsuperscript{2} In Panel A of the revised figure, BIAM-labeled SERCA2b is measured in adult rat ventricular myocytes infected with adenoviral vectors expressing LacZ, authentic wild type SERCA2b or the C674S SERCA2b mutant. As in our original report, the fraction of SERCA2b that is BIAM-labeled is decreased by HNO in LacZ- and WT-overexpressing cells, whereas the fraction of BIAM-labeled SERCA2b is decreased in cells overexpressing the C674S mutant, but is not decreased further by HNO. In Panel B, maximal calcium-stimulated calcium influx was measured under similar conditions. In contrast to our prior finding with the contaminated vector, basal HNO-stimulated calcium influx was greater in myocytes expressing WT (vs LacZ). As in our original report, HNO increased calcium influx in cells overexpressing either LacZ or WT. Likewise, as in our prior report HNO-stimulated calcium influx was abolished in cells overexpressing the C674S mutant, as compared to myocytes expressing LacZ or WT. These results with BIAM labeling and calcium influx confirm the original results shown in Figure 3 of Lancel et al,\textsuperscript{1} and do not affect the interpretation of the results indicating that a) the fraction of the reactive cysteines in SERCA2b is decreased in the C674S mutant, and b) HNO-stimulated calcium influx is abolished by overexpression of the C674S SERCA mutant. We hope that reporting our error will serve as a warning to others who use adenoviral vectors in their work.

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Figure 3 (revised). Overexpression of LacZ, wild type SERCA2b (WT) or the C674S SERCA2b mutant (C674S) in adult rat ventricular myocytes. **Top panel.** HNO decreased the fraction of BIAM-labeled SERCA2b in myocytes overexpressing LacZ or WT. The fraction of BIAM-labeled SERCA2b was decreased in myocytes overexpressing C674S vs. LacZ or WT, but was not decreased further by HNO (N=4; *P<0.05; **P<0.01; ***P<0.001). **Bottom panel.** HNO increased maximal calcium-stimulated calcium uptake in myocytes overexpressing LacZ or WT, but not in myocytes overexpressing the C674S mutant (N=4; *P<0.05; **P<0.01; ***P<0.001).

References

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SUPPLEMENT MATERIAL

Nitroxyl Activates SERCA in Cardiac Myocytes via Glutathiolation of Cysteine 674

Steve Lancel, Jingmei Zhang, Alicia Evangelista, Mario P. Trucillo, XiaoYong Tong,
Deborah A. Siwik, Richard A. Cohen, Wilson S. Colucci

Cardiovascular Medicine Section, Department of Medicine, and the Myocardial and Vascular Biology Units, Boston University Medical Center, Boston, MA

Correspondence: Wilson S. Colucci, M.D.
Cardiovascular Medicine Section
Boston University Medical Center
88 East Newton Street
Boston, MA 02118
Tel: 617-638-8071
Fax: 617-638-8081
E-mail: wilson.colucci@bmc.org
Methods

**Reagents.** Biotinylated-iodoacetamide (BIAM) was purchased from Invitrogen; Angeli’s salt (AS, batch #158304) from Cayman Chemical Company; uric acid from Calbiochem; and thapsigargin, and urea from Sigma. The SERCA2 rabbit polyclonal antibody was obtained from Novus Biologicals; the SERCA2 mouse monoclonal antibody was from Affinity Bioreagents; and the anti-glutathione monoclonal antibody was from Virogen.

**Adenoviral vectors.** Adenoviral vectors encoding human SERCA2 (WT) and human mutated SERCA2 (C674S) were constructed as previously described 1. An adenovirus encoding glutaredoxin-1 (AdGRX) was prepared as reported by Song and Lee 2. An adenovirus encoding β-galactosidase (AdLacZ) was used as the control. As previously described 3, viruses were amplified in HEK293 cells, purified with CsCl density gradient centrifugation and viral titer was determined by the use of Adeno-X rapid titer kit (Clontech) according to manufacturer’s instructions.

**Adult rat ventricular myocyte isolation.** Primary adult rat ventricular myocytes (ARVM) were prepared as previously described 4. Cells were plated in laminin-coated 60 mm dishes at the density of 30-50 cells/mm². After one hour, fresh ACCT media (albumin 2g/L, L-carnitine 2mM, creatine 5mM, taurine 5mM, P/S in 1% DMEM) was added. Cells were incubated overnight prior to experiments.

**Cell treatments.** Prior to experiments, ARVM were incubated in fresh media for 30 min. Angeli’s salt (AS) 500µM dissolved in 10 mM NaOH (or NaOH vehicle) was added for 15 min. Cells were then washed in PBS and subjected to BIAM labeling. For wash-out experiments, media containing AS was removed and replaced with fresh media for 15 min prior to cell lysis. In some experiments, cells were pre-incubated in media containing uric acid (100µM). In experiments with adenoviral overexpression, cells were infected with adenoviruses encoding glutaredoxin-1 (MOI = 100), wild-type SERCA (MOI = 10) or C674S
(MOI = 10) for 36 hours. Cells were then treated with AS or NaOH vehicle for 15 min, washed with PBS and used for BIAM-labeling or SERCA activity assay.

**Measurement of myocyte contraction/relaxation.** Cell shortening was recorded in ARVM as previously described. ARVM were maintained at 37°C, superfused with 1.2 mmol/L Ca²⁺-Tyrode solution, and electrically paced at 5 Hz via platinum wires for 20 minutes to establish steady-state conditions. Cell shortening/relaxation were measured using video edge detection (SoftEdge Acquisition System and IonWizard, IonOptix Inc). Percent cell shortening (% CS) was calculated as diastolic cell length minus systolic cell length normalized to the diastolic cell length.

**Biotinylated iodoacetamide (BIAM)-labeling.** BIAM labeling was performed as previously described. Briefly, ARVM were homogenized in RIPA buffer (in mM: NaCl 150, PIPES 100, PMSF 0.1, NaF 1, DTPA 0.1; NP40 1%, deoxycholate 0.25%, protease inhibitor set I 1%, pH 6.5; Calbiochem) containing 100 µM of biotinylated iodoacetamide (BIAM). 500 µg of total proteins were incubated with streptavidin-Sepharose beads (GE Healthcare) for 2 hours at 4°C. After 4 washes, samples were diluted in Laemmli buffer containing 10 M of urea, separated by SDS-PAGE, and SERCA2 was detected by immunoblotting using Odyssey scanner (LICOR Biosciences).

**Detection of S-glutathiolated SERCA.** After AS treatment, cells were washed in PBS and harvested in RIPA buffer. Rabbit polyclonal SERCA antibody (0.5 µL) was added to 200 µg of protein. After 2 hours at 4°C, A/G-Sepharose beads (Santa-Cruz) were added and incubated for 2 hours. Then, beads were washed three times with lysis buffer, proteins were eluted with 2X Laemmli buffer and separated by SDS page. After transfer, membranes were stained simultaneously for SERCA (rabbit polyclonal antibody) and glutathione (mouse monoclonal antibody). Blots were then incubated with goat anti-mouse or goat anti-rabbit
polyclonal antibodies labeled with near-infrared dyes (IRDye 800CW or IRDye 680; Licor) and quantitated using an Odyssey Two-Color Infrared Imaging System (Licor Biosciences).

**Measurement of SERCA activity by $^{45}Ca^{2+}$ uptake.** ARVM were homogenized on ice by sonication in Tris-sucrose homogenization buffer (8% (w/v) sucrose in (in mM) Tris-HCl pH 7.0 3, PMSF 1). The homogenate was centrifuged for 5 min at 4,000 rpm. The protein concentration of the supernatant was determined by Bradford assay. Samples were pre-treated with and without 10 µM of the SERCA inhibitor, thapsigargin. Calcium uptake was initiated by the addition of sample to assay buffer (in mM: KCl 100, NaN₃ 5, MgCl₂ 6, EGTA 0.15, CaCl₂ 0.12, Tris-HCl pH 7.0 30, oxalate 10, ATP 2.5) containing 1 µCi $^{45}$CaCl₂ (New England Nuclear, Boston, MA) in a 37°C degree water bath. Aliquots of each sample taken at 30, 60, 90 s were vacuum filtered on glass filters (Whatman GF/C, Fisher Scientific, Pittsburgh, PA), washed 3 times with wash buffer (in mM: imidazole 30, sucrose 250, EGTA 0.5), and counted with a scintillation counter. SERCA activity is expressed as the initial rate of thapsigargin-sensitive $^{45}$Ca uptake as nmol/mg protein/min.

**Statistical analysis.** All data are expressed as means ± standard error of the mean. Comparisons between vehicle and AS-treated cells were performed with a Student's unpaired t test. P values of <0.05 were considered significant. For multiple comparisons, a one-way ANOVA analysis was performed followed by Bonferroni correction.
Online Figure Legends

**Online Figure I. Effect of HNO on myocyte contraction and relaxation.** ARVM were superfused with Angeli’s salt (AS; 500 uM; 15 min) and cell shortening/relaxation were measured using video edge detection at 37°C with field pacing at 5 Hz. Shown are cells infected with adenoviral constructs for Lac-z (LacZ), wild type SERCA (WT) or the C674S SERCA mutant (C674S). Open bars = vehicle control; solid bars = HNO. **Panel A. Sarcomere shortening.** In LacZ and wild type SERCA-expressing cells, AS increased sarcomere shortening by approximately 2-fold. This effect was not present in cells in which the C674S SERCA mutant was overexpressed. **Panel B. Cell relaxation.** In LacZ and wild type SERCA expressing cells, HNO decreased Tau, the time constant of relaxation; whereas this effect was absent in cells overexpressing the C674S mutant. Data are the means of 16 - 27 cells per condition. *p<0.05 vs. vehicle control.

**Online Figure II. Adenoviral overexpression of glutaredoxin-1 (GRX1) in ARVM.** Shown is a representative western-blot demonstrating an approximately 10-fold increase in GRX1 expression after a 36 hours infection with adenoviruses encoding GRX1 or galactosidase (LacZ), each at an MOI of 100. Indicated values are the measured optical densities.

**Online Figure III. Adenoviral overexpression of wild type (WT) and mutant SERCA in which cysteine 674 is replaced by serine (C674S).** Shown are representative western-blots demonstrating approximately 5-fold increases in the expression of each, at an MOI of 10.
Online Figure IV. HNO-induced SERCA oxidation is peroxynitrite-independent.

Panel A. Preincubation with uric acid (100µM, 30min) to scavenge peroxynitrite does not prevent the AS-induced decrease in SERCA BIAM-labeling († = p<0.01 and * = p<0.05 for AS-treated vs. corresponding vehicle-treated cells; n = 5). Panel B. Representative western-blot showing BIAM-labeled and total SERCA. Panel C. Preincubation with uric acid (100µM, 30min) does not prevent SERCA activation by AS (HNO). (*) = p<0.05 for HNO-treated vs. control vehicle-treated cells; † = ns vs. HNO-treated cells; n = 3).
Online References


Online Figure I.

A.

B.