Doxyccycline inducible expression of SERCA2a improves calcium handling and reverts cardiac dysfunction in pressure overload-induced cardiac hypertrophy

Jorge Suarez, Bernd Gloss, Darrell D. Belke, Ying Hu, Brian Scott, Thomas Dieterle, Yun-Kyung Kim, Maria L. Valencik, John A. McDonald, and Wolfgang H. Dillmann. Doxyccycline-inducible expression of SERCA2a improves calcium handling and reverts cardiac dysfunction in pressure overload-induced cardiac hypertrophy. Am J Physiol Heart Circ Physiol 287: H2164–H2172, 2004. First published July 15, 2004; doi:10.1152/ajpheart.00428.2004.—Delayed cardiac relaxation in failing hearts has been attributed to reduced activity and/or expression of sarco(endo)plasmic reticulum Ca2+-ATPase 2a (SERCA2a). Although constitutive overexpression of SERCA2a has proven effective in preventing cardiac dysfunction, it is unclear whether increasing SERCA2a expression in hearts with preexisting hypertrophy will be therapeutic. To test this hypothesis, we generated a binary transgenic (BTG) system that allows tetracycline-inducible, cardiac-specific SERCA2a expression. In this system (tet-on SERCA2a), a FLAG-tagged SERCA2a transgene is expressed in the presence of doxycycline (Dox) but not in the absence of Dox (2.3-fold more mRNA, 45% more SERCA2a protein). Calcium transients measured in isolated cardiac myocytes from non-banded Dox-treated BTG mice showed an accelerated calcium decline and an increased systolic Ca2+ peak. Sarco(endo)plasmic reticulum (SR) calcium loading was increased by 45% in BTG mice. In the presence of pressure overload (aortic banding), echocardiographic analysis revealed that expression of SERCA2a-FLAG caused an improvement in fractional shortening. SERCA2a-FLAG expression alleviated the resultant cardiac dysfunction. This was illustrated by an increase in the rate of decline of the calcium transient. Cell shortening and SR calcium loading were also improved in cardiac myocytes isolated from banded BTG mice after SERCA2a overexpression. In conclusion, we generated a novel transgenic mouse that conditionally overexpresses SERCA2a. This model is suitable for both long- and short-term studies of the effects of controlled SERCA2a expression on cardiac function. In addition, inducible overexpression of SERCA2a improved cardiac function and calcium handling in mice with established contractile dysfunction.

ABNORMAL CALCIUM HANDLING contributes to the contractile dysfunction observed in heart failure. Both contraction and relaxation are altered in cardiac myocytes from humans and animal models of heart failure (6, 7). Contraction and relaxation in cardiac myocytes is regulated by intracellular calcium concentration ([Ca2+]i). The major contribution to diastolic calcium lowering is made by sarco(endo)plasmic reticulum Ca2+-ATPase (SERCA2a), which pumps cytosolic calcium into the lumen of the sarcoplasmic reticulum (SR). It was demonstrated that steady-state levels of SERCA2a mRNA are reduced in human heart failure due to dilated cardiomyopathy (1, 13, 15, 22, 24, 27) and in animal models with ischemia or pressure overload-induced heart failure (3). Most functional studies indicate decreased SERCA2a activity during heart failure. However, a concomitant decrease in SERCA2a protein levels is controversial (1, 9, 10). Previous efforts focused on increasing SERCA2a expression to alleviate contractile dysfunction in animal models of heart failure. They demonstrated that constitutive SERCA2a overexpression (i.e., elevated SERCA2a levels) before pressure overload-induced heart failure prevented pressure overload-induced contractile and calcium handling abnormalities in both mice (12) and rats (19). Furthermore, cardiac-specific overexpression of a high-Ca2+-affinity mutant of SERCA2a attenuated development of pressure overload-induced cardiac hypertrophy (20). These studies show that “prophylactic” SERCA2a overexpression in a transgenic model is able to preserve cardiac function and myocyte contractile performance during pressure overload-induced heart failure. It remains unclear whether diminished contractile function and abnormalities in calcium handling of cardiac myocytes can be restored by inducible expression of SERCA2a. Although it has been reported that adenoviral gene transfer of SERCA2a improved left ventricular (LV) function in aortic-banded rats transitioning to heart failure (19), no data are available regarding calcium handling or contractility at the individual cardiac myocyte level. The lack of information at the cellular level with adenoviral gene transfer in vivo is due to technical difficulties in achieving homogenous adenoviral infection. In addition, adenoviral administration can lead to an inflammatory process that can jeopardize the specific effect of upregulated SERCA2a. This study tested the hypothesis that conditional overexpression of SERCA2a can restore normal cardiac function, calcium handling, and contractility in cardiac myocytes with established pressure overload-induced cardiac dysfunction. For this purpose, we developed an inducible double transgenic mouse model with cardiac-specific overexpression of SERCA2a upon administration of tetracycline (tet-on SERCA2a) or doxyccycline (Dox). We demonstrated

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that, in vivo, inducible SERCA2a expression restores calcium handling abnormalities and contractility of pressure-overloaded cardiac myocytes and improves global contractile function.

MATERIALS AND METHODS

Generation of transgenic mice. The generation of binary transgenic (BTG) mice is illustrated in Fig. 1A. Transgenic mice expressing a codon-optimized reverse tetracycline transactivator driven by the cardiac specific α-myosin heavy chain promoter (MHC-rtTA) have been previously described (29). The SERCA2a transgene was created by cloning the tet-transcription response element (Tet-RE) upstream of the rat SERCA2a transgene described previously (11) containing the first and second introns and a COOH-terminal FLAG tag. This construct was injected into fertilized mouse eggs to generate Tet-RE-SERCA2a lines. These two lines were crossed to generate binary transgenic mice (rTA-SERCA2a). To determine transgene integration, genomic DNA was extracted from tails of 3-wk-old mice and subjected to Southern blot analysis. Animals carrying both transgenes (MHC-rtTA and Tet-RE-SERCA2a) were used for the experiments. To induce the expression of SERCA2a-FLAG, animals received Dox in their drinking water (200 mg/l) for 7 days. Animals (mice) were handled according to the animal welfare regulations of the University of California-San Diego, which are in accordance with National Institutes of Health guidelines for human treatment of laboratory animals, and the experimental protocol was approved by the Animal Subjects Committee of this institution.

RNase protection and RT-PCR analysis. Hearts from wild-type (WT) animals and transgenic mice were dissected after deep anesthesia, and the ventricle was frozen in liquid nitrogen and stored at −80°C until extraction of RNA. Total RNA from ventricular tissues was prepared using the RNaseasy Kit from Qiagen. Contaminating DNA was removed by DNase I digestion of the RNA preparation when still bound to the column. Hybridization conditions for RNase protection, generation of radiolabeled antisense RNA transcripts, and processing of the RNA/RNA hybrids were previously described by our group (5). Both the SERCA2a probe and a control transcript (GAPDH) were analyzed in one hybridization reaction. The protected fragments were separated on 6% acrylamide sequencing gels. Densitometry of the autoradiographs yielded digital values for both the endogenous and transgenic protected fragments, which were corrected for their UTP content. Data from at least three independent experiments were statistically evaluated to determine the relative expression levels for the endogenous and transgenic SERCA2a mRNAs. The probe for SERCA2a was generated by PCR with the transgenic SERCA2a-FLAG construct as a template. The transgenic SERCA2a mRNA protected a 15-bp longer stretch of the radiolabeled cRNA probe compared with endogenous SERCA2a mRNA. The oligonucleotides for the probe had the sequence 5′-TCAGAATTCTACTTTTGATCTTCCTAGC-3′ for the upstream sense oligonucleotide and 5′-ATAAGATCCCTTGATGTCCTCGAGCTATGC-3′ for the downstream antisense oligonucleotide. The sense oligonucleotide matched the rat SERCA2a cDNA sequence (GenBank accession no. NM_017290) from position 3329, whereas the downstream oligonucleotide was part of the FLAG tag. The PCR fragment was cleaved with BamHI and EcoRI and cloned into pBKS II–. The radiolabeled cRNA was generated off the linearized pBKS II– clone using T7 RNA polymerase. RT-PCR was performed using the one step RT-PCR kit (Novagen) and the protocol recommended by the manufacturer. The primer for SERCA2a-FLAG was the same as described above for RNase analysis.

Echocardiography. Transthoracic echocardiography was performed as previously described (28). For acquisition of in vivo cardiac function, mice were anesthetized with isoflurane, intubated with a tracheal cannula, and placed in a prone position on an ultrasonic imaging table. Echocardiograms were obtained using a 15-MHz mechanical transducer. Pulsed-wave Doppler interrogation of the mitral valve was performed with higher gain settings to detect reverse flow. M-mode and two-dimensional images were obtained from the parasternal long-axis, short-axis, and apical views. Images were acquired at 50 frames/s and analyzed offline. The LV ejection fraction was calculated as

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\frac{V_{\text{LV}} - V_{\text{LV, end systole}}}{V_{\text{LV}}}
\]

where \(V_{\text{LV}}\) is the LV volume at end-diastole and \(V_{\text{LV, end systole}}\) is the LV volume at end-systole.

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Fig. 1. Characterization of the inducible sarcotendoplasmic reticulum Ca2+-ATPase (SERCA2a) transgenic mouse model. A: illustration of the transgenic constructs used in this study. Line 1 indicates the codon-optimized reverse tetracycline transactivator (rtTA) driven by α-myosin heavy chain promoter (α-MHC). Line 2 shows the FLAG-tagged SERCA2a containing the first and second introns and driven by the tetracycline response element (TRE) and the human cytomegalovirus promoter (PhCMV). B: Western blot determining expression of SERCA2a-FLAG in cardiac tissue of wild-type (WT) and binary rTA-SERCA2a transgenic (BTG) mice being placed on or off doxycycline (Dox). Only BTG mice that received Dox expressed SERCA2a-FLAG. C: Western blot determining expression of SERCA2a-FLAG by different mouse tissues after 7 days of treatment with Dox. Expression was cardiac specific.
functional data, we used an Apoee CX (ATL Interspec) echocardiography system. For image acquisition, mice were anesthetized with 2.5% Avertin (10 μg/body wt). The mice were placed in the left lateral decubitus position, and the transducer was placed on the left hemithorax. Care was taken not to apply excessive pressure on the chest to avoid bradycardia. The two-dimensional parasternal short-axis view was used as a guide, and a LV M-mode tracing was obtained close to the papillary muscle level with a sweep speed of 100 mm/s. Pulsed Doppler tracings of the estimated LV outflow tract velocity were obtained in a modified parasternal long-axis view at a sweep speed of 100 mm/s. M-mode and Doppler tracings were recorded on a videotape for offline analysis on an Agilent Sonos 5500 system. After calibration of this system, LV end-diastolic and end-systolic internal diameters (LVEDD and LVESD, respectively) were measured in three consecutive heart cycles using the American Society of Echocardiography leading-edge method (21). LV fractional shortening (FS) was calculated as FS (%) = (LVEDD – LVESD)/LVEDD × 100. Using the mean aortic ejection time (ET) from three consecutive heart cycles obtained from the Doppler tracings of the LV outflow tract, we calculated the velocity of circumferential fiber shortening (V_{cf}) as V_{cf} (circumferences/s) = (πLVEDD – πLVESD)/(ET × πLVEDD). Being sensitive to acute changes in loading conditions, mean V_{cf} provides an approach for in vivo assessing myocardial contractility under basal conditions without acute changes in arterial pressure (14). In addition, we calculated posterior wall (PW) thickening as another parameter of contractility. It is defined as PW = (PW thickness in systole – PW thickness in diastole)/PW thickness in diastole and expressed as a percentage.

**Western blot analysis.** Ventricular tissue or isolated cardiac myocytes were homogenized with a Polytron homogenizer. Protein content was measured by the Bradford method (Bio-Rad) and adjusted for equal loading. Protein extracts from heart tissue (20 μg) were separated by a 4–12% Bis-Tris–HCl-buffered polyacrylamide gel (Invitrogen; Carlsbad, CA) and subjected to Western blotting for SERCA2a and phospholamban (PLB). Anti-FLAG antibody (FLAG-M2 Sigma) was used to identify transgenic SERCA2a expression. Total SERCA2a (transgenic SERCA2a-FLAG and endogenous SERCA2a) was assessed with an antibody targeted to the NH2-terminal (Santa Cruz Biotechnology). Endogenous SERCA2a was measured with a rabbit COOH terminal-targeted polyclonal antibody (8) that does not recognize transgenic SERCA2a-FLAG. The relative amount of endogenous SERCA2a protein was subtracted from the relative amount of total SERCA2a. The result was assumed as the transgenic SERCA2a content. It was divided by the total SERCA2a. The result was considered the proportion of SERCA2a-FLAG to total SERCA2a protein. A mouse monoclonal antibody was used for PLB (Affinity BioReagents). The blot was also probed by a mouse monoclonal α-actin antibody or a mouse porin (voltage-dependent anion channel) antibody as an internal control to ensure equivalent protein loading and protein integrity. Signals on the films were digitized on a 350-dots/in. scanner, and densities of the bands were evaluated using NIH Image.

**Generation of cardiac hypertrophy and heart failure by ascending aortic constriction.** Mice were anesthetized with intraperitoneal ketamine-xylazine. (50:2.5 mg/kg), intubated, and ventilated with room air. Aortic constriction (AC) was performed via a left thoracotomy by placing a ligature (6-0 Ethalon) securely around the ascending aorta and a blunted 26-gauge needle and then removing the needle. After banding, the thoracic cavity was closed; the mice were extubated and placed in recovery cages vented with 100% oxygen until they were fully recovered from anesthesia.

**Isolation of adult ventricular cardiomyocytes.** Calcium-tolerant adult cardiomyocytes were isolated from ventricular tissue of mice by standard enzymatic digestion. Briefly, isolated hearts were perfused using a Ca2+-free Tyrode solution containing (in mM) 126 NaCl, 4.4 KCl, 1.2 MgCl2, 0.12 NaH2PO4, 4.0 NaHCO3, 10 HEPES, 30 2,3-butanediol monoxime, 5.5 glucose, 1.8 pyruvate, and 5.0 taurine (pH 7.3) for 5 min, followed by 0.9–1.0 mg/ml collagenase (type II, Worthington) for 20 min. Hearts were transferred to tubes containing fresh collagenase for an additional 10 min in a 37°C water bath. The heart tissue was mechanically dispersed and rinsed with gradually increasing extracellular calcium to 1 mM. The cells were plated on 24 × 50-mm, no. 1 glass coverslips treated with laminin as described previously (25).

**Cell shortening measurements (edge detection).** This technique has been described previously (11). Contractile studies of myocytes were performed with a video edge motion detector (Crescent Electronics; Sandy, UT) interfaced to a standard CCTV video camera (Panasonic; Tokyo, Japan), which was attached to a Nikon Diaphot microscope (Melville, NY). A ×40 Zeiss phase two objective was used. Output from the edge detector was interfaced to the RCGC reference channel of the photon Technologies photometry system to record data. Calibration was achieved by focusing the microscope on 26-mm microspheres (Du Pont, Wilmington, DE); voltage output from the video system was linear with distance. Cells were stimulated to contract at 0.3 Hz. Edge detection records were obtained 100 points/s. Edge detection measurements were performed on myocytes at room temperature in Tyrode solution containing 2 mM CaCl2 on cells that were not loaded with indo-1.

**Indo-1 fluorescence.** Indo-1-facilitated Ca2+ transients were performed as described previously (4, 25). In brief, cells plated on the cover glass were mounted in a superfusion chamber and loaded with indo-1 (3 μM indo-1-AM) via 20-min incubations at room temperature in an atmosphere of 5% CO2–95% air. The dispersing agent, Pluronic F-127 (Calbiochem; La Jolla, CA), was also present during indo-1 loading at a final concentration of 0.02 mg/ml. Chambers were rinsed to remove excess indo-1-AM and mounted on a Nikon Diaphot epifluorescence microscope equipped with a ×40 fluor objective interfaced to a Solamere Technologies (Salt Lake City, UT) dual-emission lamp, with the excitation wavelength set to 365 nm via a filter. Fluorescence emission was split and directed to two photomultiplier tubes through 20-nm band-pass filters centered at 405 and 485 nm, respectively. In addition, an aperture mechanism allowed fluorescence to be collected from a selected portion of the field, which was always positioned over the cytoplasmic region of individual cells. Data were simultaneously collected from each emission channel at a rate of 100 Hz. Fluorescence measurements were performed by superfusing myocytes with Tyrode buffer with 2 mM CaCl2 containing 25 mM HEPES (pH 7.3) at room temperature beginning 20 min after loading with indo-1. For each cover glass, measurements were typically carried out for 10–20 s on an individual cell. During this time period, there was minimal photobleaching of indo-1. This was repeated with additional cells in other fields so that a total of up to 20–30 cells were surveyed. Indo-1 fluorescence data are reported here as ratios of fluorescence simultaneously obtained from the 405- and 485-nm channels, providing for relative comparisons of the cytoplasmic calcium concentration between experimental treatments.

**SR Ca2+ load.** Experiments were performed at room temperature as described by Shannon et al. (23). In brief, cells were superfused with normal Krebs solution and paced at 0.3 Hz for at least 20 times, and the solution was then rapidly switched to 0 Na, 0 Ca2+ normal Tyrode. After at least 30 s, 10 mM caffeine was added to cause SR calcium release. The difference between the basal and peak total systolic Ca2+ concentration in the presence of caffeine is indicative of SR calcium content.

**Statistical analysis.** Data are expressed as means ± SE. A Student’s t-test or ANOVA followed by a Student-Newman-Keuls test was used to make comparisons between groups. A value of $P < 0.05$ was considered statistically significant. A minimum of three independent experiments was performed for all components of the study.
RESULTS

Generation of SERCA2a-inducible transgenic mice. A rat SERCA2a cDNA with a COOH-terminal FLAG tag was cloned in front of the Tet-RE to generate Tet-RE-SERCA2a transgenic mice (Fig. 1A). Tet-RE-SERCA2a transgenic mice were crossed with homozygous α-MHC-rtTA transgenic mice (JAM8686) expressing a codon-optimized reverse tetracycline transactivator driven by the α-MHC promoter (29). BTG mice received Dox in their drinking water for 7–10 days. Offspring carrying the Tet-on-SERCA2a transgene were identified by the inducible expression of the SERCA2a-FLAG transgene at both the protein and mRNA level (Figs. 1B and 2A, respectively). As shown in Figs. 1B and 2, no expression of SERCA2a-FLAG was observed in the absence of Dox in BTG mice. Expression of SERCA2a-FLAG protein was cardiac specific, as assessed by Western blot (Fig. 1C). RNase protection assays demonstrated 2.3-fold more SERCA2a-FLAG mRNA relative to endogenous SERCA2a mRNA in the ventricles of Dox-treated BTG mice. Total SERCA2a protein levels increased only by 45% (Fig. 3); interestingly, endogenous SERCA2a was downregulated to 45% with respect to control values. Therefore, SERCA2a-FLAG protein constituted 70% of the total SERCA2a (not shown). In consequence, transgenic SERCA2a-FLAG protein expression predominated over endogenous SERCA2a.

Effect of inducible SERCA2a overexpression on cardiac function and calcium handling. Dox-induced expression of the SERCA2a transgene resulted in increased contractile function as assessed by echocardiography, with enhanced FS in BTG animals treated with Dox versus WT mice (43.8 ± 0.6, n = 4, vs 38.9 ± 0.8, n = 6, respectively, P < 0.002; Fig. 4A). PW thickening was also increased after the induction of SERCA2a transgene expression (Fig. 4B).

Calcium transients in isolated WT versus BTG cardiac myocytes (Dox) showed an accelerated calcium decline [half-time (t1/2) for WT 146 ± 11 ms vs. BTG 91 ± 6 ms, P < 0.05, n = 20 cells/group; Fig. 5] and a higher systolic Ca2+...
peak (indo-1 ratio for WT 0.80 ± 0.016 vs BTG 0.86 ± 0.020, 
*P < 0.005, n = 20, not shown). Additionally, SR calcium load 
was increased by 45% in BTG isolated myocytes compared 
with WT isolated myocytes (not shown). No change in the time 
to peak calcium occurred with SERCA2a overexpression. As a 
control, transients were compared in WT mice with or without 
Dox, and no differences were observed (Fig. 5).

Reversion of calcium handling abnormalities and impaired 
contractility in pressure-overloaded mouse hearts by inducible 
SERCA2a transgene expression. We tested the hypothesis that 
the abnormal calcium handling and contractile function of the 
pressure-overloaded heart can be rescued by inducible 
SERCA2a expression. Pressure overload was induced by as-
cending AC in BTG mice (see MATERIALS AND METHODS). Three 
weeks after AC, cardiac function was evaluated by echocardi-
graphy to obtain baseline pressure overload functional data. 
After baseline analysis, SERCA2a expression was either initi-
ated with Dox administration (treated group) or not initiated 
(untreated group). After 7 days, a second echocardiography 
was then performed, and mice were killed for cardiac myocyte 
isolation. Nonbanded BTG mice were used as controls. The 
results of this experiment are shown in Fig. 6. AC mice 
presented 100% increase in the heart weight-to-body weight 
ratio compared with nonbanded mice (Fig. 6A). Untreated AC 
BTG mice showed decreased FS and Vcf compared with 
nonbanded (control) BTG mice (Fig. 6, B and C). In treated 
mice, both the decrease in FS and Vcf were significantly 
improved and returned to values observed in the control group 
after SERCA2a overexpression was induced by Dox adminis-
tration (Fig. 6, B and C).

In a separate series of experiments, we determined whether 
or not the beneficial effects of increased SERCA2a expression 
on cardiac function observed in vivo by echocardiography 
could also result in an improvement of calcium handling and 
contractile function at the level of the individual cardiac 
myocyte. SERCA2a overexpression in AC BTG mice im-
proved fractional cell shortening, rate of contraction, and rate 
of relaxation (Fig. 7, A and B). This effect of SERCA2a 
induction on improving cell contractility was consistent with 
an improvement in calcium transients. For instance, the rate of 
calcium decline returned to control values in cells from treated 
AC BTG mice (Fig. 8), and SR calcium loading increased by 
70% (AC + Dox 1.038 ± 0.181 vs. AC 0.612 ± 0.063, indo-1 
ratio, *P < 0.05, n = 17 cells/group, not shown). However, the 
time to peak calcium remained prolonged in treated AC BTG 
isolated myocytes.
Fig. 7. Influence of in vivo SERCA2a transgene expression on contractility of cardiac myocytes isolated from AC mice. A: typical recording of cell shortening of a cardiac myocyte from an AC mouse and a myocyte from an AC mouse that received Dox for 7 days. The rate of contraction and relaxation is also shown (dL/dt). L/L_o, ratio of length to optimal length. B: fractional cell shortening (%), rate of contraction (+dL/dt), and rate of relaxation (-dL/dt) were improved in myocytes from AC mice that received Dox. *P < 0.05 (n = 5 for each group).

Fig. 8. Improvement of calcium transients of cardiac myocytes of AC mice after in vivo SERCA2a transgene expression. A: superimposed tracings of averaged cytosolic Ca^{2+} transients in cardiac myocytes isolated from WT mice treated with Dox for 7 days (control + Dox), AC BTG mice, and AC BTG mice treated 7 days with Dox. B: SERCA2a overexpression returned calcium decline to normal values. Time to peak remained prolonged after SERCA2a induction. Myocytes were electrically paced at 0.3 Hz. Values are means ± SE of at least 20 myocytes/group. *P < 0.05.
CARDIOVASCULAR RESPONSES TO SERCA2a OVEREXPRESION IN THE HEART

DISCUSSION

In this study, we report for the first time that conditional expression of a SERCA2a transgene in mice with pressure overload-induced decrease of contractile function is able to rescue the diminished contractile function of cardiac myocytes and the accompanying abnormal calcium handling. To accomplish this, we used a novel BTG mouse model based on the tet-on system. This system allowed us to treat pressure-overloaded hearts by inducing tightly controlled SERCA2a overexpression with Dox. We were not able to detect leakiness of the transgene by Western blot, RNase protection, or RT-PCR in the absence of Dox.

It is well established that the increase in cytosolic Ca\textsuperscript{2+} levels during systole occurs by release of Ca\textsuperscript{2+} from the SR through the ryanodine receptor via the process of Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release leading to systolic contractile function (2). Relaxation is initiated by a lowering of [Ca\textsuperscript{2+}], mediated primarily by pumping of Ca\textsuperscript{2+} back into the SR by SERCA2a or expulsion of Ca\textsuperscript{2+} out of the cell, largely by the sarcolemmal Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger.

Heart failure can be accompanied by alterations in both systolic contraction and diastolic relaxation, and previous evidence assigns abnormal calcium handling an important role in these contractile abnormalities. Downregulation of SERCA2a activity or content is considered an important contributor to the impaired diastolic relaxation. It has been demonstrated that in pathophysiological conditions with a reduction in SERCA2a function, overexpression of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger or its activation by downregulating phospholemman can compensate for the diastolic relaxation (17). In the long term, a deficiency in calcium reuptake by the SR also may lead to diminished SR calcium loading. This results in diminished calcium release during systole and diminished calcium loading for contraction. SERCA2a overexpression has shown to be protective against the pressure overload-induced decrease in contractile function maintaining contractile properties and calcium homeostasis in the cardiac myocytes (12, 19). Currently, there are no reports showing that overexpression of SERCA2a in hearts with established cardiac dysfunction can reestablish calcium homeostasis and contractility at the cellular level. One theory is that pressure overload induces structural abnormalities in the apparatus linked to calcium handling (i.e., the T tubule). These abnormalities change the distance between L-type Ca\textsuperscript{2+} channels and the foot structure of the ryanodine receptor or the general organization of the SR resulting in abnormal Ca\textsuperscript{2+} flux. In this study, we demonstrated that the timed induction of SERCA2a transgene expression produced an increase in cardiac performance as assessed by echocardiography in control mice. Similar results were reported previously using an adenoviral gene transfer approach (18). In addition, we used a pressure-overload model to generate cardiac dysfunction in mice. Cardiac cells obtained from untreated AC mice showed altered calcium transients characterized by a prolonged calcium decline. Contractile properties of these cells were also altered showing diminished fractional cell shortening and a slower rate of contraction and relaxation. Conditional expression of a SERCA2a rescued this phenotype and returned

SERCA2a was confirmed only in banded AC BTG mice treated with Dox (Fig. 9).

Effect of SERCA2a induction on the PLB-to-SERCA2a ratio.

The PLB-to-SERCA2a ratio has been suggested as a key regulator of SERCA2a activity (16). As expected, cardiac myocytes of untreated AC BTG mice showed a statistically significant decrease of SERCA2a protein levels compared with nonbanded control myocytes (AC 11,901 ± 27 arbitrary units vs. control 12,482 ± 105 arbitrary units, P < 0.05, n = 5 for each group; Fig. 9). An increase of SERCA2a expression in treated mice was confirmed by immunoblotting. Myocytes from treated AC BTC mice have higher total SERCA2a levels relative to untreated AC BTG mice; however, endogenous SERCA2a was similar in both groups (Fig. 9). PLB protein levels were increased and SERCA2a protein levels were decreased after AC. Therefore, banded mice had a higher PLB-to-SERCA2a ratio (Fig. 9). Induction of SERCA2a in AC BTG mice returned the ratio to control values (Fig. 9).
calcium handling toward normal values. The improvement in contraction may be attributed to the higher SR calcium loading found in cells expressing the SERCA2a transgene.

We found only a moderated (5%) downregulation of SERCA2a protein in pressure overload. However, we observed a marked decrease in calcium decline and cell relengthening (relaxation). This apparent discrepancy may be explained by a decrease in SERCA2a activity rather than protein expression. As previously demonstrated, an increased PLB-to-SERCA2a ratio may downregulate SERCA2a activity (16). Untreated AC mice had the expected increase in PLB (135%), which increased the PLB-to-SERCA2a ratio, leading to a decrease in SERCA2a activity. In our treated mice, we rescued this alteration by increasing total SERCA protein by about 20%. This increase in SERCA2a expression restored the PLB-to-SERCA2a ratio to control values. It is of interest to note that transgenic SERCA2a accounts for about 70% of total SERCA2a, demonstrating that the protein encoding the SERCA2a transgene is effectively incorporated into the longitudinal SR.

We found a 2.3-fold increase in SERCA2a mRNA in transgenic mice compared with control mice. This increase in mRNA did not result in an equivalent increase in SERCA2a protein expression, which was only 45% higher in BTG mice. These results are in agreement with previous reports indicating that overexpression of SERCA2a is limited by posttranscriptional events such as an accelerated degradation of SERCA2a protein formed in excess that could not be anchored in the SR membrane (11). In support of this idea, experiments in cardiac myocytes using SERCA2a adenovalir gene transfer showed that, although SERCA2a mRNA levels continuously increase with viral titer, protein levels become saturated (26). Our results support a posttranscriptional regulation because endogenous SERCA2a mRNA levels were not changed; however, endogenous SERCA2a was decreased in BTG mice expressing SERCA2a-FLAG. We found downregulation of SERCA2a protein in AC mice. Although the induction of SERCA2a-FLAG by Dox increased total SERCA2a protein levels, values never reached the induction achieved in control nonbanded mice. These results are consistent with studies showing lower SERCA2a overexpression in banded mice after adenovalir gene transfer compared with control mice (18). In addition, SERCA2a overexpression in transgenic mice was also decreased by aortic stenosis-induced cardiac hypertrophy (12).

In conclusion, conditional overexpression of SERCA2a protein in cardiac myocytes of mice with a pressure overload-induced decline in contractile function and abnormal calcium handling rescued the contractile dysfunction by normalizing Ca^{2+} flux in cardiac myocytes. Therefore, at the cellular level, increasing SERCA2a expression is an effective therapeutic strategy after pressure overload.

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hearts overexpressing SERCA2a show improved contractility under baseline conditions and pressure overload. 


