Published in final edited form as:
Circ Res. 2009 March 27; 104(6): 720–723. doi:10.1161/CIRCRESAHA.108.188441.

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**, and **Wilson S. Colucci**
Cardiovascular Medicine Section, Department of Medicine, and the Myocardial and Vascular Biology Units, Boston University Medical Center, Mass.

Abstract

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.

Keywords

sarcoplasmic reticulum ATPase; SERCA; nitroxyl; glutathiolation; cardiac myocytes

Nitroxyl (HNO), the 1-electron reduced and protonated form of nitric oxide (NO), exerts a bioactivity profile that differs markedly from NO1‚2 and other reactive nitrogen species such as peroxynitrite.3 In the cardiovascular system, HNO derived from Angeli's salt (AS) exerts inotropic and lusitropic effects in the myocardium4 and causes relaxation of vascular smooth muscle.5‚6 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).[1] In vascular smooth muscle cells SERCA activity can be increased by NO-induced *S*-glutathiolation.[7] Accordingly, we hypothesized that in cardiac myocytes HNO can activate SERCA via *S*-glutathiolation.

Correspondence to Wilson S. Colucci, MD, Cardiovascular Medicine Section, Boston University Medical Center, 88 E Newton St, Boston, MA 02118. wilson.colucci@bmc.org.

Disclosures
None.

Materials and Methods

In all experiments, adult rat ventricular myocytes (ARVMs)[8] 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.[1] 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 6.59 and to cysteine 674 of SERCA, in particular.[10,11] Oxidative modification of SERCA thiols was assessed using biotinylated iodoacetamide (BIAM).[7,9] 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[1] 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[7] and p21 Ras[12,13] 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.[13] 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.[7]

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*<0.01, n=3), 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 cysteines[14] 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 μmol/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 al[17] 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Sources of Funding

Supported by NIH grants HL-061639 and HL-064750 (to W.S.C.); NIH grant HL31607 (to R.A.C.); and the National Heart, Lung, and Blood Institute–sponsored Boston University Cardiovascular Proteomics Center (contract no. N01-HV-28178 to R.A.C. and W.S.C.). S.L. was supported by La Fondation pour la Recherche Médicale grant SPE20051105207.

References

1. Tocchetti CG, Wang W, Froehlich JP, Huke S, Aon MA, Wilson GM, Di BG, O'Rourke B, Gao WD, Wink DA, Toscano JP, Zaccolo M, Bers DM, Valdivia HH, Cheng H, Kass DA, Paolocci N.

Nitroxyl improves cellular heart function by directly enhancing cardiac sarcoplasmic reticulum Ca2+ cycling. Circ Res 2007;100:96–104. [PubMed: 17138943]

2. Paolocci N, Saavedra WF, Miranda KM, Martignani C, Isoda T, Hare JM, Espey MG, Fukuto JM, Feelisch M, Wink DA, Kass DA. Nitroxyl anion exerts redox-sensitive positive cardiac inotropy in vivo by calcitonin gene-related peptide signaling. Proc Natl Acad Sci U S A 2001;98:10463–10468. [PubMed: 11517312]
3. Katori T, Donzelli S, Tocchetti CG, Miranda KM, Cormaci G, Thomas DD, Ketner EA, Lee MJ, Mancardi D, Wink DA, Kass DA, Paolocci N. Peroxynitrite and myocardial contractility: in vivo versus in vitro effects. Free Radic Biol Med 2006;41:1606–1618. [PubMed: 17045928]
4. Miranda KM, Paolocci N, Katori T, Thomas DD, Ford E, Bartberger MD, Espey MG, Kass DA, Feelisch M, Fukuto JM, Wink DA. A biochemical rationale for the discrete behavior of nitroxyl and nitric oxide in the cardiovascular system. Proc Natl Acad Sci U S A 2003;100:9196–9201. [PubMed: 12865500]
5. Favaloro JL, Kemp-Harper BK. The nitroxyl anion (HNO) is a potent dilator of rat coronary vasculature. Cardiovasc Res 2007;73:587–596. [PubMed: 17189622]
6. Irvine JC, Favaloro JL, Kemp-Harper BK. NO-activates soluble guanylatecyclase and Kv channels to vasodilate resistance arteries. Hypertension 2003;41:1301–1307. [PubMed: 12743008]
7. Adachi T, Weisbrod RM, Pimentel DR, Ying J, Sharov VS, Schoneich C, Cohen RA. S-Glutathiolation by peroxynitrite activates SERCA during arterial relaxation by nitric oxide. Nat Med 2004;10:1200–1207. [PubMed: 15489859]
8. Communal C, Singh K, Pimentel DR, Colucci WS. Norepinephrine stimulates apoptosis in adult rat ventricular myocytes by activation of the beta-adrenergic pathway. Circulation 1998;98:1329–1334. [PubMed: 9751683]
9. Ying J, Clavreul N, Sethuraman M, Adachi T, Cohen RA. Thiol oxidation in signaling and response to stress: detection and quantification of physiological and pathophysiological thiol modifications. Free Radic Biol Med 2007;43:1099–1108. [PubMed: 17854705]
10. Bishop JE, Squier TC, Bigelow DJ, Inesi G. (Iodoacetamido)fluorescein labels a pair of proximal cysteines on the Ca2+-ATPase of sarcoplasmic reticulum. Biochemistry 1988;27:5233–5240. [PubMed: 2971396]
11. Ying J, Tong X, Pimentel DR, Weisbrod RM, Trucillo MP, Adachi T, Cohen RA. Cysteine-674 of the sarco/endoplasmic reticulum calcium ATPase is required for the inhibition of cell migration by nitric oxide. Arterioscler Thromb Vasc Biol 2007;27:783–790. [PubMed: 17234728]
12. Pimentel DR, Adachi T, Ido Y, Heibeck T, Jiang B, Lee Y, Melendez JA, Cohen RA, Colucci WS. Strain-stimulated hypertrophy in cardiac myocytes is mediated by reactive oxygen species-dependent Ras S-glutathiolation. J Mol Cell Cardiol 2006;41:613–622. [PubMed: 16806262]
13. Clavreul N, Bachschmid MM, Hou X, Shi C, Idrizovic A, Ido Y, Pimentel D, Cohen RA. S-glutathiolation of p21ras by peroxynitrite mediates endothelial insulin resistance caused by oxidized low-density lipoprotein. Arterioscler Thromb Vasc Biol 2006;26:2454–2461. [PubMed: 16931794]
14. Viner RI, Williams TD, Schoneich C. Peroxynitrite modification of protein thiols: oxidation, nitrosylation, and S-glutathiolation of functionally important cysteine residue(s) in the sarcoplasmic reticulum Ca-ATPase. Biochemistry 1999;38:12408–12415. [PubMed: 10493809]
15. Paolocci N, Jackson MI, Lopez BE, Miranda K, Tocchetti CG, Wink DA, Hobbs AJ, Fukuto JM. The pharmacology of nitroxyl (HNO) and its therapeutic potential: not just the Janus face of NO. Pharmacol Ther 2007;113:442–458. [PubMed: 17222913]
16. Lopez BE, Rodriguez CE, Pribadi M, Cook NM, Shinyashiki M, Fukuto JM. Inhibition of yeast glycolysis by nitroxyl (HNO): mechanism of HNO toxicity and implications to HNO biology. Arch Biochem Biophys 2005;442:140–148. [PubMed: 16139238]
17. Froehlich JP, Mahaney JE, Keceli G, Pavlos CM, Goldstein R, Redwood AJ, Sumbilla C, Lee DI, Tocchetti CG, Kass DA, Paolocci N, Toscano JP. Phospholamban thiols play a central role in activation of the cardiac muscle sarcoplasmic reticulum calcium pump by nitroxyl (dagger). Biochemistry 2008;47:13150–13152. [PubMed: 19053265]

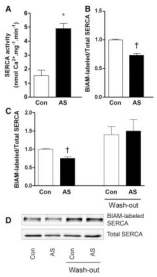

Figure 1.

HNO activation of SERCA involves reversible thiol modification. A, In ARVMs, AS (500 μmol/L, 15 minutes) increased maximal SERCA activity (**P*<0.001 vs vehicle control; n=4), measured as the initial rate of thapsigargin-sensitive $^{45}Ca^{2+}$ uptake in a postnuclear supernatant fraction. This effect was prevented by DTT (online data supplement). B, AS (500 μmol/L, 15 minutes) decreased the fraction of BIAM-labeled SERCA (†*P*<0.0001 vs vehicle control; n=14). C, This effect was reversed by subsequent washing (15 minutes) to remove AS (†*P*<0.01 vs vehicle control; n=4). D, Representative Western blot showing effects of AS and washout on BIAM-labeled and total SERCA. Data in B and D were normalized to the means of the control values.

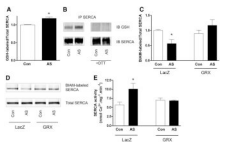

Figure 2.

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.

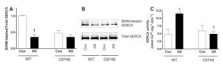

Figure 3.

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 vehicle control; †P<0.05 vs AS in wild type; n=3). Data in A were normalized to the mean of the wild-type control value.