Expression of SERCA isoform with faster Ca\textsuperscript{2+} transport properties improves postischemic cardiac function and Ca\textsuperscript{2+} handling and decreases myocardial infarction

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Talukder MA, Kalyanasundaram A, Zhao X, Zuo L, Bhupathy P, Babu GJ, Cardouel AJ, Periasamy M, Zweier JL. Expression of SERCA isoform with faster Ca\textsuperscript{2+} transport properties improves postischemic cardiac function and Ca\textsuperscript{2+} handling and decreases myocardial infarction. Am J Physiol Heart Circ Physiol 293: H2418–H2428, 2007. First published July 13, 2007; doi:10.1152/ajpheart.00663.2007.—Myocardial ischemia-reperfusion (I/R) injury is associated with contractile dysfunction, arrhythmias, and myocyte death. Intracellular Ca\textsuperscript{2+} overload with reduced activity of sarco(endo)plasmic reticulum Ca\textsuperscript{2+}-ATPase (SERCA) is a critical mechanism of this injury. Although upregulation of SERCA function is well documented to improve postischemic cardiac function, there are conflicting reports where pharmacological inhibition of SERCA improved postischemic function. SERCA2a is the primary cardiac isoform regulating intracellular Ca\textsuperscript{2+} homeostasis; however, SERCA1a has been shown to substitute for SERCA2a with faster Ca\textsuperscript{2+} transport kinetics. Therefore, to further address this issue and to evaluate whether SERCA1a expression could improve postischemic cardiac function and myocardial salvage, in vitro and in vivo myocardial I/R studies were performed on SERCA1a transgenic (SERCA1a\textsuperscript{+/+}) and nontransgenic (NTG) mice. Langendorff-perfused hearts were subjected to 30 min of global ischemia followed by reperfusion. Baseline preischemic coronary flow and left ventricular developed pressure were significantly greater in SERCA1a\textsuperscript{+/+} mice compared with NTG mice. Independent of reperfusion-induced oxidative stress, SERCA1a\textsuperscript{+/+} hearts demonstrated greatly improved postischemic (45 min) contractile recovery with less persistent arrhythmias compared with NTG hearts. Morphometry showed better-preserved myocardial structure with less infarction, and electron microscopy demonstrated better-preserved myofibrillar and mitochondrial ultrastructure in SERCA1a\textsuperscript{+/+} hearts. Importantly, intrasarclemic Ca\textsuperscript{2+} levels were significantly lower in SERCA1a\textsuperscript{+/+} hearts. The cardioprotective effect of SERCA1a was also observed during in vivo regional I/R with reduced myocardial infarct size after 24 h of reperfusion. Thus SERCA1a\textsuperscript{+/+} hearts were markedly protected against I/R injury, suggesting that expression of SERCA 1a isoform reduces postischemic Ca\textsuperscript{2+} overload and thus provides potent myocardial protection.

The sarco(endo)plasmic reticulum (SR) Ca\textsuperscript{2+}-uptake activity determines not only the speed of Ca\textsuperscript{2+} removal for relaxation but also the SR Ca\textsuperscript{2+} content and, therefore, the amount of Ca\textsuperscript{2+} released for subsequent contraction (30). The rate of active muscle relaxation is determined largely by the Ca\textsuperscript{2+} uptake function of SERCA2a (35). Acute myocardial ischemia-reperfusion (I/R) injury is associated with contractile dysfunction, arrhythmias, myocardial infarction, and sudden death (5). Although the causes of myocardial I/R injury are multifactorial, it is well known that intracellular Ca\textsuperscript{2+} overload with reduced expression and/or activity of SERCA2a plays a prominent role (32, 37, 42). High levels of oxygen-derived free radicals are generated during myocardial I/R and have been shown to damage SERCA2a, potentially contributing to cellular Ca\textsuperscript{2+} overload and myocardial injury (16, 22, 49). Thus cytotoxic free Ca\textsuperscript{2+} overload and oxidative stress, either independently or cooperatively, are major contributors to I/R-induced injury.

SERCA2a is the cardiac-specific isoform and is the major component of beat-to-beat Ca\textsuperscript{2+} cycling during excitation-contraction coupling (39). Recently, it has been reported that SERCA1a, the isoform normally expressed in fast skeletal muscle but not the heart (35), is more resistant to oxidative stress (43) and acidosis (46). SERCA1a and SERCA2a possess −84% sequence homology, and transgenic expression of SERCA1a in the mouse heart has been shown to substitute for SERCA2a both structurally and functionally with concomitant increases in SR Ca\textsuperscript{2+} uptake and cardiac contractility (19, 23, 26). Confocal microscopy demonstrated specific trafficking of SERCA1a to cardiac SR and an increased rate of Ca\textsuperscript{2+} removal from cytosol with increased intracellular Ca\textsuperscript{2+} transients in cardiomyocytes of SERCA1a-overexpressed mice (23). The striking observation is that with its unique antigenicity and equivalent upregulation, SERCA1a has twofold greater velocity of Ca\textsuperscript{2+} transport than SERCA2a (6, 40). Chronic expression of SERCA1a in vivo did not result in significant morphological differences in the hearts, with similar mortality curves seen (23).

Several studies have consistently demonstrated that increased expression of SERCA2a improves myocardial contractility and Ca\textsuperscript{2+} handling at baseline and in disease conditions including myocardial I/R (3, 7, 9, 15, 31, 33). Conversely, several investigators have controversially reported that phar-
macological inhibition of SERCA may improve postischemic function in stunned heart or in isolated myocardium following I/R (2, 12). The results with SERCA inhibitors, thapsigargin or cyclopiazonic acid, were inconsistent in terms of effectiveness, narrow dose range, and timing of administration (48). Interestingly, enhanced SR function in mice overexpressing SERCA1a recently has been shown to partially rescue the heart from hydroxyl radical-induced injury (17).

Despite its unique antigenicity and increased Ca\(^{2+}\) pumping kinetics (6, 40), it is not known whether SERCA1a overexpression can provide myocardial protection against I/R as has been shown with SERCA2a overexpression (7, 9). Therefore, to further address this issue, the aim of the present study was to test the hypothesis that expression of SERCA1a in the heart will provide cardioprotection characterized by improved postischemic contractile function, reduced intracellular Ca\(^{2+}\) overload, fewer ventricular arrhythmias, and smaller myocardial infarction.

**METHODS**

This study was reviewed and approved by the Institutional Laboratory Animal Care and Use Committee at The Ohio State University, carried out according to the approved guidelines, and conforms with the Guidelines for the Care and Use of Laboratory Animals published by the National Institutes of Health [DHHS Publication (NIH) No. 85-23, Revised 1996].

**Mice.** Details regarding the generation and characterization of SERCA1a transgenic (SERCA1a\(^{+/+}\)) mice have been described previously (26). Briefly, rat SERCA1a CDNA was linked to the mouse cardiac α-MHC promoter, and SERCA1a\(^{+/+}\) mice were generated. They were bred with FVB/N wild-type mice to establish germ line transmission. Each mouse was genotyped when it was weaned, and only respective littermates served as controls. The experiments were performed in young (16–20 wk) male mice, and tail clips were kept to reconfirm the genotypes.

**Western blot analysis.** Tail genotyping (PCR) and SERCA1a and SERCA2a expressions in cardiac homogenates were performed as described previously (26). Hearts from age-matched mice were homogenized in the lysis buffer, and protein concentrations were determined by Bio-Rad protein assay. The relative expression of SERCA2a and SERCA1a proteins in SERCA1a transgenic (SERCA1a\(^{+/+}\)) vs. nontransgenic (NTG) mice were determined by immunoblotting. Equal amounts (10 μg) of protein extract were separated by SDS-PAGE on polyacrylamide gel and then transferred to nitrocellulose membrane using a Bio-Rad transblot apparatus. Membranes were incubated with a polyclonal antibody for SERCA2a or SERCA1a (1:5,000 dilution) at room temperature for 1 h. The membranes were washed with TBS-T (20 mM Tris-HCl, 137 mM NaCl, and 0.05% Tween 20) six times (10 min each). Secondary antibodies were peroxidase-labeled anti-rabbit IgG (Kirkegaard & Perry Laboratories) at room temperature at a dilution of 1:5,000 for 45 min. After extensive washing with TBS-T, antibody signals were detected using an enhanced chemiluminescence kit (Pierce).

**Langendorff-perfused heart preparation.** Hearts were isolated from age-matched mice of both strains as described previously (41, 44). Briefly, mice were anesthetized with pentobarbital (50 mg/kg ip) and hearts were excised, aortas were cannulated, and hearts were perfused in a Langendorff mode at a constant pressure of 80 mmHg with a modified Krebs-Henseleit buffer (KHB) equilibrated with 95% O\(_2\)-5% CO\(_2\) at 37°C. The constituents of KHB were (in mM) 120 NaCl, 5.9 KCl, 25 NaHCO\(_3\), 1.2 MgCl\(_2\), 2.5 CaCl\(_2\), 0.5 EDTA, and 16.7 glucose. A fluid-filled balloon was inserted into the left ventricle (LV) across the mitral valve and connected to a pressure transducer permitting continuous measurement of pressure (LVP). Hearts were immersed in a water-jacketed bath maintained at 37°C, and the LV balloon was filled with water to yield a LV diastolic pressure of 3–6 mmHg. Coronary flow was continuously monitored via a Doppler flow probe (T206; Transonic Systems, Ithaca, NY) placed in the aortic perfusion line. Aortic pressure and LV developed pressure (LVPD) were recorded on a PowerLab/400 multichannel data acquisition system (ADInstruments; Castle Hill, Australia). The LVP signal was digitally processed (using PowerLab Chart software version 4.2; ADInstruments) to yield diastolic and systolic pressures as well as heart rate. Hearts having unexpected arrhythmias during equilibration were excluded from the study. There was no preischemic arrhythmia with SERCA1a\(^{+/+}\) hearts; however, 2 of 26 NTG hearts developed preischemic arrhythmias and were excluded from the study.

Following 30 min of equilibration, hearts underwent 30 min of global ischemia, followed by 45 min of reperfusion. At the end of reperfusion, hearts were processed for myocardial infarct size measurement, histopathological examination, Western blot analysis, and electron microscopy. One subset of hearts underwent only 5 min of reperfusion to measure free radical generation.

**Criteria used to determine arrhythmias.** Ventricular tachyarrhythmias, such as ventricular fibrillation (VF) and anterioventricular tachycardia (VT), with episodes of mechanical alternans are common occurrences with reperfusion after 30 min of global ischemia. Because these hearts were un paced, a change in heart rate reflects an important pathophysiological impact of reperfusion on these hearts. Identification of rhythm abnormalities was performed from synchronized recordings of LVP traces and heart rate; therefore, differentiation of supraventricular and ventricular arrhythmias was not possible in relation to the P wave. According to Merillat et al. (28) and Kawahara et al. (21), VF was defined as 1) the development of chaotic, irregular, rapid LVP recordings, 2) the loss of pulsatile LVP, and 3) the loss of grossly observable regular ventricular contraction. VT was defined as a rapid and regular cyclic change in LVP with smaller amplitude than control. Mechanical alternars (34) characterized by alternate large and small contractions were defined according to the previously described hallmark s such as 1) impaired and incomplete relaxation of the strong contraction, 2) higher end-diastolic pressure of the strong contraction, and 3) smaller peak systolic pressure of the weak contraction. When we recorded abnormal contractile rhythms for >30 min of reperfusion, we considered them as persistent rhythm abnormalities.

**Light microscopy.** Standard hematoxylin and eosin (HE) staining was used for morphological evaluation. Hearts were fixed in 10% neutral buffered formalin and embedded in paraffin, and serial cross sections (6 μm) were made for staining. Digital images of each slide were randomly taken for morphometric evaluation using Spot Basic Software 4.0.4 (Diagnostics International).

**Electron microscopy.** Small tissue blocks (~1 mm\(^3\)) were cut from the LV free wall and fixed in 2.5% glutaraldehyde plus 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) overnight at 4°C. The samples were rinsed three times in 0.1 M sodium cacodylate buffer, postfixed in 1% osmium tetroxide in the same buffer for 1 h at room temperature, and stained in 1% uranyl acetate for 1 h. The samples were then dehydrated in ascending concentrations of alcohol, treated with propylene oxide, embedded in Spur resin, and heat polymerized. After polymerization, ultrathin sections (~70 nm) were cut using a Leica electron microscope UC6 ultramicrotome, mounted on uncoated copper grids, and stained in 2% uranyl acetate followed by Reynolds’s lead citrate. Samples were examined in a Philips CM12 transmission electron microscope at 80 KV.

**Electron paramagnetic resonance spectroscopy and spin trapping.** Spin-trapping measurements of oxygen radical generation from SERCA1a\(^{+/+}\) and NTG hearts were performed as described previously (44, 45). Hearts were infused with 50 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO), and effluent was sampled before ischemia and during the first 3 min of reperfusion. Relative quantitation of radical signals was performed by double integration.

**Immunohistochemistry for nitrotyrosine.** Immunohistochemistry for nitrotyrosine were performed as described previously (44, 45,
were estimated by loading the isolated beating hearts with a Ca2⁺ homogenates (100 mCi/ml) were incubated at 37°C in 1.5 ml of reaction mixture containing 40 mM imidazole, pH 7.0, 100 mM KCl, 5 mM MgCl₂, 5 mM NaCl, 5 mM potassium oxalate, 1 μM ruthenium red, and 0.5 mM EGTA to yield a free Ca2⁺ concentration in the range of 0.03 to 3 μM (containing 1 μCi/mmol ⁴⁴Ca) as determined using Calcium Titration Program computer software. The uptake reaction was started by adding 5 mM ATP to the reaction mixture. After 1 min, 300-μl samples were vacuum-filtered through Millipore (0.45 μm HAWP) nitrocellulose membrane, and the vesicles remaining on the filters were washed, dissolved, and then processed for liquid scintillation counting. The Ca²⁺ concentration required for one-half of the maximum velocity for Ca²⁺ uptake was determined by nonlinear curve fitting using GraphPad Prism 4.0 software.

Rhod-2 spectrofluorometry. Intracellular free Ca²⁺ (Ca²⁺i) changes were estimated by loading the isolated beating hearts with a Ca²⁺-sensitive fluorescence probe, rhod-2 AM (Molecular Probes, Eugene, OR), as previously described with slight modification (10, 11, 27). Rhod-2 AM is membrane permeable and becomes Ca²⁺ sensitive and trapped in the cytosol when deesterified to rhod-2 intracellularly. Because the amplitude of rhod-2 fluorescence transients depends on Ca²⁺i (11), the estimation of its value allows monitoring the Ca²⁺ changes over time.

A fiber optic probe was gently positioned against the LV wall to obtain emission signal from the heart. To suppress motion-induced artifacts and minimize the effects from the curvature of the epicardium, the distance between heart and cable surface was adjusted by monitoring tissue autofluorescence before rhod-2 loading. Rhod-2 was loaded after spontaneous cardiac contractility became regular (20 min) and no clear signs of damage were evident. Rhod-2 (50 μg) was added to 25 μl of DMSO, thoroughly mixed, and diluted up to 2 ml with perfusion buffer constituting 25 μg/ml Rhod-2 dye (2 ml) was added without recirculation through a parallel infusion line just above the aortic cannula. During bolusing, the bolus line was opened and the other line was closed. Dye loading was followed by a 10-min washout period with normal KHB to remove any extracellular dye. LVDP and heart rate were monitored during loading and the washout period. Typically, there was a 30–50% decline in LVDP during rhod-2 loading, followed by complete recovery within 5 min of dye-free perfusion. Hearts having arrhythmias during the washout period were excluded from the study.

Fluorescence was excited with a 150-W xenon arc lamp through excitation/emission filters in a modified tissue fluorometer (C&L Instruments, Hummelstown, PA), and the light was directly focused on the photomultiplier tube (PMT). To reduce light interference, the tissue chamber was housed in a solid dark metal box. The area of the light guide facing the heart was 28 mm², providing a complete observation window of the whole heart. The PMT shutters were kept closed except during data acquisition to minimize photobleaching and photooxidation of rhod-2. The PMT output was collected via personal computer using an analog-to-digital converter. The emitted signal was detected, digitized, and recorded at a rate of 125 Hz for analysis (FluorMeasure version 2.7 acquisition software). The excitation/emission parameter for rhod-2 is 531 ± 20 nm/593 ± 20 nm. The sampling time was set at 8 ms per data point per filter. After 10 min of washout (37°C), Ca²⁺ fluorescence data was acquired. The heart was then subjected to 30 min of global ischemia, and Ca²⁺ fluorescence data was collected at end ischemia just before reperfusion. Upon reperfusion, gradual loss and washout of rhod-2 occurred, precluding subsequent measurement of Ca²⁺ fluorescence.

Ca²⁺ was calculated according to previous literature (10) with rhod-2 calibration in the isolated heart, that is: Ca²⁺ = Kd(F – Fmin)/(Fmax – F), where Kd (710 nM) is the dissociation coefficient for rhod-2 and F is the fluorescence signal detected by PMT at a specific time point, Fmax is the maximal fluorescence after digitonin treatment, and Fmin is the blank fluorescence. The Fmax was acquired before rhod-2 loading, and Fmin was obtained after 15 μM digitonin (Sigma) infusion at the end of each experiment. The calculated myocardial Ca²⁺ in our experimental conditions with control FB/N mice was 370 ± 6 and 753 ± 22 nM in diastole and systole, respectively. These results agree well with those previously reported with rhod-2 (11) and aequorin (14) in the mouse heart. The diastolic or mean ischemic levels of rhod-2 fluorescence were used to evaluate Ca²⁺ changes and were expressed as relative units (RU).

In vivo myocardial I/R. In vivo myocardial I/R was performed as described previously (47). Briefly, mice were anesthetized with a mixture of intraperitoneal ketamine (55 mg/kg) and xylazine (15 mg/kg). After adequate anesthesia and aseptic preparations, mice were intubated and ventilated with room air by a MiniVent (type 845; Harvard Apparatus). The respiratory rate was maintained at 100 breaths/min with a tidal volume of 0.25 ml for a 25-g mouse. After the chest was opened and the heart visualized, the left anterior descending (LAD) coronary artery was ligated 2 mm below the tip of the left auricle with a 7-0 silk ligature. Occlusion was confirmed by the dramatic change in color (red to pallor) and restricted ventricular motion. After 30 min of LAD artery occlusion, the knot was released to start reperfusion, and reperfusion was confirmed by return of the pink-red color and motion of the anterior wall of the LV. The chest was closed in layers with subeutaneous administration of Penicillin G Procaine. Buprenorphine (0.1 mg/kg, <0.5 ml in volume) was given subcutaneously to reduce postoperative acute pain. The rectal temperature of the mouse was maintained at 37°C by a thermo heating pad. When mice resumed a normal breathing pattern and started walking, the ventilator was taken off and mice were transferred to a clean cage with free access to food and water.

Myocardial infarct size measurement. In vitro myocardial infarction was measured by 2,3,5-triphenyltetrazolium chloride (TTC; Sigma) staining of heart sections as reported previously (44). Briefly, the heart was immediately removed after I/R, wrapped in polyethylene, and frozen for 10 min for hardening. The heart was then serially sectioned into transverse slices (1 mm) with a heart slicer (Zivic laboratories) and incubated in 1% TTC in phosphate-buffered saline for 15 min at room temperature to demarcate the viable (brick red) and infarcted (pale) myocardium. Heart slices are then fixed overnight in 10% neutral buffered formaldehyde for better color contrast and digitally imaged. Computerized planimetry (with image analysis software Meta Vue, version 6.0) of each section was used to determine the percent infarction from the total cross-sectional area of the LV.

In vivo myocardial infarction was measured at 24 h postreperfusion as previously described with slight modification (47). Mice were anesthetized, intubated, and ventilated, and the chest was opened along the previous incision line and the left main coronary artery was ligated at the same location as before. Evans blue dye (0.2 ml of a 4.0% solution) was injected directly into the inferior vena cava for visualization of the nonischemic zone. The area of the myocardium not stained with Evans blue was defined as the area at risk (AAR). The heart was then rapidly excised, wrapped in polyethylene, frozen for 10 min for hardening, serially sectioned along the short axis (1 mm thick) with a heart slicer, and incubated with 1% TTC for 15 min at room temperature for demarcation of the viable and nonviable myocardium.
within the AAR. Both sides of each myocardial slice were photographed, and the area of infarction, AAR, and nonrisk area were determined by computerized planimetry as described above.

Data analysis. All results are means \pm SE. Data were analyzed using either two-tailed Student’s t-test for paired data from the same experiment and unpaired data from different experiments or ANOVA followed by Fisher’s post hoc test. Values of \( P < 0.05 \) were considered to be statistically significant.

RESULTS

Characterization of SERCA1a and SERCA2a expression. Transgenic expression of SERCA1a was confirmed by tail biopsy genotyping and Western blot analysis of cardiac homogenates. Consistent with previous data (26), PCR results demonstrated the distinct band of SERCA1a gene in the transgenic mice, whereas no band was seen in the NTG mice (Fig. 1A). Similarly, SERCA1a\(^{+/+}\) hearts demonstrated prominent SERCA1a protein expression (Fig. 1B), and this was associated with a concomitant decrease in SERCA2a (Fig. 1C). In SERCA1a\(^{+/+}\) hearts, the total amount of SERCA was markedly increased compared with NTG hearts. As demonstrated previously with quantitative immunoblotting (26), the level of SERCA1a appeared to be twice the level of SERCA2a in NTG, whereas the level of SERCA2a was decreased by \(-50\%\).

Pre- and postischemic cardiac function in perfused hearts. The functional characteristics of the isolated-perfused hearts are summarized in Table 1. There were no significant differences in body weight (32 \pm 1 g vs. 30 \pm 1 g, \( n = 8/\)group) or heart weight-to-body weight ratio (0.49 \pm 0.01 vs. 0.48 \pm 0.01\%, \( n = 8/\)group) between SERCA1a\(^{+/+}\) and NTG mice (Table 1). The intrinsic heart rates were identical; however, baseline coronary flow, LVDP, and maximal rates of contraction (+dP/dt) and relaxation (−dP/dt) were significantly higher in SERCA1a\(^{+/+}\) than in NTG hearts.

Figure 2, A and B, shows the time course of the recovery of LVDP and LVEDP in SERCA1a\(^{+/+}\) and NTG hearts subjected to 30 min of global ischemia and 45 min of reperfusion. Upon reperfusion, improved recovery of postischemic LV function was seen in SERCA1a\(^{+/+}\) hearts with significantly higher LVDP (Fig. 2A) and greater recovery in both +dP/dt and −dP/dt, maximal rates of contraction and relaxation, respectively; LVEDP, LV end-diastolic pressure; PI, preischemia. Beating time was determined as the time to initiation of functional contraction. \(* P < 0.01; † P < 0.001 vs. NTG. ‡ P < 0.001 vs. ischemia."

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Table 1. Hemodynamic parameters before and after in vitro myocardial I/R

Fig. 1. Characterization of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) gene and protein expression in nontransgenic (NTG) and SERCA1a transgenic (SERCA1a\(^{+/+}\)) mice. A: PCR analysis with tail sample from offspring of transgenic breeders. Genomic DNA with SERCA1a primer shows a distinct band of SERCA1a gene in the transgenic mice but not in the NTG mice. B and C: immunoblots for SERCA1a (B) and SERCA2a protein levels (C) in cardiac homogenates.
structurally normal (Fig. 3, A and B). After I/R, frequent interstitial edema, nuclear vacuolation, and contraction band-type changes (arrow) were evident in NTG hearts as early as the first minute of reperfusion (Fig. 3C) and persisted for 45 min (Fig. 3E), but only modest changes were seen in SERCA1a+/− hearts (Fig. 3, D and F).

Myocardial ultrastructure before and after in vitro I/R. To further correlate myocardial infarction and histology findings with myocardial ultrastructure, we performed electron microscopic examination. Concordant with light microscopy, no qualitative differences in myocardial ultrastructure were seen between nonischemic SERCA1a+/− and NTG hearts (Fig. 4, A and B). Orderly distributed myofibrils with normal sarcomere and dark mitochondria containing tightly arranged cristae were seen in both strains. However, after I/R, NTG hearts showed markedly severe ultrastructural damage (Fig. 4C) with thinning, discontinuation, or lysis of myofibrils associated with intracellular edema and misregistration of Z bands, whereas little change was seen in SERCA1a+/− hearts (Fig. 4D). Most of the mitochondria in NTG hearts were swollen and larger in size with abnormal cristae and loss of matrix, whereas no abnormalities were seen in SERCA1a+/− hearts.

Electron paramagnetic resonance measurements of free radical generation. To determine the magnitude of free radical generation in postischemic hearts, we performed electron paramagnetic resonance (EPR) and spin-trapping studies using the spin trap DMPO. Before ischemia, no radical generation was seen in either strain (Fig. 5, A and B, top tracings). However, over the first minute of reperfusion, both SERCA1a+/− and NTG hearts exhibited radical signals consisting of a 1:2:2:1 quartet of DMPO-OH. The reperfusion-associated increase in radical generation was not different between the NTG and SERCA1a+/− hearts (Fig. 5, A and B, bottom tracings).

Immunohistochemistry for nitrotyrosine. Immunohistochemical staining of nitrotyrosine, a marker of peroxynitrite formation (32, 33) was performed in nonischemic and postischemic hearts. In positive controls with peroxynitrite infusion, strong dense red nitrotyrosine staining of the vascular bed and weaker staining within the myocytes was seen throughout the heart (Fig. 6A). There was no staining in the negative control (Fig. 6B). In nonischemic myocardium, there was no positive staining for nitrotyrosine (Fig. 6, C and D). In postischemic myocardium, focal positive nitrotyrosine staining was detectable upon reperfusion within the myocytes of both SERCA1a+/− and NTG hearts at 1 min of reperfusion (Fig. 6, E and F). The intensity of nitrotyrosine staining in reperfused myocardium was similar in both strains.

Rhod-2 spectrofluorometry for myocardial Ca2+ and SR Ca2+ uptake activity. To study Ca2+ in the isolated beating mouse heart, we measured rhod-2 Ca2+ fluorescence before (preischemia) and after 30 min of ischemia. Figure 7 shows representative rhod-2 Ca2+ fluorescence tracings in both NTG (A) and SERCA1a+/− hearts (B) during preischemia and after ischemia. Figure 7C shows average systolic and diastolic rhod-2 Ca2+ fluorescence tracings after 10 min of rhod-2 loading and washout. Preischemic peak systolic rhod-2 Ca2+ fluorescence was significantly higher (954 ± 28 vs. 753 ± 40 RU, P < 0.05, n = 5), whereas diastolic Ca2+ fluorescence was significantly lower (295 ± 11 vs. 376 ± 4 RU, P < 0.05, n = 5) in the SERCA1a+/− hearts compared with NTG hearts (Fig. 7C). Although a sharp rise and rapid fall of amplitude for rhod-2 Ca2+ fluorescence transients were seen in both strains before ischemia, with the onset of ischemia the amplitude of these transients decreased as diastolic values increased, with only minimal transients seen after 30 min of global ischemia. After 30 min of ischemia, the Ca2+ fluorescence values were...
markedly elevated in both strains with decreased or no rhythmic amplitude, and mean Ca\textsuperscript{2+} fluorescence (Fig. 7D) was significantly less in the SERCA1a\textsuperscript{+/+} hearts compared with NTG hearts (1,147 ± 45 vs. 1,528 ± 93 RU, P < 0.05, n = 5).

The ATP-dependent Ca\textsuperscript{2+} uptake was determined in NTG (Fig. 7E) and SERCA1a\textsuperscript{+/+} (Fig. 7F) cardiac homogenates from nonischemic (30-min equilibration) hearts and ischemic (30-min global ischemia) hearts. The rate of SR Ca\textsuperscript{2+} uptake in SERCA1a\textsuperscript{+/+} hearts was increased significantly over a wide range of free Ca\textsuperscript{2+} concentration (pCa 7.5 to 5.5) at both preischemia and ischemia. The maximal velocity of SR Ca\textsuperscript{2+} uptake was less affected by ischemia in SERCA1a\textsuperscript{+/+} hearts (181 to 28 nmol·mg\textsuperscript{-1}·min\textsuperscript{-1}, or ~13%) than that observed in NTG hearts (40 to 28 nmol·mg\textsuperscript{-1}·min\textsuperscript{-1}, or ~29%).

Myocardial infarction after in vivo I/R. Myocardial infarction (Fig. 8, A–C) was assessed 24 h after 30 min of LAD ligation. Under anesthesia, both the NTG and SERCA1a\textsuperscript{+/+} mice had blood pressure between 70 and 75 mmHg and heart rate between 335 and 400 beats/min, as reported previously (18). Although NTG and SERCA1a\textsuperscript{+/+} hearts exhibited similar values (~65% in both strains) for AAR/LV (Fig. 8B), much smaller infarct size was observed in the SERCA1a\textsuperscript{+/+} hearts with little discernable infarction (Fig. 8, A and C). The calculated infarct area per AAR was 12 ± 4.5% in NTG hearts and 0.6 ± 0.4% in SERCA1a\textsuperscript{+/+} hearts (P < 0.05). Of note, the FVB/N strain has been shown to be relatively resistant to in vivo postischemic injury with smaller infarction seen than in other mouse strains, such as C57BL/6 (29).

DISCUSSION

The major goal of this study was to determine whether cardiac expression of SERCA1a can protect the heart against I/R injury and the mechanism of this protection. We observed that SERCA1a\textsuperscript{+/+} expression resulted in 1) accelerated and markedly improved postischemic myocardial contractile function, 2) diminished incidence of abnormal ventricular rhythms upon reperfusion, 3) significantly smaller myocardial infarct size, and 4) significantly reduced accumulation of intracellular Ca\textsuperscript{2+} in the postischemic hearts compared with NTG mice. Concordant with these in vitro findings, we also observed much less myocardial infarction in SERCA1a\textsuperscript{+/+} mice compared with NTG mice subjected to in vivo regional I/R. Thus these results verify the hypothesis that upregulation of SERCA function is beneficial for postischemic myocardial recovery and salvage, and they suggest that expression of SERCA1a could substitute for SERCA2a under conditions where SERCA2a expression and/or activity are reduced. The mech-
anism of protection was shown to be primarily due to enhanced Ca²⁺ handling with prevention of posts ischemic cytosolic Ca²⁺ overload.

**Beneficial role of SERCA upregulation in cardiac protection.** Whereas increased SERCA2a expression improves Ca²⁺ cycling and cardiac function in different experimental conditions (3, 15, 31, 33), the use of SERCA1a to improve cardiac contractility has been only recently attempted (8). This is the first study to investigate the efficacy of cardiac SERCA1a overexpression in protecting the heart against I/R injury both in vitro and in vivo. We chose to study the effect of SERCA1a, since it has been shown to have higher Ca²⁺ transport velocity than SERCA2a and is associated with faster rates of contraction and relaxation in adult cardiomyocytes compared with equivalent SERCA2a overexpression (3, 6, 26, 40). Importantly, diastolic intracellular Ca²⁺ is decreased with SERCA1a expression (8) compared with SERCA2a overexpression (31).

Consistent with previous reports on various myocardial preparations (17–19, 23, 26), we also observed markedly enhanced baseline contractile function (LVDP) with significantly faster rates of contraction and relaxation in adult cardiomyocytes compared with equivalent SERCA2a overexpression (3, 6, 26, 40). Importantly, diastolic intracellular Ca²⁺ is decreased with SERCA1a expression (8) compared with SERCA2a overexpression (31).

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**Fig. 4.** Representative electron micrographs of nonischemic and postischemic hearts of two separate experiments. In nonischemic hearts, normal myocardial ultrastructure is shown in both NTG (A) and SERCA1a⁺/⁻ mice (B). After 30 min of ischemia and 45 min of reperfusion, grossly distorted structures of myofibrils and mitochondria are noted in NTG (C) but not SERCA1a⁺/⁻ hearts (D). Bar, 1 μm; magnification, ×12,500.

**Fig. 5.** Electron paramagnetic resonance (EPR) spin trapping measurement of free radical generation in nonischemic and postischemic hearts. Hearts were infused with 50 mM DMPO before ischemia and upon the first 45 s of reperfusion following 30 min of global ischemia. A: a positive control heart infused with 1 mM peroxynitrite. B: a negative control of the same slice without anti-nitrotyrosine primary antibody. There is an absence of positive red staining in the nonischemic NTG (C) and SERCA1a⁺/⁻ hearts (D); however, focal positive red staining (arrow) is shown after 1 min of reperfusion in both NTG (E) and SERCA1a⁺/⁻ hearts (F). Magnification, ×400; n = 3/group.

**Fig. 6.** Representative immunostaining for nitrotyrosine product in isolated nonischemic and postischemic heart slices. Hearts were obtained before ischemia (nonischemia) and at the first minute of reperfusion following 30 min of global ischemia. A: a positive control heart infused with 1 mM peroxynitrite. B: a negative control of the same slice without anti-nitrotyrosine primary antibody. There is an absence of positive red staining in the nonischemic NTG (C) and SERCA1a⁺/⁻ hearts (D); however, focal positive red staining (arrow) is shown after 1 min of reperfusion in both NTG (E) and SERCA1a⁺/⁻ hearts (F). Magnification, ×400; n = 3/group.
in favor of more free SERCA1a pump, and this in addition to the faster kinetics of SERCA1 likely explains the enhanced baseline contractile function in SERCA1a
hearts.

Myocardial I/R injury is associated with severe arrhythmias, contractile dysfunction, and myocardial death (5, 32, 42); therefore, reduction of arrhythmias and limitation of myocardial infarction are of paramount importance. With reperfusion following 30 min of global ischemia, we observed that SERCA1a
hearts started to beat much earlier with diminished incidence of arrhythmias and postischemic contracture compared with NTG hearts (Table 1 and Fig. 2B). This improved postischemic contractile recovery in SERCA1a
hearts was associated with a 2.5-fold increase in the recovery of LVDP (Fig. 2A) and an ~2-fold decrease in myocardial infarct size compared with NTG hearts (Fig. 2E). Concordantly, LVEDP after 45 min of reperfusion was ~2-fold lower in SERCA1a
hearts (Fig. 2B), and both maximal +dP/dt and −dP/dt were 8- and 6-fold higher respectively in SERCA1a
hearts (Table 1). The decreased infarct size and improved contractile recovery in SERCA1a
hearts are due to greater preservation of myocardial integrity with less myocardial structural damage seen by light and electron microscopy (Figs. 3 and 4). These striking differences between SERCA1a
and NTG hearts during reperfusion clearly show that SERCA1a expression imparts prominent myocardial protection despite the 50% reduction in constitutive SERCA2a.

Consistent with the isolated heart data, our studies in an in vivo regional model of I/R also showed markedly smaller infarct size in SERCA1a
hearts than in NTG hearts, demonstrating that expression of SERCA1a protects against myocardial injury in the complex in vivo situation. Thus these findings further extend our understanding that posts ischemic

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Fig. 7. Rhod-2 spectrofluorometry of intracellular free Ca2+
(Ca2+) in relative units (RU), and sarcoplasmic reticulum (SR) Ca2+
uptake activity during preischemia and ischemia. A and B: representative rhod-2 Ca2+
fluorescence signals for NTG and SERCA1a
hearts, respectively. C: averaged peak and diastolic rhod-2 Ca2+
signals in NTG and SERCA1a
hearts before ischemia. D: bar graphs show average diastolic rhod-2 Ca2+
levels during preischemia and mean rhod-2 Ca2+
levels at 30 min of ischemia. *P < 0.05 vs. NTG; n = 5/group. E and F: ATP-dependent SR Ca2+
uptake activity in NTG and SERCA1a
hearts, respectively. The rate of SR Ca2+
uptake increased significantly over a wide range of free Ca2+
concentration (pCa 7.5 to 5.5) at both preischemia and ischemia. The average of 3 separate experiments, each performed in duplicate, is shown. IS, ischemia; PI, preischemia.
myocardial recovery is closely related to the functional levels of SERCA and that preservation or augmentation of SR Ca\(^{2+}\) uptake activity can profoundly protect the heart and prevent contractile dysfunction and myocyte death.

Together, our findings with SERCA1a expression are consistent with earlier studies showing that overexpression of SERCA2a confers myocardial protection with enhanced contractile function (9). The observed cardioprotective effect of SERCA1a is not model specific, because prominent reduction of infarct size occurred in both in vitro and in vivo models.

**SERCA overexpression and myocardial Ca\(^{2+}\) handling.** Intracellular Ca\(^{2+}\) overload is one of the major mechanisms of myocardial I/R injury (32, 37, 42). Cardiac contracture observed with ischemia and reperfusion is mainly due to a rise in intracellular Ca\(^{2+}\) levels (1, 32). It has been reported that SERCA1a\(^{+/+}\) hearts can tolerate supraphysiological levels of calcium without any signs of Ca\(^{2+}\) overload or failure (18).

![Fig. 8. Measurement of infarct size in hearts subjected to in vivo regional myocardial ischemia and reperfusion. Evans blue infusion was performed to visualize the nonrisk region and TTC staining to visualize infarction (A). Percentages of LV area at risk (AAR/LV) and myocardial infarct size over the area at risk (IA/AAR) are shown in B and C, respectively. Values are means ± SE. *P < 0.05 vs. NTG; n = 5/group.](image)

Importantly, LVEDP rapidly decreased upon reperfusion in SERCA1a\(^{+/+}\) hearts, whereas a further rise was seen in NTG hearts (Fig. 2B). This salutary effect is likely due to increased Ca\(^{2+}\) uptake activity in SERCA1a\(^{+/+}\) hearts, because we noted significantly lower both preischemic diastolic and mean ischemic (30 min) rhod-2 Ca\(^{2+}\) fluorescence in SERCA1a\(^{+/+}\) hearts compared with NTG hearts (Fig. 7D). Thus our results directly show for the first time that the overexpression of SERCA improves intracellular Ca\(^{2+}\) handling during acute myocardial ischemia.

In this context, we observed that the rate of SR Ca\(^{2+}\) uptake in SERCA1a\(^{+/+}\) hearts was much higher than in NTG hearts both at baseline and following 30 min of ischemia (Fig. 7, E and F). Consistent with prior reports that Ca\(^{2+}\)-ATPase activity is decreased following ischemia (22), we also observed that the maximal velocity of SR Ca\(^{2+}\) uptake was decreased in postischemic NTG hearts with 25% loss of activity compared with 15% loss of activity in SERCA1a\(^{+/+}\) hearts. Together, these data suggest that expression of SERCA1a is effective in preserving SR Ca\(^{2+}\) uptake during I/R, and thus in decreasing intracellular Ca\(^{2+}\) overload.

SERCA upregulation and postischemic reactive oxygen and nitrogen species. The burst of reactive oxygen species during reperfusion is another key central mechanism of reperfusion injury. Potent oxidants and free radicals such as superoxide anion, hydroxyl radical, and peroxynitrite are formed during postischemic reperfusion and reach their highest concentrations during the first minute of reperfusion (4, 49). Oxygen-derived free radicals have been reported to damage SERCA, potentially contributing to Ca\(^{2+}\) overload with concurrent myocardial damage and ventricular arrhythmias upon reperfusion (16, 22). With EPR spin-trapping studies, similar DMPO-OH adduct signals, indicative of superoxide-derived hydroxyl radicals (49), were seen in both NTG and SERCA1a\(^{+/+}\) hearts over the first minute of reperfusion (Fig. 5), and the observed levels of radical generation were indis-
tistinguishable between the two strains. Importantly, despite comparable radical generation, SERCA1a+/− hearts exhibited enhanced recovery of postischemic contractile function and smaller infarct size. Consistent with our findings, a recent study has shown that SERCA1a expression can protect the heart from hydroxyl radical-induced injury (17). Thus it is evident that the postischemic functional recovery in SERCA1a+/− hearts would be less affected by oxygen radical generation during reperfusion.

It has been shown that the burst of superoxide during the early period of reperfusion reacts with nitric oxide to form the reactive nitrogen species peroxynitrite in the myocardium, subsequently aggravating reperfusion injury (4, 24, 45, 49, 50). Interestingly, it has been reported that exogenous peroxynitrite-induced nitration of SERCA2a is associated with a parallel loss of SERCA2a activity in skeletal muscle, whereas SERCA1a did not become nitrated (43). Recently, it has been reported that increased nitration of SERCA2a protein causes impaired cardiac relaxation in heart failure patients (25). Although we observed a similar degree of nitrosylation staining in the postischemic hearts of both strains (Fig. 6, E and F), SERCA1a+/− hearts displayed improved postischemic contractile recovery with significantly lower LVEDP. Thus the enhanced ability to pump back Ca2+ into the SR and the resultant lower cytosolic Ca2+ levels do not affect the magnitude of free radical generation or peroxynitrite-mediated nitration.

Implications of SERCA modulation in cardioprotection. Since defective SR Ca2+ handling plays a major role in myocardial IR-induced contractile dysfunction and arrhythmias (32, 37, 42, 48), the SR has recently been proposed as a critical primary target for reperfusion protection (36). Although currently available drugs do not improve SR Ca2+ uptake, in both transgenic mice and adenovirus-mediated cardiac myocytes, overexpression of SERCA2a or SERCA1a resulted in increased Ca2+ uptake activity, Ca2+ transient amplitude and SR Ca2+ content with increased contractility (3, 8, 13, 26, 32, 40). In this study, we have demonstrated that expression of the higher-velocity SERCA1a isoform not only can substitute for SERCA2a with enhanced SR Ca2+ uptake activity (19, 23, 25) but also strongly protects the heart against IR injury with efficient Ca2+ removal capacity under oxidative stress. On the basis of our overall findings, we can understand the mechanisms by which SERCA overexpression enhances myocardial contractility under normal physiological conditions and how it is effective in preserving contractile function following IR (Fig. 9). In conclusion, the present study provides clear direct evidence that cardiac expression of SERCA1a, independent of the effect of oxidant stress that accompanies reperfusion, confers potent myocardial protection against IR injury by reducing intracellular Ca2+ overload.

REFERENCES


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