

Hydrogen Peroxide–Mediated SERCA Cysteine 674 Oxidation Contributes to Impaired Cardiac Myocyte Relaxation in Senescent Mouse Heart

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Background—A hallmark of aging of the cardiac myocyte is impaired sarcoplasmic reticulum (SR) calcium uptake and relaxation due to decreased SR calcium ATPase (SERCA) activity. We tested the hypothesis that H₂O₂-mediated oxidation of SERCA contributes to impaired myocyte relaxation in aging.

Methods and Results—Young (5-month-old) and senescent (21-month-old) FVB wild-type (WT) or transgenic mice with myocyte-specific overexpression of catalase were studied. In senescent mice, myocyte-specific overexpression of catalase (1) prevented oxidative modification of SERCA as evidenced by sulfonation at Cys674, (2) preserved SERCA activity, (3) corrected impaired calcium handling and relaxation in isolated cardiac myocytes, and (4) prevented impaired left ventricular relaxation and diastolic dysfunction. Nitroxyl, which activates SERCA via S-glutathiolation at Cys674, failed to activate SERCA in freshly isolated ventricular myocytes from senescent mice. Finally, in adult rat ventricular myocytes in primary culture, adenoviral overexpression of SERCA in which Cys674 is mutated to serine partially preserved SERCA activity during exposure to H₂O₂.

Conclusion—Oxidative modification of SERCA at Cys674 contributes to decreased SERCA activity and impaired myocyte relaxation in the senescent heart. Strategies to decrease oxidant levels and/or protect target proteins such as SERCA may be of value to preserve diastolic function in the aging heart. (*J Am Heart Assoc.* 2013;2:e000184 doi: 10.1161/JAHA.113.000184)

Key Words: aging • catalase • left ventricular diastolic dysfunction • oxidative stress

Cardiac aging is characterized by left ventricular (LV) diastolic dysfunction that is due, at least in part, to slowed relaxation of cardiac myocytes.¹ A mechanism that may contribute to slowed myocyte relaxation is decreased sarcoplasmic reticulum (SR) calcium cycling due to decreased SR calcium ATPase (SERCA) activity.¹ The mechanism responsible for decreased SERCA activity in aging is not known. However,

because the expression levels of SERCA protein are not consistently decreased in aging,^{1–5} a decrease in SERCA protein, per se, does not appear necessary. Another potential mechanism for decreased SERCA activity is oxidative post-translational modification of the protein. Studies in skeletal and cardiac muscle have shown extensive age-related oxidation of SERCA.^{6,7} In this regard, we have shown that the activity of SERCA can be regulated by oxidative posttranslational modification of Cys674: Reversible S-glutathiolation increases SERCA activity, whereas irreversible oxidative sulfonation is associated with decreased activity.^{8,9} Furthermore, in cardiac myocytes, we found that redox-stimulated SERCA activation is mediated by S-glutathiolation of Cys674,¹⁰ and in related experiments we noticed that redox-stimulated SERCA activation is absent in myocytes from senescent (versus young) mice (Figure S1), thus suggesting that Cys674 may be oxidized in senescent myocytes.

These observations led to our hypothesis that impaired myocyte relaxation in aging is due, at least in part, to oxidation of SERCA leading to decreased SERCA activity. This thesis is consistent with the demonstration that transgenic overexpression of catalase prevents impairments in myocyte calcium handling and myocyte relaxation in senescent mice.^{11–13} However, prior studies of the effects of catalase overexpression in aging did not measure

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SERCA activity or assess for oxidative modifications of SERCA. Therefore, we assessed SERCA activity and oxidative posttranslational modification in myocardium from young and senescent mice. Diastolic dysfunction in senescent mice was associated with a decrease in SERCA activity but no change in SERCA protein level. Using a sequence-specific antibody, we found that aging was associated with oxidation (sulfonation) of cardiac SERCA at Cys674. Transgenic overexpression of catalase prevented SERCA Cys674 oxidation and normalized SERCA activity in senescent mice. Likewise, slowed relaxation and calcium reuptake in myocytes freshly isolated from senescent mouse hearts were corrected by catalase overexpression. Finally, in cardiac myocytes in primary culture, mutation of Cys674 to serine partially protected SERCA from oxidative inactivation, thereby supporting the functional importance of Cys674 oxidation in senescent heart.

Methods

Experimental Animals

Young (5-month-old) and senescent (21-month-old) transgenic mice having myocyte-specific overexpression of catalase (Line 742; 60× catalase activity; FVB/N)¹⁴ and age- and gender-matched WT (FVB/N) mice were used in this study. The protocol was approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

Two-dimensional, M-Mode, and Doppler Echocardiography for LV Structure and Systolic Function

LV dimensions and systolic function were measured in non-anesthetized mice using an Acuson Sequoia C-256 echocardiograph machine equipped with a 15-MHz linear transducer (model 15L8), as we have described.¹⁵ LV diastolic function was assessed by transmitral and tissue Doppler echocardiography using a VisualSonics Vevo 770 high-resolution imaging system equipped with a 30-MHz RMV-707B transducer, as we have described.¹⁶ Briefly, mice were anesthetized with isoflurane at a concentration of 2.5% for induction and then 1.5% for maintenance by a facemask during the entire procedure. Pulsed-wave Doppler images were collected with the apical 4-chamber view to record the mitral Doppler flow spectra. Peak early and late mitral inflow velocities, ratio of peak early-to-late diastolic transmitral velocity, deceleration time of early filing, and isovolumetric relaxation time were measured. Tissue Doppler images were collected with the use of parasternal short-axis views. Early diastolic myocardial tissue velocity was measured. Doppler spectra were recorded for 12 to 14 cardiac cycles; at least 5 consecutive cardiac cycles were selected for the measurements, and the values were averaged in accordance with the American Society of Echocardiography

guidelines.¹⁷ All data analysis was performed offline using customized Vevo 770 Analytic software.

Isovolumic Langendorff Measurements

Mice were heparinized (100 U IP) and anesthetized with sodium pentobarbital (150 mg/kg IP). The heart was excised and perfused at a constant pressure of 80 mm Hg at 37°C as previously described.¹⁸ The perfusate was equilibrated with 95% O₂ and 5% CO₂ (pH 7.4) and contained (in mmol/L) NaCl 118, NaHCO₃ 25, KCl 5.3, CaCl₂ 1.2, MgSO₄ 1.2, glucose 10, and pyruvate 0.5. Hearts were paced at 7.5 Hz throughout the protocol. A water-filled balloon was inserted into the left ventricle to record ventricular pressure. After stabilization, an LV pressure-volume (P-V) relationship was obtained by stepwise increases of the balloon volumes, until the maximum LV developed pressure was reached for each heart.

Organ Weight and Histology

The mice were killed at 5 or 21 months of age. The heart, left ventricle with septum, lung, and liver were weighed. LV samples were fixed in 10% buffered formalin, embedded with paraffin, and then sectioned. Sections were stained with hematoxylin and eosin and examined under a light microscopy (BX 40; Olympus). Five random fields from each of 4 sections per animal were analyzed, and 60 myocytes per animal were measured. The quantification of myocyte cross-sectional area was determined using NIH ImageJ software. To assess fibrosis, sections were stained with Masson's trichrome kit (Sigma) and examined under a light microscope (BX 40).

Myocyte Contraction, Relaxation, and Intracellular Calcium Concentration

Myocytes were isolated as we have described previously.¹⁹ Briefly, mice were heparinized and anesthetized with pentobarbital (50 mg/kg), and hearts were rapidly excised, cannulated, and perfused with Ca²⁺-free Tyrode solution (3 minutes) followed by a solution containing collagenases B and D (Roche) and protease XIV (Sigma) until digestion was complete. Tissue was dissociated using forceps and filtered through a 250- μ m filter. Myocytes were exposed to solutions of increasing Ca²⁺ concentration and plated in culture chambers (Cell MicroControls) for contractility studies.

Myocyte contraction, relaxation, and intracellular calcium transients were measured as we have described previously.²⁰ Briefly, freshly isolated myocytes were loaded with 0.5 μ mol/L Fura 2-AM (Invitrogen) diluted in 1.2 mmol/L Ca²⁺ Tyrode buffer containing 500 μ mol/L probenecid for 15 minutes at 37°C. The cells were washed with buffer and then incubated for an additional 30 minutes at 37°C to allow complete

deesterification of intracellular acetoxymethyl esters. After rinsing, cells were paced at 5 Hz at 37°C for 3 minutes. Myocyte shortening was measured using a video-based edge-detection system (IonOptix). The calcium transient was measured with Fura 2 fluorescence amplitude using the 360/380 nm ratio. Contraction, relaxation, and calcium measurements were acquired simultaneously in 1 cell at a time, in from 15 to 25 cells per heart with 6 hearts in each experimental group.

Immunoblotting for SERCA Protein

Hearts were homogenized in lysis buffer (1% Triton X-100, 0.5% Nonidet P-40, Tris 10 mmol/L, EDTA 1 mmol/L, EGTA 1 mmol/L, NaCl 150 mmol/L, PMSF 0.4 mmol/L, sodium orthovanadate 0.2 mmol/L, and leupeptin 1 g/L). Protein concentration was determined using the Bradford assay (Bio-Rad). Equal amounts of total protein were separated by SDS-PAGE onto 10% gels and transferred to nitrocellulose membrane. The following primary antibodies were used: mouse monoclonal anti-SERCA2 (Affinity Bioreagents) and rabbit polyclonal anti-GAPDH antibody (Abcam). Protein-antibody complex was detected by using infrared-dye conjugated goat polyclonal antibody IRDye 680 or IRDye 800 (LICOR Biosciences) and scanned with use of the LI-COR Odyssey Infrared Imaging System.

SERCA Activity in Myocardium and Cultured Myocytes

SERCA activity was measured in mouse myocardium using calcium-stimulated, thapsigargin-inhabitable ⁴⁵Ca uptake in a crude homogenate that contains SR vesicles, via a modification of published methods, as we have described previously.¹⁰ LV tissues were homogenized on ice in Tris-sucrose homogenization buffer (8% sucrose [w/v] in Tris-HCl 3 mmol/L, pH 7.0, PMSF 1 mmol/L, 1% Protease Inhibitor Cocktail I [Calbiochem]). The homogenate was centrifuged for 5 minutes at 1520g. The protein concentration of the supernatant was determined by use of the Bradford assay. Samples were pretreated with and without 10 μmol/L thapsigargin, the SERCA inhibitor. Calcium uptake was initiated by the addition of sample to assay buffer (in mmol/L: KCl 100, Na₃ 5, MgCl₂ 6, EGTA 0.15, CaCl₂ 0.12, Tris-HCl 30, pH 7.0, oxalate 10, ATP 2.5, ruthenium red 0.01) containing 1 μCi ⁴⁵CaCl₂ (New England Nuclear) in a 37°C water bath. Aliquots of each sample taken at 30, 60, and 90 seconds were vacuum filtered on glass filters (Whatman GF/C; Fisher Scientific), washed 3 times with wash buffer (in mmol/L: imidazole 30, sucrose 250, EGTA 0.5), and counted with a scintillation counter. SERCA activity is expressed as the initial rate of thapsigargin-sensitive ⁴⁵Ca uptake as nmol/mg protein per minute.

SERCA activity was also measured in cultured adult rat ventricular myocytes (ARVMs), as we have described previously.^{10,21} The cells were homogenized on ice via sonication in Tris-sucrose homogenization buffer (8% [w/v] sucrose in Tris-HCl 3 mmol/L, pH 7.0, PMSF 1 mmol/L). The homogenate was centrifuged for 5 minutes at 1520g. The protein concentration of the supernatant was determined by use of the Bradford assay. SERCA activity was then measured exactly as described for myocardium except that, due to limited tissue, calcium content was measured at a single time point (90 seconds).

Immunohistochemistry for Cys674-Sulfonated SERCA, 3-Nitrotyrosine, and 4-Hydroxy-2-Nonenal

LV tissue sections (4 μm) were blocked with 10% goat serum in phosphate-buffered saline, incubated with (1) rabbit anti-3-nitrotyrosine (NY) polyclonal antibody, (2) mouse anti-4-hydroxy-2-nonenal (HNE) monoclonal antibody, or (3) rabbit polyclonal anti-SERCA antibody raised against a peptide containing the sulfonated Cys674 residue.²² Sections were then incubated with goat biotin-conjugated anti-rabbit IgG or goat biotin-conjugated anti-mouse IgG (Vector Laboratory). The sections were incubated with avidin and biotinylated horseradish peroxidase macromolecular complex (Vector Laboratory) and stained with 3-amino-9-ethylcarbazole (Vector Laboratory) and hematoxylin (Vector Laboratory). For negative control, the primary antibody was omitted instead of normal rabbit IgG or normal mouse IgG. The samples were examined under a light microscope (BX 40).

SERCA Expression in ARVMs

ARVMs in primary culture were isolated and cultured as previously described.¹⁰ WT SERCA and SERCA in which Cys674 was mutated to serine (Cys674Ser) were overexpressed in ARVMs using adenoviral vectors as previously described.⁸ SERCA adenoviruses were screened for proper constructs through extraction of viral DNA using the RedExtract-N-Amp Tissue PCR kit (Sigma). Extracted DNA was subjected to PCR using a forward primer specific to the viral promoter and a reverse primer located beyond the Cys674 codon: 5'-ACCGTCAGATCCGCTAGAGA-3' and 5'-GCCACAATGGTGGAGAAGTT-3'. PCR products were cleaned using a Qiagen QiaQuick PCR Purification Kit and then sequenced using the sequencing primer: 5'-GATCACTGGGGACAACAAGG-3'. SERCA adenovirus was purified using the double cesium chloride purification technique. Tissue culture infectious dose and total viral particle units were determined. E1A contamination of the purified SERCA adenovirus was excluded as described previously.²³ ARVMs were infected with adenoviruses encoding LacZ (multiplicity of infection=10), WT SERCA (multiplicity of infection=10),

or Cys674S (multiplicity of infection=10) for 36 hours. Using these conditions, we previously demonstrated equivalent levels of WT and mutant SERCA expression in ARVMs.¹⁰ Cells were then treated with H₂O₂ (100 μmol/L; 20 minutes), washed with PBS, and used for the SERCA activity assay.

Statistical Analysis

Results are presented as mean±SEM. The statistical significance of differences among multiple groups was determined using Kruskal–Wallis ANOVA and Dunn test. A Mann–Whitney test was used to compare 2 groups. P–V curves were analyzed by use of repeated-measures 2-way ANOVA. *P*<0.05 values were considered significant. All analyses were performed using GraphPad Prism 5.

Results

Myocyte-Specific Overexpression of Catalase Prevents LV Hypertrophy and Diastolic Dysfunction in Senescent Mice

LV weight, wall thickness, and chamber size were increased in senescent WT mice compared with young WT mice (Table). In mice with myocyte-specific overexpression of catalase, there was partial amelioration of these age-related structural changes with decreases in LV wall thickness and mass (Table).

Histological analysis showed increases in myocyte cross-sectional area and interstitial fibrosis in senescent WT mice (Figure S2). In transgenic mice with cardiac-specific overexpression of catalase, myocyte hypertrophy was prevented and interstitial fibrosis was decreased, indicating amelioration of the aging phenotype. In senescent WT mice, immunohistochemical analysis showed marked increases in 2 markers of oxidation in the myocardium: HNE and NY (Figure S3). Catalase overexpression decreased the levels of HNE and NY to those observed in young mice, confirming that oxidative damage in the aging heart is sensitive to a remarkable extent to catalase.

Mitral inflow and tissue Doppler echocardiography in senescent WT mice showed prolongation of LV isovolumetric relaxation time and deceleration time of early filling in association with decreases in the ratio of peak early-to-late diastolic transmitral velocity (Figure 1A through 1C) and early diastolic myocardial tissue velocity (Figure 1D), a pattern typical of abnormal relaxation.¹⁷ The abnormalities in diastolic function were prevented by catalase overexpression in senescent mice (Figure 1A through 1D).

Consistent with the Doppler findings, isovolumic balloon–in–left ventricle Langendorff studies showed a marked leftward/upward shift in the end-diastolic P–V relationship and a rightward/downward shift in LV peak developed P–V relationship in senescent WT mice (versus young WT mice) (Figure 2), indicating LV diastolic and systolic dysfunction in senescent WT mice. Catalase overexpression prevented both diastolic and systolic dysfunction in the senescent hearts (Figure 2).

Table. Echocardiographic Data and Organ Weights

	Y-WT	Y-CAT	S-WT	S-CAT
n	8	8	10	11
IVSTd, mm	0.63±0.02	0.62±0.03	0.83±0.02	0.74±0.01
PWTd, mm	0.91±0.01	0.91±0.01	1.46±0.04***	1.26±0.03*†
LV EDD, mm	2.96±0.03	3.00±0.04	3.39±0.10**	3.27±0.05*
LV ESD, mm	1.14±0.02	1.15±0.03	1.44±0.08**	1.24±0.04*†
LV FS, %	61.2±0.6	62.3±0.8	57.7±1.2	62.1±0.7
LV EF, %	91.4±0.2	91.3±0.5	88.5±1.3	91.5±0.5
BW, g	36.2±0.8	38.8±4.2	40.1±2.8	45.3±2.8
HW, mg	135.9±3.6	143.9±11	193.3±17.9*	175.3±8.8*†
HW/BW, mg/g	3.75±0.02	3.76±0.24	4.71±0.36*	3.94±0.12†
LV W, mg	94.1±2.3	95.9±6.8	131.2±13.8*	122.1±6.5*
LV W/BW, mg/g	2.60±0.03	2.51±0.11	3.28±0.11*	2.74±0.09†
Lung W, wet/dry	4.18±0.19	4.53±0.13	4.74±0.07	4.57±0.07
Liver W, wet/dry	3.03±0.02	3.21±0.05	3.33±0.07	3.23±0.03

Values are mean±SEM. BW indicates body weight; CAT, catalase; EDD, end-diastolic dimension; EF, ejection fraction; ESD, end-systolic dimension; FS, fractional shortening; HW, heart weight; IVSTd, interventricular septal thickness at diastole; LV, left ventricular; PWTd, posterior wall thickness at diastole; S, senescent; W, weight; WT, wild-type; Y, young. **P*<0.05, ***P*<0.01, ****P*<0.001 versus Y-WT or Y-CAT group; †*P*<0.05 versus S-WT group.

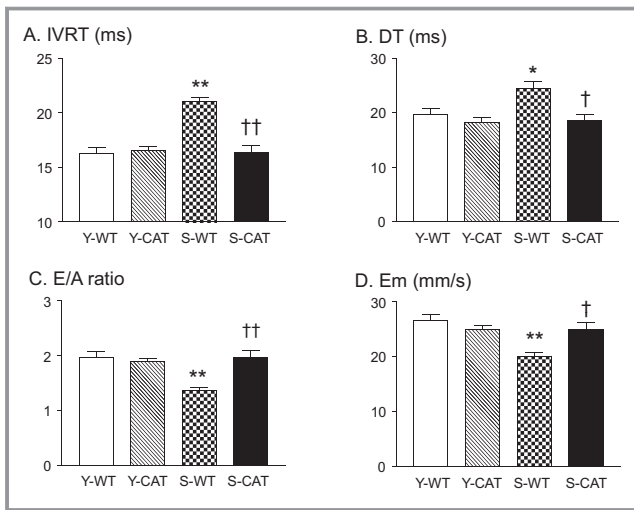


Figure 1. Doppler echocardiographic measures of left ventricular (LV) diastolic function in young (5 months; Y) and senescent (21 months; S) wild-type (WT) and transgenic catalase-overexpressing (CAT) mice. In senescent WT mice, there were increases in isovolumic relaxation time (IVRT; A) and deceleration time (DT; B); and decreases in the ratio of early-to-late diastolic mitral inflow velocity (E/A; C) and myocardial peak early diastolic velocity (E_m ; D) indicative of diastolic dysfunction. Diastolic dysfunction was not present in senescent mice overexpressing catalase. Values are mean \pm SEM; n=8-11; * P <0.05, ** P <0.01 versus Y-WT or Y-CAT mice. † P <0.05, †† P <0.01 versus S-WT mice.

Catalase Preserves SERCA Activity and Prevents SERCA Oxidation in Senescent Myocardium

Maximal SERCA activity was decreased by 38% in senescent (versus young) WT mice (Figure 3A). Overexpression of catalase prevented the age-related decrease in SERCA activity (Figure 3A). The expression of SERCA protein was not decreased in senescent (versus young) WT myocardium, and was not affected by the overexpression of catalase in young or senescent mice (Figure S4). We previously developed an antibody that is specific for SERCA sulfonated at Cys674.²² Immunohistochemistry using this antibody revealed a marked increase in staining of Cys674-sulfonated SERCA in senescent (versus young) WT myocardium that was prevented by catalase overexpression (Figure 3B and 3C).

Cys674Ser-Mutated SERCA Resists H₂O₂-Mediated Oxidation and Inhibition In Vitro in Cardiac Myocytes

We previously showed that Cys674 accounts for the majority of physiologically reactive cysteines in SERCA and is required for glutathiolation-mediated enzyme activation.^{8,10} We further showed that brief exposure (20 minutes) of ARVMs to H₂O₂ (100 μ mol/L) in vitro inhibited SERCA activity and caused oxidation of SERCA cysteines.²¹ Therefore, the observation

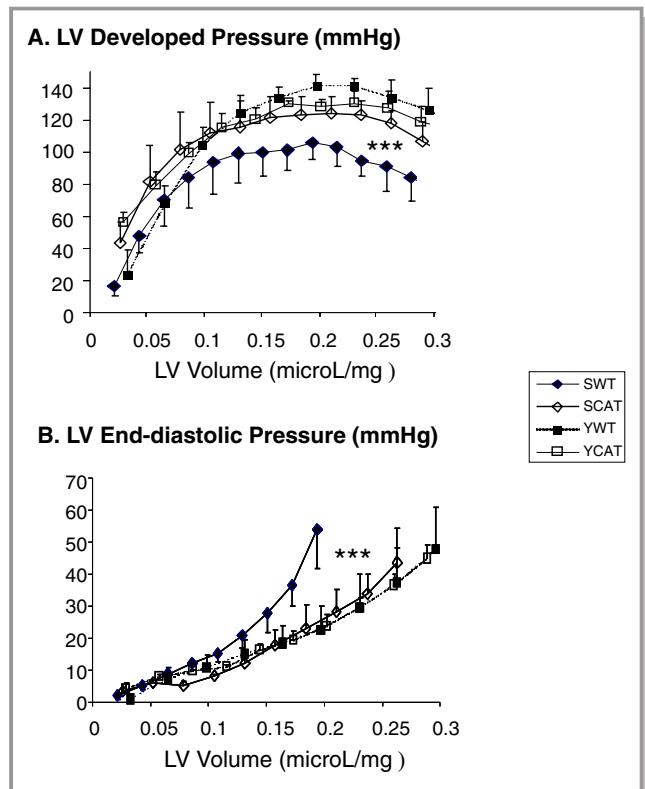


Figure 2. Isovolumic balloon-in-left ventricle Langendorff measurements of left ventricular (LV) developed pressure (A) and LV end-diastolic pressure (B) in young wild-type (Y-WT), young catalase-overexpressing (Y-CAT), senescent WT (S-WT), and senescent catalase-overexpressing (S-CAT) mice. The x-axis depicts LV balloon volume in μ L/mg of LV weight. Values are mean \pm SEM; n=4; *** P <0.001 versus Y-WT or Y-CAT mice by repeated-measures 2-way ANOVA.

that decreased SERCA activity in senescent myocardium is associated with oxidation (sulfonation) of SERCA at Cys674 suggests that oxidative modification of Cys674 may be of pathophysiological relevance. To test the relevance of Cys674 oxidation, in cultured ARVMs we overexpressed WT SERCA or SERCA in which Cys674 was mutated to serine (Cys674Ser), as we have previously described.^{10,24} In myocytes overexpressing WT SERCA, exposure to H₂O₂ (100 μ mol/L, 20 minutes) inhibited SERCA activity by \approx 60% (Figure 3D). In cells overexpressing Cys674Ser SERCA, the ability of H₂O₂ to inhibit SERCA activity was diminished by approximately half (Figure 3D), indicating increased resistance of Cys674Ser SERCA to oxidative inactivation.

Myocyte-Specific Overexpression of Catalase Prevents Age-Related Dysfunction and Calcium Dysregulation in Isolated Myocytes

To determine whether catalase improves relaxation at the level of the cardiac myocyte, we assessed myocyte function

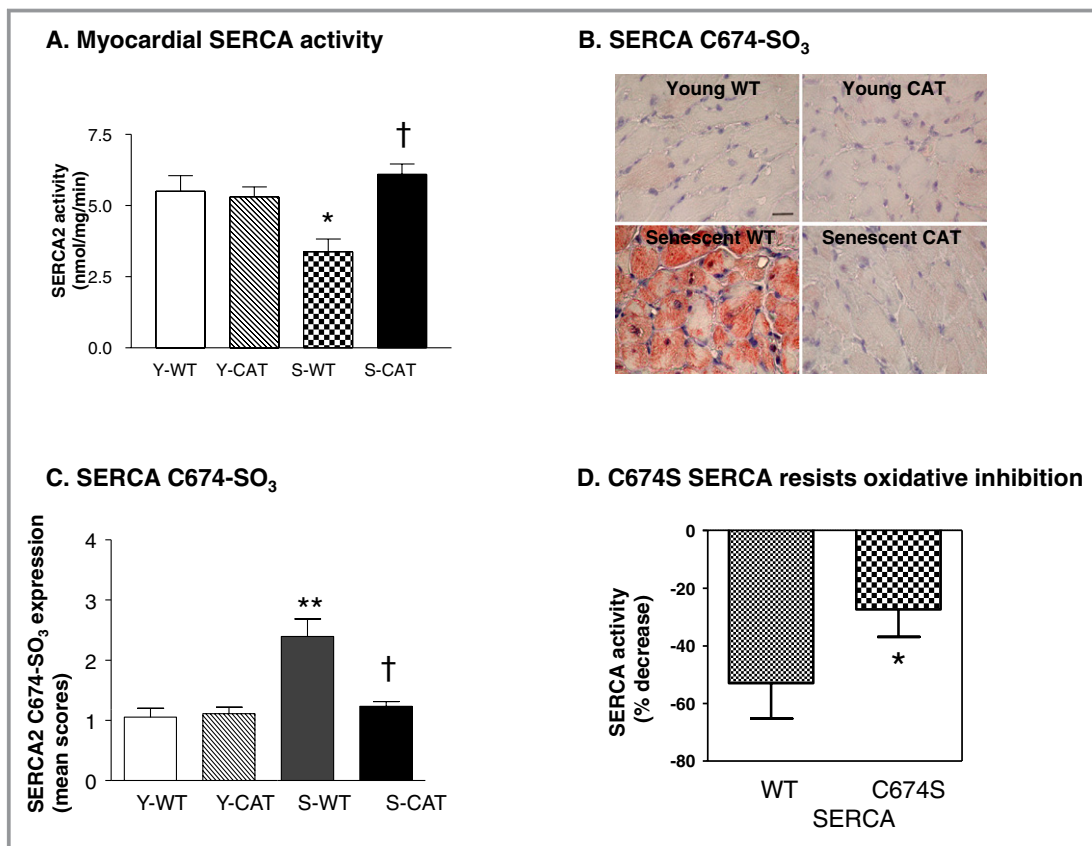


Figure 3. A, Maximal calcium-stimulated SERCA activity in myocardial SR vesicles from young wild-type (Y-WT), young catalase overexpressing (Y-CAT), senescent WT (S-WT), and senescent catalase overexpressing (S-CAT) mice. Values are mean \pm SEM; n=3. * P <0.05 versus Y-WT or Y-CAT mice. † P <0.05 versus S-WT mice. B, Representative photomicrographs of myocardium subjected to immunohistochemical staining with a site-specific antibody that recognizes SERCA that is sulfonated on Cys674 (SERCA C674-SO₃), and (C) shows the quantification of myocardial SERCA Cys674-SO₃ as per (B). Values are mean \pm SEM; n=3-4; ** P <0.01 versus Y-WT or Y-CAT mice. † P <0.05 versus S-WT mice. D, The H₂O₂-mediated decrease in SERCA activity in adult rat ventricular myocytes overexpressing wild-type (WT) SERCA or SERCA in which Cys674 was mutated to serine (Cys674Ser). Values are mean \pm SEM; n=3; * P <0.05 versus WT SERCA. SERCA indicates sarcoplasmic reticulum calcium ATPase.

and intracellular calcium concentration in isolated ventricular myocytes. In myocytes from senescent WT mice, sarcomere shortening, maximal rate of shortening ($-dL/dt$), and maximal rate of relengthening ($+dL/dt$) were decreased by 42%, 41%, and 50%, respectively (Figures 4 and S5). Likewise, in myocytes from senescent WT mice, intracellular diastolic calcium was increased by 18%, the calcium transient amplitude was decreased by 40%, and the time constant of cytosolic calcium decline (τ), an index of SERCA activity, was prolonged by 25%. In senescent mice, overexpression of catalase improved all of these measures of myocyte function and calcium handling (Figures 4 and S5).

Discussion

In this study we demonstrate that (1) decreased SERCA activity in senescent myocardium is associated with oxidative sulfonation of SERCA at Cys674, a cysteine known to be

involved in the regulation of SERCA activity, and (2) both Cys674 oxidation and SERCA inhibition are prevented by overexpression of catalase. We further show in cardiac myocytes in vitro that (1) redox activation of SERCA is decreased in myocytes from senescent mice and (2) Cys674 is oxidized by H₂O₂ and participates in H₂O₂-mediated SERCA inhibition, thus supporting the functional relevance of Cys674 sulfonation observed in the senescent heart. While it is likely that other oxidations on SERCA also contribute to decreased activity in the senescent heart, our observations support the thesis that impaired myocyte relaxation in the aging heart is due, in part, to oxidation of SERCA at Cys674.

SERCA in the Senescent Heart

Our finding of a decrease in SERCA activity in senescent hearts is consistent with most prior studies in rats and mice,^{1,12,25,26} although it should be noted that at least 1 study

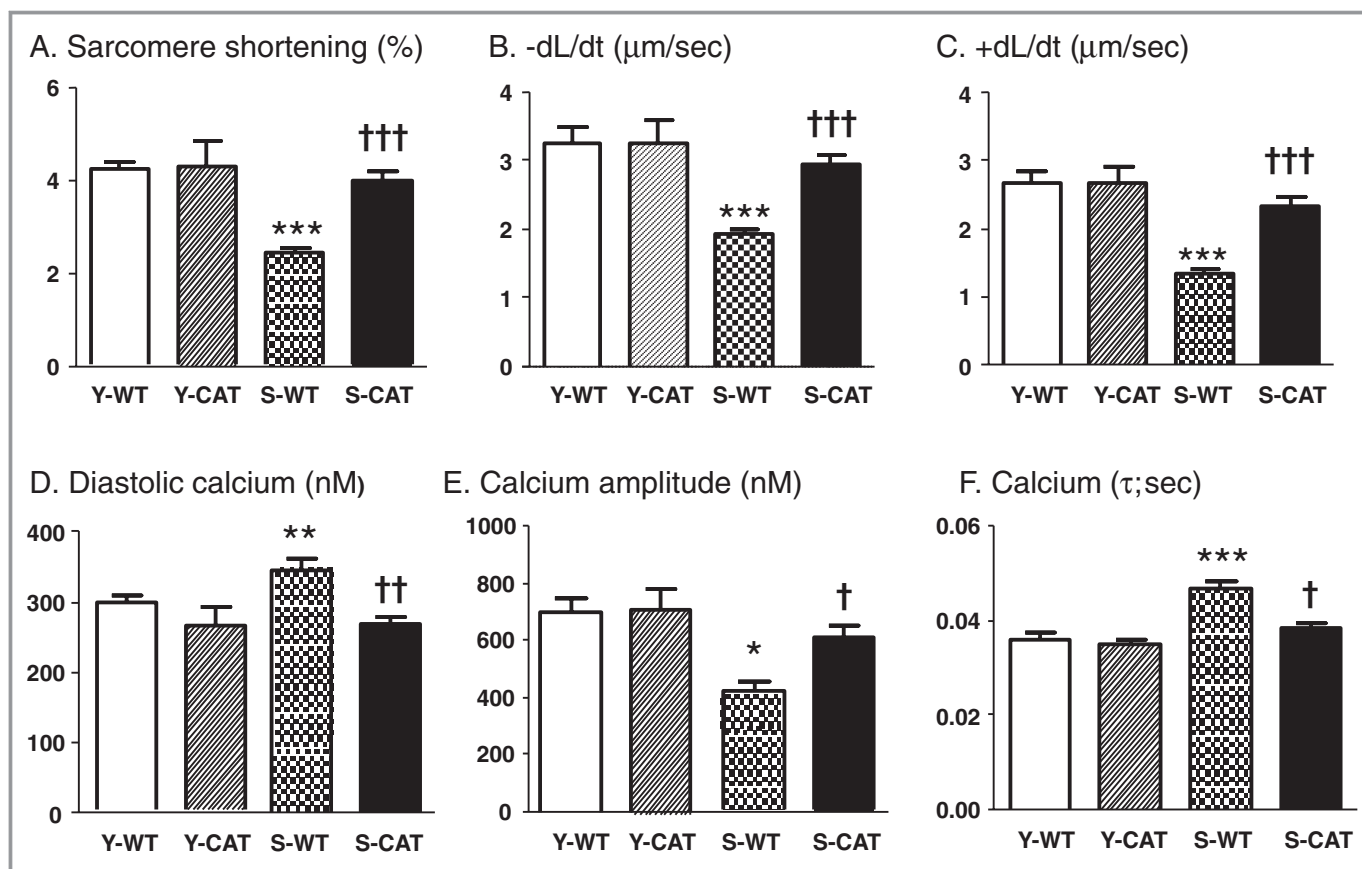


Figure 4. Sarcomere shortening (A), the maximal rate of shortening ($-dL/dt$; B), the maximal rate of relengthening ($+dL/dt$; C), diastolic calcium (D), calcium amplitude (E), and the time constant of diastolic calcium decline (τ ; F) in young wild-type (Y-WT, $n=4$), young catalase (Y-CAT, $n=3$), senescent WT (S-WT, $n=6$) and senescent catalase (S-CAT, $n=6$) mice. Values are mean \pm SEM; * $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus Y-WT or Y-CAT mice. † $P<0.05$, †† $P<0.01$, ††† $P<0.001$ versus S-WT mice.

in senescent sheep did not find a decrease in SERCA activity, raising questions about species-related differences.²⁷ The functional relevance of this decrease in SERCA activity is supported by the finding of impaired calcium reuptake and relaxation in myocytes isolated from the senescent heart, as observed previously by ourselves¹⁹ and others.¹² While some studies have observed decreased levels of SERCA protein in the senescent heart,^{1,11,12} our current data and those from several laboratories,¹⁻⁵ demonstrate that a decrease in SERCA protein expression does not occur consistently in senescent hearts. Thus, a decrease in protein expression does not appear necessary for decreased SERCA activity in the senescent heart.

Another possibility for decreased SERCA activity is an alteration in phospholamban (PLN) expression or activity. Changes in PLN expression or activity regulate SERCA activity by shifting the EC_{50} for calcium but without affecting maximal calcium-stimulated activity, as measured in this study.²⁸ Conversely, for the same EC_{50} , changes in maximal activity reflect directionally similar changes in activity within the physiological range.²⁸ Therefore, (1) a change in PLN activity

would not explain the changes in myocardial SERCA activity that we observed and (2) the observed changes in maximal SERCA activity would be predicted to reflect directionally similar changes in activity in the physiological range. Thus, it is likely that the observed changes in maximal activity reflect similar changes in activity within the physiological calcium range. This premise is supported by the observation that the rate of calcium reuptake, which is a reasonable reflection of SERCA activity, changed in parallel with maximal SERCA activity. Finally, while we cannot comment on PLN activity, prior studies in mouse heart have not observed an increase in PLN expression or activity with aging.^{11,29}

Oxidative Posttranslational Modification of SERCA

The finding of decreased SERCA activity in the presence of normal protein expression is highly suggestive of a posttranslational event that modifies enzyme activity. While SERCA can be regulated by phosphorylation,^{26,30} the ability of catalase overexpression to preserve SERCA activity, as well as

relaxation and calcium reuptake in isolated myocytes, strongly suggests that in senescent heart the posttranslational modification of SERCA is mediated by reactive oxygen species and, in particular, H₂O₂. Increased oxidative stress is well recognized in the senescent heart and has been implicated in the pathophysiology of cardiac aging.^{11,12,31–33} Markers of protein and lipid oxidation (NY and HNE, respectively) were markedly increased in senescent hearts, and the increases were prevented by catalase overexpression, indicating that H₂O₂ is an important oxidant in the senescent heart and confirming the antioxidant effectiveness of catalase overexpression in our mice.

SERCA Cys674 Sulfonation

We previously developed and validated a site-specific antibody that recognizes SERCA sulfonated at Cys674.²² Using this antibody for immunohistochemistry, we demonstrate that cardiac aging is associated with oxidation (sulfonation) of SERCA on Cys674. While several oxidative modifications of SERCA have been observed in senescent myocardium,^{6,7} the functional significance of these modifications is not known. Cys674 is located in the hinge region of SERCA, which is thought to be involved in regulating the passage of calcium and is accessible from the cytosolic compartment.³⁴ Recent work has indicated that the Cys674 thiol plays an important role in regulating SERCA activity: In arterial smooth muscle nitric oxide–induced relaxation is mediated by S-glutathiolation of SERCA Cys674, whereas decreased S-glutathiolation and decreased activation of SERCA by nitric oxide in atherosclerotic aorta are associated with sulfonation at Cys674.⁸ Likewise, in mice with heart failure, we found that decreased SERCA activity in the myocardium is associated with SERCA sulfonation at Cys674 in the absence of a decrease in protein expression.²⁰

Recently, we used a SERCA mutant in which Cys674 was replaced by serine (Cys674Ser) to show that glutathiolation of Cys674 is necessary for the redox activation of SERCA by nitroxyl anion.¹⁰ In studies leading to the current project, oxidation of Cys674 was implicated by the finding that SERCA activity could not be stimulated by nitroxyl in myocytes from senescent mice (Figure S1). This observation is consistent with the thesis that SERCA oxidation at Cys674 contributes to decreased SERCA activity and calcium handling in the senescent heart. We previously found that exposure to H₂O₂ causes rapid inhibition of SERCA activity that is associated with oxidative modification of cysteines.²¹ Therefore, to test the role of Cys674 oxidation in inhibiting myocyte SERCA activity, we overexpressed WT SERCA and the Cys674Ser SERCA mutant in cultured ARVMs. Because the Cys674S SERCA mutant cannot be oxidized at Cys674, it provides an opportunity to test the functional conse-

quence of Cys674 oxidation. As we previously observed in native cardiac myocytes,²¹ H₂O₂ exposure markedly inhibited SERCA activity in myocytes overexpressing WT SERCA. By comparison, the ability of H₂O₂ to inhibit SERCA activity was attenuated by approximately half in myocytes overexpressing the Cys674Ser mutant, suggesting that oxidation of Cys674 accounts for approximately half of the decrease in activity caused by exposure to H₂O₂. Thus, the functional importance of Cys674 in mediating the effects of H₂O₂ on SERCA, together with the identification of catalase-sensitive Cys674 sulfonation in the senescent heart, supports our thesis that SERCA oxidation at Cys674 contributes to decreased SERCA activity and impaired myocyte relaxation in the aging heart.

Implications

The ability of cardiac-specific catalase to correct age-related abnormalities in SERCA activity and calcium handling and relaxation in the cardiac myocyte support the thesis that oxidation of SERCA contributes to slowed relaxation of cardiac myocytes in senescent heart. However, it is important to recognize that there are multiple other abnormalities in the aging heart that may contribute to impaired relaxation (eg, increased interstitial fibrosis or altered of titan function). This study does not allow assignment of the relative importance of decreased SERCA activity versus other potential mechanisms in causing diastolic dysfunction. However, because overexpression of SERCA has been shown to improve LV diastolic function in aging hearts,³⁵ it is likely that the ability of catalase to prevent the oxidative inactivation of SERCA contributed to improvement in myocyte relaxation and, at least in part, to improvement in diastolic function in our senescent mice. These observations therefore suggest that strategies to decrease oxidant levels and/or protect SERCA from oxidation at particular sites (eg, Cys674) may be of value in the amelioration of cardiac aging. Because impaired SERCA activity and SERCA oxidation are present in other pathophysiological settings such as heart failure,³⁶ it is also possible that oxidative modification of SERCA will be relevant to other conditions that are associated with impaired SERCA activity.

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Disclosures

None.

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