Short Communication

Oxidative Posttranslational Modifications Mediate Decreased SERCA Activity and Myocyte Dysfunction in Gaq-Overexpressing Mice

Steve Lancel,* Fuzhong Qin,* Shannon L. Lennon, Jingmei Zhang, XiaoYong Tong, Michael J. Mazzini, Y. James Kang, Deborah A. Siwik, Richard A. Cohen, Wilson S. Colucci

Background: Myocyte contractile dysfunction occurs in pathological remodeling in association with abnormalities in calcium regulation. Mice with cardiac myocyte–specific overexpression of Gaq develop progressive left ventricular failure associated with myocyte contractile dysfunction and calcium dysregulation.

Objective: We tested the hypothesis that myocyte contractile dysfunction in the Gaq mouse heart is mediated by reactive oxygen species, and in particular, oxidative posttranslational modifications, which impair the function of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA).

Methods and Results: Freshly isolated ventricular myocytes from Gaq mice had marked abnormalities of myocyte contractile function and calcium transients. In Gaq myocardium, SERCA protein was not altered in quantity but displayed evidence of oxidative cysteine modifications reflected by decreased biotinylated iodoacetamide labeling and evidence of specific irreversible oxidative modifications consisting of sulfonation at cysteine 674 and nitration at tyrosines 294/295. Maximal calcium-stimulated SERCA activity was decreased 47% in Gaq myocardium. Cross-breeding Gaq mice with transgenic mice that have cardiac myocyte-specific overexpression of catalase (a) decreased SERCA oxidative cysteine modifications, (b) decreased SERCA cysteine 674 sulfonation and tyrosine 294/295 nitration, (c) restored SERCA activity, and (d) improved myocyte calcium transients and contractile function.

Conclusions: In Gaq-induced cardiomyopathy, myocyte contractile dysfunction is mediated, at least in part, by 1 or more oxidative posttranslational modifications of SERCA. Protein oxidative posttranslational modifications contribute to the pathophysiology of myocardial dysfunction and thus may provide a target for therapeutic intervention. (Circ Res. 2010;107:228-232.)

Key Words: cardiac myocytes ■ sarcoplasmic reticulum ATPase ■ SERCA ■ oxidative modification

Myocyte contractile dysfunction occurs in several models of pathological remodeling including pressure overload\(^{1-4}\) and after myocardial infarction.\(^{5-8}\) Although myocyte dysfunction appears to be caused, at least in part, by abnormalities in calcium regulation,\(^{9}\) the underlying mechanism remains unclear. There is evidence that reactive oxygen species (ROS) mediate some aspects of pathological myocardial remodeling including myocyte hypertrophy and apoptosis.\(^{10-12}\) Mice with cardiac myocyte–specific overexpression of Gaq develop progressive left ventricular (LV) dilation and failure\(^{13}\) associated with myocyte contractile dysfunction and calcium dysregulation.\(^{14}\) These mice have increased oxidative stress in the myocardium,\(^{15}\) and recently, we demonstrated that concomitant myocyte-specific expression of catalase ameliorated pathological LV remodeling, inhibited myocyte hypertrophy and apoptosis, and preserved LV contractile function.\(^{16}\) Accordingly, we tested the hypothesis that myocyte contractile dysfunction in the Gaq mouse heart is also mediated by ROS, and in particular, involves oxidative posttranslational modifications (OPTM) that impair the function of sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA).\(^{17}\)

Methods
An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

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Briefly, transgenic mice with cardiac myocyte-specific overexpression of Gaq (Gaq-40 mice, FVB/N) and wild-type (FVB/N) mice were crossbred with transgenic mice having myocyte-specific overexpression of catalase, as we previously described.16 Myocytes were isolated, and contraction and intracellular calcium transients were measured, as we have described previously.18 SERCA2 activity was measured using calcium-stimulated, thapsigargin-inhibitable calcium uptake in an SR membrane preparation by a modification of published methods, as we have described.21 BIAM-labeling, immunoblotting, and immunohistochemical detection of SERCA OPTM are available in the Online Data Supplement. All data are presented as means±SEM.

**Results**

**Concurrent Myocyte-Specific Catalase Overexpression Ameliorates Contractile Dysfunction and Calcium Dysregulation in Myocytes From Gaq Mice**

Ventricular myocytes were isolated from mice with myocyte-specific overexpression of Gaq and myocyte contractile function and intracellular calcium transients were assessed, as we have described.19 In myocytes from Gaq mice (versus wild type), the amplitude of cell shortening was decreased by 53%, and the rates of myocyte shortening and relaxation were reduced by 62% and 63%, respectively (Figure 1A through 1C). Likewise, in Gaq myocytes, the calcium transient amplitude was decreased by 37%, and the rates of rise and decline were decreased by 34% and 33%, respectively (Figure 1D through 1F). Gaq mice were crossbred with mice that have myocyte-specific overexpression of catalase, as we have described. In myocytes from Gaq/catalase mice, the amplitude of cell shortening and the rates of cell shortening and relaxation were improved (Figure 1A through 1C) and the abnormalities in calcium transient amplitude and kinetics were ameliorated (Figure 1D through 1F).

**Expression of Calcium Regulating Proteins in Gaq Myocardium**

The protein levels of SERCA, the ryanodine receptor (RyR), phospholamban (PLB), and the sodium/calcium exchanger (NCX) were determined by immunoblotting. RyR protein expression was decreased by 47% in Gaq mice, whereas the expression of SERCA, PLB, and NCX was unchanged (Table). The decrease in RyR protein was associated with a 42% decrease in mRNA (Online Figure I), suggesting that the

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**Non-standard Abbreviations and Acronyms**

- **BIAM**: biotinylated iodoacetamide
- **LV**: left ventricular
- **NCX**: sodium/calcium exchanger
- **OPTM**: oxidative posttranslational modifications
- **PLB**: phospholamban
- **RyR**: ryanodine receptor
- **ROS**: reactive oxygen species
- **SERCA**: sarcoplasmic reticulum $\text{Ca}^{2+}$ ATPase

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Figure 1. Abnormal contractile function and intracellular calcium transients in cardiac myocytes from Gaq-overexpressing mice are ameliorated by cross-breeding with mice that overexpress catalase in the myocardium. Ventricular myocytes were isolated from wild-type (WT), Gaq (Gq), or Gaq/catalase (GqCat) mice. A, Cell shortening (% of baseline). B, Velocity of contraction ($-dL/dt$). C, Velocity of relaxation ($+dL/dt$). D, Calcium transient amplitude (delta of the ratio [R] of fluorescence 360/380 nm). E, Rate of calcium transient rise (+dR/dt). F, Rate of calcium transient decline (-dR/dt). *$P<0.05$ vs WT; **$P<0.01$ vs WT; †$P<0.05$ vs Gaq; ††$P<0.01$ vs Gaq; 5 to 10 cells per heart, 4 to 5 hearts per group.
decrease in protein was mediated at the transcript level. Concurrent expression of catalase in Gaq/catalase mice had no effect on RyR protein or transcript levels (Table and Online Figure I).

**OPTM of SERCA in Gaq Myocardium**

To test whether OPTM may contribute to contractile dysfunction in Gaq myocytes, oxidative thiol modifications of SERCA and RyR were assessed using biotinylated iodoacetamide (BIAM), as we have described. Compared with wild type, the fraction of BIAM-labeled SERCA in Gaq was decreased by 36% (Figure 2A and 2B), whereas BIAM-labeling of RyR was unchanged (data not shown). We have developed antibodies directed at SERCA that are sulfonylated at cysteine 674 or nitrated at tyrosine 294/295. Using these antibodies, immunohistochemistry revealed increased staining for both OPTM diffusely over myocytes in Gaq hearts (Figure 2C and 2D). In myocardium from Gaq/catalase mice (compared with Gaq mice), there was (1) increased BIAM labeling of SERCA (Figure 2A and 2B), indicating a decrease in cysteine oxidation, (2) decreased sulfonylation of SERCA cysteine 674 (Figure 2C), and (3) decreased nitration of SERCA tyrosine 294/295 (Figure 2D).

**Decreased SERCA Activity in Gaq Myocardium Is Restored by Catalase**

To assess the functional consequence of the observed OPTM, SERCA activity was measured using maximal calcium-stimulated calcium uptake in sarcolemmal membranes, as we have described. SERCA-mediated calcium uptake was reduced by 47% in Gaq membranes (Figure 3). In myocytes from Gaq/catalase mice, maximal calcium-stimulated SERCA activity was restored to wild-type levels (Figure 3).

**Table. Total Protein Expression of Calcium-Handling Proteins in WT and Gaq Mice**

<table>
<thead>
<tr>
<th>Protein</th>
<th>WT</th>
<th>Gaq</th>
<th>Gaq/Cat</th>
</tr>
</thead>
<tbody>
<tr>
<td>SERCA</td>
<td>1±0.07</td>
<td>1.05±0.07</td>
<td>0.99±0.11</td>
</tr>
<tr>
<td>RyR</td>
<td>1±0.09</td>
<td>0.53±0.01*</td>
<td>0.54±0.10*</td>
</tr>
<tr>
<td>PLB</td>
<td>1±0.05</td>
<td>1.23±0.31</td>
<td>ND</td>
</tr>
<tr>
<td>NCX</td>
<td>1±0.08</td>
<td>0.95±0.15</td>
<td>ND</td>
</tr>
</tbody>
</table>

WT indicates wild-type; and ND, not done. Total protein is expressed as the ratio of the protein of interest/GAPDH, normalized to the average value in the WT group. Data are the means of 4 hearts in each group (*P<0.05 vs WT mice).
Discussion

We used the Gq mouse model of dilated cardiomyopathy to examine the role of protein OPTM in mediating myocyte contractile dysfunction. Prior work from Satoh et al and our group demonstrated increased oxidative stress in the myocardium of these mice. Likewise, our finding of contractile dysfunction and abnormal calcium regulation in Goq myocytes confirms and extends the prior report by Yatani et al. Recently, we demonstrated that concurrent cardiac myocyte–specific overexpression of catalase improved cardiac function in these mice. Accordingly, we hypothesized that catalase would improve myocyte function and thereby allow the identification of catalase-sensitive OPTM of myocyte proteins involved in the pathophysiology of contractile dysfunction.

Our initial finding was that cardiac myocyte–specific overexpression of catalase ameliorated the abnormalities in cardiac myocyte contractile function and calcium regulation. This indicates that a catalase-sensitive pathway is involved in mediating myocyte dysfunction and directed our attention to proteins involved in calcium handling. Of the proteins primarily involved in myocyte calcium regulation, only the expression of RyR was decreased. However, the decreases in RyR protein and its mRNA were not affected by catalase and thus are not responsible for the effect of catalase on myocyte function that we observed.

SERCA activity was decreased in Goq myocardium, consistent with prior observations in this and other models of heart failure. However, the expression of SERCA was not decreased, which is also consistent with prior observations in this mouse. Of note, SERCA activity was corrected by concurrent catalase expression, suggesting that OPTM of SERCA might be responsible for decreased SERCA activity. This thesis was further supported by 3 observations. First, in Goq myocardium, there was a decrease in the quantity of BIAM binding to SERCA, which indicates oxidative modification of the most reactive SERCA cysteine, cysteine 674, and potentially other cysteines. Importantly, the quantity of BIAM binding to SERCA was restored toward normal in Goq/catalase mice, confirming that the modification was oxidative in nature. Second, there was immunohistochemical evidence of sulfonylation of SERCA at cysteine 674. This OPTM is noteworthy because we have shown that sulfonylation of SERCA cysteine 674 in atherosclerotic aortic smooth muscle is associated with decreased activity. Third, there was immunohistochemical evidence of nitration of SERCA tyrosine 294/295. Sulfonylation of SERCA at cysteine 674 and nitration of tyrosine at 294/295 provide evidence of irreversible oxidation by elevated oxidants. As with BIAM binding, both SERCA sulfonylation and nitration were markedly decreased in Goq/catalase mice. These findings thus directly identify 2 specific OPTM of SERCA in the Goq mouse and demonstrate that both can be prevented by catalase. This observation implicates H2O2 in the oxidation of SERCA cysteine 674 and the nitration of SERCA tyrosines. H2O2 probably is derived through dismutation of superoxide that is produced by mitochondria and/or oxidases.

Although we have identified 2 specific irreversible OPTM that are associated with decreased SERCA activity, we cannot exclude phosphorylation of calcium-regulating proteins caused by oxidative regulation of a phosphatase. However, we think it is unlikely that this mechanism could explain our primary observation (correction of maximal calcium-stimulated SERCA activity) because (1) SERCA is not known to have regulatory phosphorylation sites; and (2) SERCA activity was measured using maximal calcium stimulation, which is not sensitive to PLB. On the other hand, oxidative regulation of phosphorylation might contribute to other aspects of contractile dysfunction in this model.

Prior studies in mice have implicated abnormal calcium handling, and in particular, decreased SERCA function in the pathophysiology of myocardial contractile dysfunction. Likewise, numerous studies have identified decreased SERCA activity in failing human myocardium, which, in some cases, has been associated with normal SERCA protein levels. In preliminary studies, we have found immunohistochemical evidence that SERCA is sulfonylated at cysteine 674 in myocardium from patients with heart failure caused by dilated cardiomyopathy but not in myocardium from patients without heart failure (unpublished data), thus suggesting that our findings in the Goq mouse are relevant to human disease.

Novel findings of this study include the demonstration of (1) multiple specific oxidative modifications of SERCA; (2) the relationship between SERCA OPTM and reduced SERCA activity and altered cellular calcium handling; and (3) the rescue of OPTM, SERCA activity, and cellular calcium handling by myocyte-specific overexpression of catalase. Taken together, our data suggest that cardiac myocyte contractile dysfunction in the Goq mouse is mediated, in part, by catalase-sensitive OPTM of SERCA. These observations suggest that OPTM caused by H2O2 contribute to myocardial dysfunction in pathological states, such as heart failure, that are associated with increased oxidant levels in the heart.

Sources of Funding

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Disclosures

None.

References

What New Information Does This Article Contribute?

- Myocyte-specific overexpression of catalase prevents OPTM of SERCA, restores SERCA activity, and improves myocyte calcium dysregulation and contractile dysfunction.

Novelty and Significance

- Myocyte-specific overexpression of catalase prevents OPTM of SERCA, restores SERCA activity, and improves myocyte calcium dysregulation and contractile dysfunction.

We tested whether myocyte dysfunction in Gαq mice is mediated by OPTM of SERCA. In Gαq myocardium there were specific OPTM of SERCA associated with reduced SERCA activity and impaired calcium-related myocyte function. Myocyte-specific overexpression of catalase prevented OPTM of SERCA and rescued SERCA activity and isolated myocyte function. Thus, myocyte contractile dysfunction in Gαq-induced cardiomyopathy is mediated, at least in part, by OPTM of SERCA. More broadly, these observations suggest that protein OPTM may contribute to the pathophysiology of myocardial dysfunction in heart failure and other conditions associated with increased myocardial reactive oxygen species and may provide a novel therapeutic target.
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Myocyte Dysfunction in Gαq-Overexpressing Mice

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Michael J. Mazzini, Y. James Kang, Deborah A Siwik, Richard A. Cohen
and Wilson S. Colucci

Methods

Experimental animals. Transgenic mice with cardiac myocyte-specific overexpression of Gαq (Gαq-40 mice, FVB/N) ¹ and WT (FVB/N) mice were cross-bred with transgenic mice having myocyte-specific overexpression of catalase (Line 742; 60X catalase activity; FVB/N) ², as we previously described ³. For the current experiments, animals were studied at 20-22 weeks of age. The protocol was approved by the Institutional Animal Care and Use Committee at Boston University School of Medicine.

Myocyte isolation. Myocytes were isolated as we have described previously ⁴. Briefly, mice were heparinized and anesthetized with pentobarbital (50mg/kg), hearts were rapidly excised, cannulated and perfused with Ca²⁺-free Tyrode (3 min) 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 250μm filter. Myocytes were exposed to
solutions of increasing Ca\(^{2+}\) concentration, and plated in culture chambers (Cell MicroControls, Norfolk, VA) for contractility studies.

**Myocyte contraction and calcium transients.** Myocyte contraction and intracellular calcium transients were measured as we have described previously\(^4\). Briefly, freshly isolated myocytes were loaded with 0.5\(\mu\)M Fura2-AM (Invitrogen) diluted in 1.2mM Ca\(^{2+}\) tyrode buffer containing 500\(\mu\)M probenecid for 15min at 37°C. After rinsing, cells were paced at 5Hz at 37°C for 3 min. Myocyte shortening was measured using a video-based edge-detection system (IonOptix, Milton, MA). The calcium transient was measured with Fura2 fluorescence amplitude using the 360/380nm ratio. Contraction and calcium measurements were acquired simultaneously from 5-15 cells per heart with 4-6 hearts in each experimental group.

**RT-PCR.** Hearts were lysed in TRIzol reagent (Invitrogen) using a tissue homogenizer. Following a 5 min-incubation at room temperature, chloroform was added and samples were centrifuged at 12,000\(g\) for 15min at 4°C. The upper aqueous phase containing RNA was collected and purified using the PureLink Micro-to-Midi Total RNA Purification System (Invitrogen) according manufacturer’s instructions. RyR2 mRNA was measured by real-time reverse transcriptase polymerase chain reaction (RT RT-PCR) using iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad). The RyR2 primers were GTCACGGATGCTCAGTCTCA (forward) and GGAGACTCCATGACCCTTCA (reverse). Results were normalized to ribosomal 18S mRNA using CGGCGACGACCATTCGAAC and GAATCGAACCCTGATTCCCGTC as forward and reverse primers, respectively.
BIAM-labeling and Western-Blotting. Hearts were homogenized in RIPA buffer (in mM: PIPES 100, DTPA 0.1, NaCl 150, NaF 1, PMSF 0.1; NP40 1%, deoxycholate 0.25%, protease inhibitor set 1, 1%, pH 6.5) containing 100µM of biotinylated iodoacetamide (BIAM, Invitrogen). Lysates were passed through PD-10 Sephadex-G25 columns (GE Healthcare) to remove the excess of BIAM. 1mg of total proteins was incubated with streptavidin-Sepharose beads (GE Healthcare) overnight at 4°C. After 4 washes, samples were eluted in Laemmli buffer containing 10M urea and separated by SDS-PAGE. After transfer on nitrocellulose membrane, the following primary antibodies were used: mouse monoclonal anti-RyR, anti-SERCA2, anti-PLB (Affinity Bioreagents), anti-NCX (Abcam) antibodies and rabbit polyclonal anti-GAPDH antibody (Abcam). Protein-primary antibody complex was detected by using infrared-dye conjugated goat polyclonal antibody IRDye 680 or IRDye 800 (LICOR Biosciences) and scanned with LI-COR Odyssey Infrared Imaging System.

Immunohistochemical detection of SERCA OPTM. LV myocardial samples were fixed in 10% neutral-buffered formalin, embedded with paraffin, and then sectioned (4µm thick). The paraffin sections were then deparaffinated and rehydrated in xylene and in decreasing concentrations of ethanol. Blocked with 10% normal goat serum, sections were subsequently incubated with primary antibody rabbit polyclonal anti-SERCA antibody raised against a peptide containing the sulfonylated cysteine 674 residue (C674) or the nitrated tyrosine 294/295 residue; and then incubated with a goat biotin-conjugated anti-rabbit secondary antibody. AEC was used as substrate. Slides were counterstained with hematoxylin. Slides were examined under an Olympus BX 40 microscope. We have previously demonstrated the specificity of these antibodies by using blocking peptides. For the anti-SERCA C674-SO$_3$H antibody, pre-
incubating with the antigenic C674-SO₃H SERCA peptide (CLNARC(SO₃H)FARV), eliminated staining of the 110kDa band detected in pig cardiac SR treated with 1 mM peroxynitrite. However, pre-incubating the antibody either with the peptide of the same sequence in which cysteine-674 has a reduced thiol (CLNARCFARV), or a peptide containing a scrambled sequence of the antigenic peptide containing the cysteine sulfonic acid (CRAFNC(SO₃H)VRAL) did not block the detection of the 110 kDa band. These results indicate that the anti-SERCA C674-SO₃H antibody detects only SERCA protein with cysteine sulfonic acid in a sequence-specific manner. We have validated the SERCA-SO₃H antibody using the sequence-specific peptide and the antigenic scrambled peptide in mouse aortic tissue (unpublished data). For the anti-SERCA2 di-nY-294,295 antibody, specificity was confirmed by treating sections for 40 min with sodium dithionite (100 mmol/l in 100 mmol/l sodium borate, pH 9.0) to reduce nitrotyrosine to aminotyrosine or by preincubating the antibodies with free SERCA2 di-nY-294,295 peptide (antibody:peptide, 1:5 by weight, respectively).

**SERCA activity.** SERCA2 activity was measured using calcium-stimulated, thapsigargin-inhibitable calcium⁴⁵ uptake in an SR membrane preparation, by a modification of published methods ⁷,⁸, as we have described ⁹. LV tissues were homogenized on ice in Tris-sucrose homogenization buffer (8% (w/v) sucrose in (in mM) Tris-HCl pH 7.0 3, PMSF 1, 1% Protease Inhibitor Cocktail I (Calbiochem)). 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, Ruthenium Red 0.01) containing 1 µCi ⁴⁵CaCl₂ (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 mean ± S.E.M. The groups were compared by ANOVA, and differences between groups were assessed using a sequential Bonferroni procedure. After application of Bonferroni correction, significance was achieved with P<0.05 for comparisons with control.
Figure Legend

Online Figure I. Decreased ryanodine receptor (RyR) protein expression in myocardium of Gaq mice is not affected by cross-breeding with mice that overexpress catalase in the myocardium.

Panel A. Representative immunoblot for RyR protein. Panel B. Mean values for RyR protein normalized to GAPDH (*p<0.05 vs. WT; n = 4). Panel C. RyR mRNA, assessed by real-time RT-PCR. Shown are mean data normalized to 18S RNA from 3 hearts per group (*p<0.05 vs. WT).
References


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Online Figure I.

A. 

B. 

C. 

Online Figure I.