Cysteine-674 of the Sarco/Endoplasmic Reticulum Calcium ATPase Is Required for the Inhibition of Cell Migration by Nitric Oxide

Jia Ying, XiaoYong Tong, David R. Pimentel, Robert M. Weisbrod, Mario P. Trucillo, Takeshi Adachi, Richard A. Cohen

Objectives—Nitric oxide inhibits smooth muscle cell migration after arterial injury, but the detailed mechanism is not fully understood. The sarco/endoplasmic reticulum calcium ATPase (SERCA) lowers cell Ca\(^{2+}\) by increasing intracellular Ca\(^{2+}\) uptake and inhibiting extracellular Ca\(^{2+}\) influx. Our previous studies showed that NO causes cyclic GMP-independent arterial relaxation by increasing SERCA activity by inducing reversible S-glutathiolation at cysteine-674. Because Ca\(^{2+}\) is an important second messenger for cell migration, we hypothesized that NO also inhibits cell migration through redox regulation of SERCA activity via cysteine-674.

Methods and Results—To test our hypothesis, overexpression of either wild type (WT) or mutant SERCA in which cysteine-674 was mutated to serine was accomplished by stable transfection of HEK 293 or adenoviral expression in rat aortic smooth muscle cells (VSMCs). In the cell models expressing mutant SERCA, biotinylated-iodoacetamide (BIAM) and biotinylated-glutathione labeling of SERCA was decreased, and NO failed to increase SERCA activity or decrease Ca\(^{2+}\) influx, thus validating that the expression of mutant SERCA prevents its redox-dependent activation. In the absence of NO, fetal bovine serum stimulated migration of both cell types expressing WT or C674S SERCA at similar rates. The NO donor S-nitrosoenacillamine inhibited migration of cells with WT SERCA, but had no effect on the migration of either HEK cells or VSMCs with C674S SERCA. The same result was obtained in VSMCs in which endogenous NO was produced by iNOS induced by interleukin (IL)-1\(\beta\). Blocking cyclic GMP did not prevent the inhibition of migration by NO.

Conclusions—In cells overexpressing SERCA, the cyclic GMP-independent, redox regulation of SERCA cysteine-674 is required for the inhibition of cell migration by both exogenous and endogenously generated NO. (Arterioscler Thromb Vasc Biol. 2007;27:783-790.)

Key Words: nitric oxide ■ SERCA ■ migration ■ S-glutathiolation

The sarcoplasmic/endoplasmic reticulum Ca\(^{2+}\) ATPase (SERCA) plays an important role in maintaining intracellular Ca\(^{2+}\) homeostasis through its ability to pump cytosolic Ca\(^{2+}\) into the SR/ER.\(^{3}\) Nitric oxide (NO) stimulates SERCA activity, lowers the cytosolic Ca\(^{2+}\) level, inhibits Ca\(^{2+}\) influx, and induces vascular smooth muscle relaxation. In our previous studies, we proposed a molecular mechanism by which NO causes cyclic GMP-independent vascular relaxation by increasing SERCA activity by a thiol redox-dependent mechanism.\(^{2}\) Briefly, NO increases SERCA activity by inducing the reversible S-glutathiolation of specific cysteines in SERCA, predominantly on the most reactive thiol on cysteine-674. Formation of this adduct with normal SERCA increased Ca\(^{2+}\)-uptake into intracellular stores, but did not occur in SERCA in lysates of HEK cells transiently transfected with a cysteine-674 SERCA mutant. In a pathological model of atherosclerosis, cysteine-674 was irreversibly oxidized attributable to prolonged oxidative stress, thereby impairing the NO-induced S-glutathiolation, activation of SERCA, and arterial relaxation.\(^{2}\)

NO serves many vascular protective roles. For instance, after artery injury NO inhibits smooth muscle cell migration and proliferation.\(^{3,4}\) Because intracellular Ca\(^{2+}\) is an important second messenger mediating cell migration, the hypothesis of the present studies is that inhibition of smooth muscle cell migration by NO may occur through its stimulation of SERCA activity. Indeed, it has been reported that adenoviral-mediated overexpression of SERCA inhibits neointima formation after balloon injury.\(^{5}\)

Although redox regulation of SERCA by S-glutathiolation may cause acute cyclic GMP-independent arterial relaxation, it is unknown if redox-dependent regulation of SERCA is involved in more chronic effects of NO such as those on cell migration. To better understand the impact of this regulation
on cellular activity, cell model systems were devised in which SERCA is mutated at cysteine-674 to serine, thus lacking the reactive thiol. We demonstrated in this study that C674 is required for NO-mediated stimulation of SERCA activity and inhibition of Ca\(^{2+}\) influx in cells, and that this regulation is essential for NO-induced inhibition of Ca\(^{2+}\) influx and cell migration. The effect of NO on cell migration did not rely on cyclic GMP. Furthermore, we showed that in addition to exogenous NO donors, endogenous NO produced from inducible NO synthase inhibits smooth muscle cell migration through the redox regulation of SERCA cysteine-674. These studies indicate that redox regulation of SERCA cysteine-674 may be responsible for cyclic GMP-independent regulation of complex cellular processes by NO.

**Methods**

**Construction and Selection of HEK 293 Cells Stably Transfected With SERCA WT or SERCA C674S Mutant**

Full-length human SERCA 2b constructed in pcDNA 3.1 was a gift from Dr Jonathan Lytton (Department of Biochemistry and Molecular Biology, University of Calgary, Alberta, Canada). The mutagenesis of cysteine-674 was performed as previously described using the Stratagene mutagenesis kit. Briefly, the primers for C674S were: 5’-CTGCCATGACGCCGGCTTTTTGCTGAGTTAGAC-3’ (boldface type indicates codon in which mutation occurred, underline indicates mutated nucleotide). Migration studies of transiently transfected cells were made difficult because of increased death of cells attributable to the transfection itself. Therefore, stably transfected cell lines were developed. Both SERCA 2b WT and C674S vectors were digested by endonuclease BgII and then transfected into HEK 293 cells using lipofectamine (Invitrogen). Cells were cultured in 60 mm dishes in Dulbecco modified essential medium (DMEM, Invitrogen) with 10% FBS until they were 25% confluent and transfected colonies were selected by adding 1 mg/mL gentamicin (Invitrogen) to the medium. Medium containing gentamicin was changed every 3 days until visible cell clones formed. Surviving cell clones were individually transferred into 24-well culture plates. SERCA protein expression in all stably transfected cell clones was assessed by immunoblot of SERCA in cell lysates. SERCA WT and C674S clones were chosen to undergo further experiments based on approximately similar SERCA expression and cell morphology.

**Construction of Adenoviral Expression Vectors for SERCA 2b WT and SERCA 2b C674S**

The inserts of SERCA 2b WT and SERCA 2b C674S were excised from pcDNA3.1 using PME-1 and ligated into the EcoRV site of pshuttle-CMV. The correct direction was confirmed by enzyme restriction analysis as well as sequencing. pshuttle-SERCA 2b WT and C674S were linearized and then recombined with pAdEasy in BJ5183 cells and colonies were screened for the appropriate constructs using restriction enzyme analysis. The appropriate cosmids were cut by PAC-1 digestion and transfected into HEK-293 cells. Adenoviral colonies were chosen and amplified as previously described.

**Rat Aorta Smooth Muscle Cell (VSMC) Culture**

VSMCs from passage 8 to 12 were used and cultured in DMEM medium with 10% FBS as described.

**BIAM Labeling of SERCA C674**

The methods for biotinylated-IAM labeling of the reactive C674 in SERCA followed those previously reported with modifications and can be found in the online supplement (available at http://atvb.ahajournals.org).

**Biotinylated GSH Ester and Detection of S-Glutathiolation of SERCA**

The biotinylated GSH ester was prepared as previously described, and methods can be found in the online supplement.

**SERCA \(^{45}\)Ca\(^{2+}\) Uptake Activity**

Maximal SERCA activity at saturating ATP and ambient Ca\(^{2+}\) concentrations greater than 1 μmol/L was measured by \(^{45}\)Ca\(^{2+}\) uptake in a protocol modified from a previous report, and detailed methods can be found in the online supplement.

**Ca\(^{2+}\) Influx**

Ca\(^{2+}\) influx in stably transfected HEK 293 cells was assessed by monitoring the intensity of fura-2 fluorescence using a spectrofluorimeter (Hitachi F-4500) using a protocol modified from previous studies, detailed methods are found in the online supplement.

**Migration Assay in HEK 293 Cells Stably Transfected With SERCA 2b WT or SERCA 2b C674S Mutant**

HEK 293 cells that were stably transfected with SERCA 2b WT or SERCA 2b C674S mutant were seeded into 6-well plates at a density of 2×10⁴ cell per well. The plate was coated with bovine fibronectin (10 μg/mL) for 30 minutes before the cells were plated. After culturing in DMEM with 10% FBS for 2 days, the cell monolayer reached 100% confluence. Cells were pretreated with mitomycin C (25 μg/mL) for 30 minutes. The NO donors, S-nitroso-N-acetylpenicilliamine (SNAP, 200 μmol/L) or Diethylentetriamine NONOate (Deta-NONOate, 300 μmol/L), were added 5 minutes before the scratch injury by replacing DMEM with the same medium containing the donors. These NO donors were chosen because their half-life in aqueous solution at 37°C provides a nearly constant NO concentration of 1 to 1.5 μmol/L over the 6-hour period for the migration measurements as calculated previously. The cell monolayer was scratched using a plastic 200 μL pipette tip. After the injury, cells were gently rinsed with PBS to remove unattached cells and incubated with DMEM with 10% FBS with or without SNAP or Deta-NONOate. The scratch was marked at 3 sites that were photographed (40×) at indicated times using a Nikon Diaphot 300 microscope. The width of the scratch line was measured at each of the 3 sites and averaged at each time point using SPOT Advanced software.

**Migration Assay in Adenoviral Infected VSMCs**

VSMCs were seeded into 6-well cell culture plates in DMEM with 10% FBS. When 100% confluent, cells were transfected with SERCA 2b WT, SERCA 2b C674S mutant, or GFP control adenovirus in DMEM with 0.1% FBS for 2 days. The concentration of virus was adjusted to obtain similar expression of SERCA (supplemental Figure I, available online at http://atvb.ahajournals.org, and Figure 5). SNAP (200 μmol/L) was added to selected wells 5 minutes before the injury. ODQ (10 μmol/L) was added to selected wells 1 hour before the injury. Migration was assessed as for the HEK cells. IL-1β (5 ng/mL) was used to induce iNOS expression, which was detected in cell lysates by SDS PAGE with an anti-iNOS antibody (1:1000, Transduction Laboratories). VSMCs were infected 2 days before the migration assay with adenovirus to express SERCA. IL-1β with or without N6-(1-iminoethyl)-l-lysine (L-NIL, 10 μmol/L) was added to DMEM for 24 hours before the migration assay. No evident changes in growth and differentiation of either HEK and SMC were noted secondary to infection with the SERCA mutant compared with SERCA WT or GFP infected cells.

**Data Analysis**

All experiments were repeated at least 3 times unless otherwise indicated. Data are expressed as means±SEM. The bands on...
immunoblots were quantified by densitometry (Molecular Analyzer, Biorad). Statistics were analyzed with SPSS 13.0 as indicated for each experiment, and statistical significance was accepted for a probability value less than 0.05. Paired comparisons within one cell type treated with or without NO donor or IL-β were analyzed with a paired t test. An unpaired t test for independent samples was used for comparisons made between cells overexpressing WT SERCA and C674S SERCA assuming equal variance within the groups. When comparisons were made among multiple groups, an ANOVA followed by a post hoc S-N-K test was used. In the HEK cell migration assay a one-way repeated measures ANOVA was used to compare the differences accumulated over time.

Results

Validation of Stably Transfected HEK 293 Cell Lines Overexpressing WT and C674S SERCA Mutant

Initial migration studies were performed in HEK 293T cells stably transfected with SERCA WT and SERCA C674S. WT and C674S transfected cell clones were chosen on the basis of similar SERCA expression and cell morphology to undergo further experiments. As shown in Figure 1A, the clones of cells expressing WT had somewhat less SERCA than those expressing SERCA C674S. These differences in SERCA expression persisted during passage of the stably transfected cells.

Iodoacetamide is an alkylating reagent that binds preferentially to reactive thiolate anions at pH 6.5.1 Previous studies showed that iodoacetamide and its labeled analogues bind primarily to SERCA C674.12,13,14,15 Here, we used biotinylated iodoacetamide (BIAM) to validate the expression of the SERCA C674S mutant. As shown in Figure 1B (left), the amount of lysate from each of the two cell lines was adjusted so that equal amounts of total SERCA protein was used for each BIAM labeling experiment. Figure 1B (right) and summary data (bar graph) show that equal amounts of total SERCA protein were labeled approximately 4-fold more by BIAM in HEK cells expressing WT SERCA than in those expressing SERCA C674S mutant. This indicates that the mutant transfected cells largely lack C674.

NO Increases SERCA Activity in HEK Cells Expressing SERCA WT, but not SERCA C674S

Previous studies showed that peroxynitrite and glutathione stimulated SERCA activity by approximately 50% in lysates of HEK cells transiently overexpressing WT SERCA, but not in lysates of cells expressing SERCA C674S.2 To confirm that the stimulation of SERCA activity by NO in intact cells depends on SERCA cysteine-674, SERCA activity was assayed 1 minute after adding NO (10 μmol/L) to both cell lines. As shown in Figure 2A, baseline maximal SERCA activity was less in HEK cells transfected with WT SERCA compared with those expressing C674S mutant SERCA. Although the difference is not statistically significant, it is likely attributable to the lower SERCA expression in HEK cells expressing SERCA WT used for these assays (Figure 1A) and the fact that SERCA activity was measured immediately on cell lysis and protein could not be adjusted to assay equal amounts of SERCA. Nevertheless, NO increased SERCA activity in cells expressing WT SERCA, but had no effect in cells expressing SERCA C674S, indicating that SERCA C674 is required for stimulation of SERCA activity by NO in intact HEK cells overexpressing SERCA.

NO Increases Reversible S-Glutathiolation on WT but not C674S Mutant SERCA

The NO-mediated increase in SERCA activity is mediated by reversible S-glutathiolation of cysteine-674, and the binding of SERCA C674S mutant to GSH Sepharose beads was diminished compared with WT SERCA protein.2 To confirm that SERCA C674S is S-glutathiolated in intact HEK cells in response to NO, biotinylated GSH ester was used to label GSH pools in cells transfected with either WT SERCA or SERCA C674S, and NO gas was added to the cells. After equalizing the amount of SERCA WT and SERCA C674S protein as shown in Figure 1B (left), S-glutathiolated SERCA was pulled down with streptavidin-Sepharose beads, separated by SDS PAGE, and immunoblotted with anti-SERCA antibody. As shown in Figure 1B (right), S-glutathiolation of WT was increased by NO, as indicated by increased labeling with BIAM, but not in cells expressing SERCA C674S.
2B, some S-glutathiolation of SERCA was present in the absence of NO and did not differ in cells expressing WT or mutant SERCA. This is consistent with previous observations showing that several cysteine residues in SERCA in intact arteries are S-glutathiolated under basal conditions. NO significantly increased S-glutathiolation of WT SERCA, but had no effect on SERCA C674S, demonstrating the importance of C674 for NO-induced S-glutathiolation of SERCA in intact HEK cells. Taken together, these results demonstrate that the NO-induced S-glutathiolation of SERCA and increase in SERCA activity requires cysteine-674 in HEK cells with stably overexpressed SERCA.

NO Decreases Calcium Influx in HEK Cells Expressing SERCA WT, but not SERCA C674S Mutant

Previous studies showed that NO decreases intracellular calcium via SERCA by sequestering cytoplasmic Ca$^{2+}$ into intracellular stores and inhibiting Ca$^{2+}$ influx. To test directly whether cysteine-674 is required for NO to inhibit Ca$^{2+}$ influx, carbachol-induced release of intracellular Ca$^{2+}$ in the absence of extracellular Ca$^{2+}$ and the increase in Ca$^{2+}$ occurring with Ca$^{2+}$ influx were measured with fura-2 in HEK cells overexpressing WT or C674S SERCA. As shown in Figure 3A and summarized in Figure 3B, carbachol-induced Ca$^{2+}$ release from intracellular stores as well as Ca$^{2+}$ influx were not significantly different in HEK cells overexpressing WT and C674S SERCA. However, NO significantly prevented the increase in intracellular Ca$^{2+}$ associated with Ca$^{2+}$ readition in HEK cells overexpressing WT SERCA, but had no effect in cells overexpressing C674S SERCA. Therefore, cysteine-674 is required for NO-induced inhibition of Ca$^{2+}$ influx. Thus cysteine-674 is required for NO-induced S-glutathiolation of SERCA, increase in SERCA activity, and NO-induced inhibition of Ca$^{2+}$ influx, which consequently could affect Ca$^{2+}$-dependent cell functions, such as cell migration.

SERCA Cysteine-674 Is Required for NO-Mediated Inhibition of HEK Cell Migration

After validating the HEK cell model, migration was assayed in the presence and absence of the NO donor SNAP (0.2 mmol/L), which provides a nearly stable concentration of NO during the 6-hour migration assay of approximately 1 to 1.5 mmol/L. As shown in Figure 4A, the time course of serum-stimulated migration over 6 hours was similar in WT...
and C674S mutant SERCA infected cells. However, SNAP significantly inhibited the migration of HEK cells expressing WT SERCA (Figure 4A), but had no effect on the migration of cells expressing SERCA C674S mutant (Figure 4A and 4B). To exclude potential effects of the thiol of SNAP and the NO that it releases, these experiments were repeated with Deta-NONOate (0.3 mmol/L) which releases approximately the same concentration of NO as SNAP, but as the NO neutral charged radical. Supplemental Figure I shows that like SNAP, Deta-NONOate inhibited migration in HEK cells expressing WT, but not C674S mutant SERCA.

**Overexpression of WT and C674S Mutant SERCA in Rat Aortic Vascular Smooth Muscle Cells**

To further corroborate our hypothesis that SERCA C674 is required for NO to inhibit cell migration, we developed adenoviral vectors to express WT and mutant C674S SERCA in a more physiologically relevant model using dedifferentiated VSMCs that are implicated in neointimal proliferation and atherogenesis. Furthermore, the adenoviral infection avoided differences of SERCA protein expression that were unavoidable in the experiments with HEK cells expressing WT, but not C674S mutant SERCA.

**Figure 5.** Overexpression of WT and C674S mutant SERCA in rat aorta vascular smooth muscle cells (VSMCs). A, Equal amounts of SERCA were expressed in VSMCs infected with Ad-SERCA2b (WT) or Ad-C674S mutant SERCA. Control (Ctrl) protein is from uninfected VSMCs. GAPDH was detected as a loading control. B, S-glutathiolation after NO (10 μmol/L, 1 minute) was decreased in Ad-SERCA C674S infected VSMCs compared with WT. SDS PAGE of cell lysate proteins (15 μg) before streptavidin-Sepharose pull-down confirmed equal expression of SERCA in the 2 groups (left). For S-glutathiolation, a total of 800 μg cell lysate protein from each group was used for streptavidin-Sepharose pull-down, followed by SDS PAGE and immunoblot for SERCA. The bar graph summarizes densitometry data from 3 similar experiments. *P<0.05, unpaired t test between cells overexpressing SERCA WT or C674 mutant.

**Figure 4.** NO inhibits migration in HEK 293 cells expressing WT, but not C674S mutant SERCA. A, HEK 293 cells expressing WT or C674S SERCA were analyzed for their ability to migrate into a denuded area of the cell culture plate made by a scratch. Cells were incubated in 10% serum and DMEM alone or with SNAP (0.2 mmol/L). The migration distance was measured at 0, 2, 4, 6 hours after the scratch. The results are n=5 (average±SEM). *P<0.05, one-way repeated measures ANOVA. The bar graph summarizes migration distance at 6 hours. *P<0.05, paired t test between cells with or without SNAP.

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NO-induced S-glutathione labeling was also determined to confirm that the redox-active C674 was less abundant as expected in the C674S SERCA mutant infected VSMCs. As shown in Figure 5B and summarized in the bar graph below, S-glutathiolation was significantly less in C674S mutant than in WT SERCA after exposure to NO (10 μmol/L). This confirms that VSMCs transfected with SERCA C674S have decreased levels of SERCA reactive thiols available for S-glutathiolation by NO.

**NO Inhibits Cell Migration in VSMCs Infected With WT, but not C674S Mutant SERCA, via a Cyclic GMP Independent Pathway**

As in experiments in HEK cells, inhibition of VSMC migration by NO after 6 hours reached significance, and the...
inhibition by SNAP (200 μmol/L) was to a similar extent as that observed in the HEK cells. As shown in supplemental Figure IVA, SNAP inhibited the migration of both control GFP-infected VSMCs as well as those infected with WT SERCA. However, NO had no effect on the migration of cells infected with the SERCA C674S mutant. These results confirm our finding in HEK cells indicating that SERCA cysteine-674 is required for NO to inhibit cell migration.

Previous investigators found that NO inhibits VSMC migration via cyclic GMP-dependent\textsuperscript{16,17} and -independent pathways.\textsuperscript{16,18} To assess whether the inhibition of migration in rat aortic VSMCs used here is cyclic GMP-dependent, cells were treated with the guanylyl cyclase inhibitor, ODQ (10 μmol/L), which blocks the generation of cyclic GMP pathway in VSMCs by NO (10 μmol/L).\textsuperscript{19} As shown in supplemental Figure IVB, ODQ partially prevented the effect of NO on uninfected VSMCs, although the effect was not significant. In VSMCs infected with WT SERCA, there was no apparent effect of ODQ on the inhibition of migration caused by SNAP, nor did ODQ affect migration in cells infected with SERCA C674S mutant. These results indicate that inhibition of migration by NO may occur by cyclic GMP-dependent and -independent pathways in rat aortic VSMCs, but that which is dependent on SERCA C674 occurs independently of cyclic GMP.

**NO Produced Endogenously by iNOS Inhibits Migration in VSMCs Transfected With WT, but not C674S Mutant SERCA**

In smooth muscle cells, iNOS can be induced by cytokines, such as IL-1β, or lipopolysaccharide (LPS).\textsuperscript{20,21} To further test our hypothesis that inhibition of smooth muscle cell migration by NO requires SERCA cysteine-674, expression of iNOS was induced in VSMCs by IL-1β.\textsuperscript{22} After treatment of cells with IL-1β (5 ng/mL, 24 hours), iNOS was similarly expressed in control uninfected, SERCA WT, and SERCA C674S infected VSMCs (supplemental Figure V). As shown in Figure 6A, IL-1β inhibited migration of GFP transfected control and WT SERCA infected VSMC to a similar extent, but had no effect on migration of cells infected with SERCA C674S mutant. Because IL-1β could have effects on migration other than those for which NO released by iNOS is responsible, cells treated with IL-1β were treated with the iNOS inhibitor, L-N\textsuperscript{N′}-4-(1-iminoethyl) lysine hydrochloride (L-NIL, 10 μmol/L). As shown in Figure 6B, the inhibition of migration by IL-1β in VSMCs infected with WT SERCA was entirely blocked by L-NIL. These results indicate that endogenous NO produced by iNOS induced by IL-1β inhibits VSMC cell migration, and provide further evidence that SERCA C674 is essential for NO to inhibit migration.

**Discussion**

Previously, we showed that S-glutathiolation of SERCA was associated with stimulation of SERCA activity in smooth muscle cells and arteries, and presented evidence that this mechanism mediated the cyclic GMP-independent NO-induced relaxation of vascular smooth muscle.\textsuperscript{2,23} The direct stimulation of SERCA activity by its S-glutathiolation was demonstrated in purified SERCA reconstituted into phospholipid vesicles, excluding a role of upstream signaling or associated proteins.\textsuperscript{2} Our current findings in cells overexpressing SERCA suggest that SERCA, and in particular the thiol of cysteine-674, is required for regulation of cell migration by NO. We found that not only the NO donors SNAP and Deta NONOate but also endogenous NO produced by cytokine-induced iNOS inhibits cell migration through regulation of SERCA by this thiol-dependent mechanism. These findings are novel not only because they suggest that SERCA is essential for regulation of cell migration by NO, but also because a single cysteine thiol is responsible.

We established two cell models that either possessed wild-type SERCA or mutated SERCA lacking the most redox-sensitive SERCA thiol that undergoes the majority of S-glutathiolation.\textsuperscript{2} In the present study, NO-induced S-glutathiolation and stimulation of SERCA activity was demonstrated in intact HEK cells overexpressing SERCA WT but not C674S mutant, whereas previously we showed that S-glutathiolation and increased activity of SERCA in HEK cell lysates was stimulated by peroxynitrite in the presence of added glutathione.\textsuperscript{2} In addition, the decreased BIAAM labeling and incorporation of biotinylated GSH by NO further establish the lack of cysteine-674 in both HEK cells and VSMCs infected with mutant SERCA, validating their use to study the impact of redox regulation of SERCA cysteine-674 by NO on complex cellular functions.

As an important intracellular second messenger Ca\textsuperscript{2+} regulates many cellular phenomena, including cell migration.\textsuperscript{24} The major function of SERCA is to pump Ca\textsuperscript{2+} into intracellular stores. This in turn inhibits store-operated Ca\textsuperscript{2+} influx, thereby lowering intracellular Ca\textsuperscript{2+}.\textsuperscript{25} Our results using stably transfected HEK cells suggest that not only is cysteine-674...
required for NO to S-glutathiolate and stimulate SERCA activity and to inhibit extracellular Ca\(^{2+}\) influx, but also to inhibit cell migration in response to serum. In part, because of unavoidable differences in SERCA expression in the two stably transfected HEK cell lines chosen for study, and to broaden our approach, adenoviral infection of VSMC was employed. In these VSMC decreased levels of NO-induced biotin GSH labeling of the SERCA C674S mutant was verified. These studies in VSMC confirmed that SERCA cysteine-674 is required for NO to inhibit migration.

One possibility is that overexpression of C674S mutant SERCA downregulates endogenous SERCA expression. This might explain why, in cells overexpressing the C674S SERCA mutant, NO failed to stimulate the activity of native SERCA possessing C674 and to inhibit Ca\(^{2+}\) influx and migration. Although this possibility is difficult to exclude, preliminary results indicate that in noninfected VSMCs reactive oxygen species associated with hyperglycemia oxidize endogenous SERCA cysteine-674 and prevent its S-glutathiolation and inhibition of migration by NO, suggesting that redox regulation of endogenous SERCA-674 is required for normal NO action (XiaoYong Tong, unpublished results, 2006). The fact that expression of the SERCA C674S mutant did not reveal any other potential mechanism by which NO could inhibit calcium influx also is consistent with the essential role of SERCA C674 in doing so. The importance of endogenous SERCA cysteine-674 S-glutathiolation was also suggested by previous studies that showed an association between impaired NO-induced arterial relaxation of the hypercholesterolemic rabbit aorta in which SERCA C674 was oxidized by approximately 50\%.2

It has been well-documented that NO inhibits VSMC cell migration, but the detailed mechanisms have not been clarified.16,17,24,26,27 Some previous studies found that NO inhibits VSMC migration via the cyclic GMP pathway.17,24 NO activates guanylyl cyclase which produces cyclic GMP, and cyclic GMP subsequently activates protein kinase G that induces phosphorylation of many target proteins potentially involved in cell migration. However, other studies found that as yet undefined cyclic GMP-independent mechanisms were involved in inhibition of VSMC migration by NO.16,18 The difference in results is potentially attributable to passage number, breed of rat, or culture technique. In our study, ODQ prevented about half of the inhibition of VSMC migration by NO in GFP-transfected cells, consistent with both cyclic GMP-dependent and -independent effects of NO described previously.19 In VSMCs in which WT SERCA was overexpressed, ODQ had no demonstrable effect, consistent with a larger role for the redox regulation of SERCA occurring independently of cyclic GMP. The fact that NO did not significantly inhibit migration of VSMCs in which the C674S SERCA mutant was expressed also is consistent with the hypothesis that the principal cyclic GMP-independent mechanism by which NO inhibits VSMC migration is redox regulation of SERCA activity via C674.

In vivo, smooth muscle cells in the neointima express iNOS, likely arising in response to locally produced cytokines, and the NO produced acts to offset the stimuli that promote neointimal growth.4 Our studies indicate that cysteine-674 in SERCA plays an important role in inhibiting cell migration caused by IL-1β. Because the iNOS inhibitor, L-NIL, which is an analog of the NOS substrate, arginine, blocked the IL-1β-induced inhibition of migration, these studies indicate that the levels of NO produced by iNOS are sufficient to activate the SERCA-dependent mechanism which was demonstrated directly by using NO donors. Here too, the thiol on cysteine-674 was essential for the inhibition caused by endogenous NO because VSMCs transfected with the serine-674 mutant lost the response to NO.

Neointimal hyperplasia is an important pathological process in several vascular occlusive diseases, including atherosclerosis. VSMCs are normally quiescent in the vasculature, but their migration and proliferation in response to injury is associated with progressive narrowing of the arterial lumen. One key initiator of this pathology is endothelial damage, which exposes the underlying medial smooth muscle cells to cytokines, growth factors, and other plasma components, resulting in the loss of contractile characteristics and adoption of a synthetic phenotype. Dedifferentiation of the underlying smooth muscle cells precedes their migration from the media to the intima where their proliferation leads to the formation of the occlusive neointima.28 The smooth muscle cells used in this study have undergone phenotypic changes from a differentiated contractile phenotype in the rat aorta to a dedifferentiated, synthetic phenotype in culture, which is similar to the dedifferentiation of the migrating VSMCs in the neointima. Another characteristic of dedifferentiated smooth muscle cells is loss of protein kinase G, which is believed to contribute to uncontrolled neointimal growth.29 Though cultured cell models may differ with respect to the expression and activity of the cyclic GMP-protein kinase G system, the SERCA-dependent mechanism described here defines a cyclic GMP-independent mechanism by which NO can limit such neointimal expansion of smooth muscle and potentially other cell types. Indeed, loss of the cyclic GMP, protein kinase G system either in culture or in vivo29 would be expected to emphasize the role served by redox regulation of SERCA.

In summary, this study provides evidence that redox regulation of SERCA cysteine-674 is a key mechanism by which cell migration is inhibited by NO released chronically by NO donors or produced by endogenous iNOS. Because this mechanism does not depend on cyclic GMP, it may become more prominent in dedifferentiated cells such as those that lose protein kinase G and migrate into the neointima of injured arteries. Furthermore, we have demonstrated that irreversible oxidation of SERCA cysteine-674 occurs in atherosclerotic aorta,2 suggesting that loss of NO-induced inhibition of migration, like the loss of NO-induced vasodilation, might be caused by oxidation of the redox-sensitive cysteine-674 of SERCA.

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References


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Supplemental Methods:

**BIAM labeling of SERCA C674:**

Briefly, cells were lysed in buffer A (Tris-HCL 50 mmol/L pH 8.5, NaCl 150 mmol/L, MgCl₂ 5 mmol/L, DETA-PAC 50 μmol/L, PMSF 2 mmol/L, Triton 0.5% and NEM 100 μmol/L) on ice for 50 min. Pretreatment with this low concentration of NEM was used to minimize incorporation of BIAM label into protein that was partially denatured during cell lysis according to previous studies. The excess NEM was removed by gel filtration using Biospin 6 columns (Biorad). The cell lysate was then incubated with BIAM (1 mmol/L) in buffer B (MES 50 mmol/L pH 6.5, NaCl 150 mmol/L, MgCl₂ 5 mmol/L, DETA-PAC 50 μmol/L, PMSF 2 mmol/L and Triton X-100 1%) in the dark at 25°C for 30 minutes. The labeling reaction was terminated by adding β-mercaptoethanol to a final concentration of 50 mmol/L. The excess reagent was removed by a Biospin 6 column. Finally, 500 μg cell lysate was incubated with 50 μL streptavidin-Sepharose beads overnight at 4°C. The beads were rinsed 3 times using buffer C (Tris-HCl 125 mmol/L pH 7.4, NaCl 500 mmol/L, MgCl₂ 5 mmol/L and 2% SDS), the BIAM labeled proteins were released by Laemmli buffer containing 5 mol/L urea and 5% β-mercaptoethanol at 55°C for at least 30 min. Proteins were separated by SDS-PAGE, and SERCA was detected by immunoblot using a IID8 monoclonal anti-SERCA antibody (Affinity Bioreagent).

**Biotinylated GSH ester and detection of S-glutathiolation of SERCA**

The cells were preincubated in physiological salt solution (PSS, KCl 4.7 mmol/L, CaCl₂ 2.5 mmol/L, NaCl 118.3 mmol/L, KH₂PO₄ 1.2mmol/L, MgSO₄ 0.6 mmol/L, NaHCO₃
25 mmol/L, and dextrose 5.5 mmol/L) containing biotinylated GSH ester (250 μmol/L) for 1 h. Then NO gas (10 μmol/L) prepared as previously described 3 was added to a dish of cells while mixing for 1 min. Cells were lysed immediately in a cell lysis buffer (Cell Signaling) containing NEM (10 mmol/L) to block further thiol reactions. Excess reagents were removed with Biospin 6 column. Cell lysate protein 500 μg was incubated with 50 μL streptavidin-Sepharose beads overnight at 4°C. The beads were rinsed 3 times using buffer C, and the S-glutathiolated proteins were released by elution buffer (buffer A with 10 mmol/L DTT) at 55°C for at least 30 minutes. Each sample was diluted in Laemmli buffer containing 5% β-mercaptoethanol, separated by SDS-PAGE, and SERCA was detected by immunoblotting.

**SERCA \(^{45}\)Ca\(^{2+}\) Uptake Activity**

HEK cells were incubated at 37°C for 1 h in PSS. In some plates NO (10 μmol/L) was added for 1 min. PSS was then immediately removed and cells were scraped into 150 μL of ice cold buffer consisting of sucrose (0.34 mol/L), Tris (3 mmol/L, pH 7.0), diethylenetriaminepentaacetic acid (50 μmol/L), and reduced glutathione (0.5 mmol/L). The cell homogenate was centrifuged at 4000 rpm for 4 min at 4°C. The post nuclear supernatant fraction was sonicated for 10 s before being assayed. To determine SERCA-dependent uptake, paired samples were treated with thapsigargin (10 μmol/L, 20 minutes) at 37°C. \(^{45}\)Ca\(^{2+}\) uptake buffer (30 mmol/L Tris-HCl, pH 7.0, 100 mmol/L KCl, 5 mmol/L NaN\(_3\), 6 mmol/L MgCl\(_2\), 0.15 mmol/L EGTA, 0.12 mmol/L CaCl\(_2\), and 10 mmol/L oxalate) was mixed with 1 μCi \(^{45}\)Ca\(^{2+}\) and 2 mmol/L ATP at 37°C. The reaction was started by adding 50 μL protein (10 to 20 μg, total volume 250 μL). After 30 min 200 μL of this mixture was filtered through Whatman
GF/C glass filters under vacuum. The filters were rinsed 4 times with 2.5 mL wash buffer (30 mmol/L imidazole, 250 mmol/L sucrose, and 0.5 mmol/L EGTA). $^{45}\text{Ca}^{2+}$ uptake was calculated by counting the radioactivity collected by the filters and standardized by protein concentration determined by the Bradford method.

**Ca$^{2+}$ influx:**

After reaching confluence in 10 cm dishes, cells were trypsinized and resuspended at 2 $\times 10^6$ cells/mL in buffer D (NaCl 119 mmol/L, NaHEPES 20 mmol/L, KCl 4.6 mmol/L, CaCl$_2$ 1.2 mmol/L, MgSO$_4$ 1.0 mmol/L, Na$_2$HPO$_4$ 0.15 mmol/L, KH$_2$PO$_4$ 0.4 mmol/L, and NaHCO$_3$ 5.0 mmol/L, 0.1% albumin) Fura2/AM (5 $\mu$mol/L) was added at 37ºC for 40 min. After rinsing in Buffer D for 15 min at 37 ºC, cells were divided into 2 mL aliquots and kept in the dark at room temperature until use. Before measurement, cells were washed twice with Buffer D modified only by omission of Ca$^{2+}$. Two mL of cell suspension was transferred to a quartz cuvette and maintained at 37 ºC under continuous stirring. Carbachol (200 $\mu$mol/L) was added after 5 min, NO (10 $\mu$mol/L) was added after 7 min, and CaCl$_2$ (1.2 mmol/L) was added after 8 min. Changes in intracellular fura-2 fluorescence intensity were measured by alternating excitation at 340 nm and 380 nm and detecting emission at 510 nm. The calibration of the signal was performed in each sample by adding ionomycin (2 $\mu$mol/L) to obtain maximal fluorescence, and then by adding MnCl$_2$ (8 mmol/L) to obtain minimal fluorescence. Changes in Ca$^{2+}$ were analyzed and are reported as peak increases in the ratio of fura-2 fluorescence over basal Ca$^{2+}$ after subtracting background determined after adding Mn$^{2+}$. 
Supplemental references:


Supplemental figure legends:

Supplemental figure 1:
A: HEK 293 cells expressing WT or C674S SERCA were analyzed for their ability to migrate into a denuded area made by a scratch. Cells were incubated in 10% serum and DMEM alone or with Deta-NONOate (0.3 mmol/L). The migration distance was measured at 0, 2, 4, 6 h after the scratch. The results are n=6 (average ± SEM). *P<0.05, one-way repeated measures ANOVA. The bar graph summarizes migration distance at 6 h. * P< 0.05, paired t-test between cells with or without Deta NONOate.

Supplemental figure 2.
VSMC were transfected with Ad-SERCA WT or Ad-SERCA C674S for 48 h to determine the concentrations that resulted in similar levels of SERCA protein expression. The top row shows SERCA expression detected by IID8 antibody at 1:2,000. The middle and lower rows show α-actin expression using short and longer exposures. α-actin was detected by anti-α-actin.
antibody (Sigma, 1:2,000). Lane 1. Untransfected cells; 2. 12 µl purified WT SERCA virus; 3. 10 µl mutant SERCA virus; 4. 2 µl WT SERCA virus; 5. 1 µl mutant SERCA virus.

Supplemental figure 3.

SERCA expression in untransfected VSMC, or ad-WT or ad-C674S mutant SERCA transfected VSMC. SERCA was detected with a polyclonal rabbit anti-K30/A43 peptide SERCA antibody (1:1000, Bethyl Laboratories).

Supplemental figure 4.

NO inhibits migration in VSMC transfected with WT, but not C674S mutant SERCA.

A: Migration distance was measured 6 h after the scratch injury was made. Cells were transfected for 48 h with either Ad-GFP or Ad-WT or Ad-C674S SERCA2b. *P<0.05, paired t-test between cells treated or not with SNAP (0.2 mmol/L).

B: Inhibition of migration mediated by SERCA C674 is cyclic GMP-independent. Cells were treated with ODQ (10 µmol/L, 1 h) before the scratch was made. SNAP (0.2 mmol/L) was added to the medium prior to the scratch. *P<0.05, n=5, one-way ANOVA.

Supplemental figure 5.

iNOS expression after IL-1β treatment for 24 hours in VSMC transfected with Ad-WT or Ad-C674S. iNOS was detected by anti-iNOS mAb (1:1000, Transduction laboratories) and GAPDH was detected by anti-GAPDH mAb (1:5000).
Supplemental Figure 1

A

Migration distance (micrometer)

WT

B

Migration distance (micrometer)

C674S

C

Migration distance (micrometers)/6h

WT C674S
Supplemental Figure 3

SERCA

117 kDa
100 kDa

VSMC  Ad-WT  Ad-C674S
Supplemental Figure 4

A  Migration distance

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B

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* indicates statistical significance compared to the respective control condition.
Supplemental Figure 5

WT C674S WT + IL-1β C674S + IL-1β WT + IL-1β+NIL

130 kDa → -iNOS

37 kDa → -GAPDH