ONLINE SUPPLEMENT

Oxidative Post-Translational Modifications Mediate Decreased SERCA Activity and Myocyte Dysfunction in Gαq-Overexpressing Mice

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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 anesthesized with pentobarbital (50mg/kg), hearts were rapidly excised, cannulated and perfused with Ca^{2+}-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 0.5μM Fura2-AM (Invitrogen) diluted in 1.2mM Ca2+ tyrode buffer containing 500μM 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 CGGCGACGACCCATTCGAAC and GAATCGAACCCTGATTCCCCGTC 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) [5] or the nitrated tyrosine 294/295 residue [6]; 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.[5;6] For the anti-SERCA C674-SO_3H antibody, pre-

incubating with the antigenic C674-SO_3H SERCA peptide (CLNARC(SO_3H)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(SO_3H)VRAL) did not block the detection of the 110 kDa band. These results indicate that the anti-SERCA C674-SO_3H antibody detects only SERCA protein with cysteine sulfonic acid in a sequence-specific manner. We have validated the SERCA-SO_3H 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 calcium[45] 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 $^{45}CaCl_2$ (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 Gαq 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).

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Online Figure I.