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Genetic modulation of the SERCA activity does not affect the Ca^{2+} leak from the cardiac sarcoplasmic reticulum

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Summary

The Ca^{2+} content in the sarcoplasmic reticulum (SR) determines the amount of Ca^{2+} released, thereby regulating the magnitude of Ca^{2+} transient and contraction in cardiac muscle. The Ca^{2+} content in the SR is known to be regulated by two factors: the activity of the Ca^{2+} pump (SERCA) and Ca^{2+} leak through the ryanodine receptor (RyR). However, the direct relationship between the SERCA activity and Ca^{2+} leak has not been fully investigated in the heart. In the present study, we evaluated the role of the SERCA activity in Ca^{2+} leak from the SR using a novel saponin-skinned method combined with transgenic mouse models in which the SERCA activity was genetically modulated. In the SERCA overexpression mice, the Ca^{2+} uptake in the SR was significantly increased and the Ca^{2+} transient was markedly increased. However, Ca^{2+} leak from the SR did not change significantly. In mice with overexpression of a negative regulator of SERCA, sarcolipin, the Ca^{2+} uptake by the SR was significantly decreased and the Ca^{2+} transient was markedly decreased. Again, Ca^{2+} leak from the SR did not change significantly. In conclusion, the selective modulation of the SERCA activity modulates Ca^{2+} uptake, although it does not change Ca^{2+} leak from the SR.

Introduction

Cardiac muscle contraction is regulated by Ca^{2+} released from intracellular Ca^{2+} stores in a region named the sarcoplasmic reticulum (SR), a central player in excitation-contraction coupling that allows actin-myosin interactions to produce active tension [1]. Membrane depolarization opens L-type Ca^{2+} channels, thereby leading to Ca^{2+} influx through the sarcolemma. A small increase in $[Ca^{2+}]_i$ due to Ca^{2+} influx can trigger a large amount Ca^{2+} release from the SR through the Ca^{2+} release channel (ryanodine receptor, RyR) which is known as the Ca^{2+}-induced Ca^{2+} release mechanism [2]. The amplitude of the intracellular Ca^{2+} transient, which determines cardiac contractility, is largely dependent on the Ca^{2+} content in the SR [1]. Two important regulators of the Ca^{2+} content in the SR are the activity of the Ca^{2+} pump (sarco/endoplasmic reticulum Ca^{2+}-ATPase, SERCA), which actively transports Ca^{2+} from the myoplasm to inside the SR, and Ca^{2+} leak through the RyR. The amount of Ca^{2+} released from the SR is known to exhibit a steep relationship with the Ca^{2+} content in the SR, and a small change in the Ca^{2+} content can produce a large change in the amount of Ca^{2+} released [3]. This mechanism is thought to be important for changes in the activity of SERCA to modulate $[Ca^{2+}]_i$. A recent study revealed that changes in the amount of Ca^{2+} leak through the RyR also regulate the Ca^{2+} content and the amplitude

of $[Ca^{2+}]_i$ under both physiological and pathophysiological conditions [4] [5]. Under physiological conditions, Ca^{2+} leak occurs during diastole and can prevent Ca^{2+} overload upon the increased Ca^{2+} cycling (such as sympathetic nervous stimulation) [5]. Moreover, a recent report suggested that differences in the amount of Ca^{2+} leak can explain the strain differences observed in Ca^{2+} handling among mouse strains [6]. Under pathophysiological conditions, such as heart failure, excess Ca^{2+} leak from the SR decreases the Ca^{2+} content in the SR and hence reduces the amount of Ca^{2+} available to produce cardiac contractions, possibly due to the phosphorylation of the RyR [4] [7]. Although these two regulators (the SERCA activity and Ca^{2+} leak) are important for modulating cardiac contractility, the direct relationship between the SERCA activity and Ca^{2+} leak has not been fully investigated due to the lack of ideal models to estimate these two activities separately in the same experimental sample and the difficulty of selectively modulating the SERCA activity *in vitro*. The Ca^{2+} leak function has been investigated in single cardiomyocytes using Ca^{2+} spark measurement [8] and pharmacological inhibition of Ca^{2+} leak [7] and in the lipid bilayer using electrical Ca^{2+} flux measurement [9]. In contrast, the Ca^{2+} uptake function has been estimated in microsomal fraction- enriched SR using fluorescence dye or radioisotopes [10]. Therefore, it is difficult to estimate Ca^{2+} uptake and Ca^{2+} leak separately in the same

experimental sample using the above methods.

Recently, we reported a novel method to estimate Ca^{2+} uptake and Ca^{2+} leak separately in the same preparation using saponin-skinned mouse left ventricular preparations [5]. We also created two transgenic mouse models of selectively increasing or decreasing the SERCA activity [10] [11]. In the present study, we investigated the direct interaction between the SERCA activity and Ca^{2+} leak using the saponin-skinned method combined with transgenic mouse models in which the SERCA activity was selectively modulated. We also determined the changes in the level of $[Ca^{2+}]_i$ and contractions in these transgenic mouse models to estimate the relationship between the SERCA activity and Ca^{2+} leak under beat to beat conditions.

Materials and Methods

Animals

All experiments were performed in accordance with the Guidelines on Animal Experimentation of The Jikei University School of Medicine. The study protocol was approved by the Animal Care Committee of The Jikei University School of Medicine (Approval number: 19-049C1). The investigation conformed to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Transgenic (TG) mice with cardiac-specific overexpression of rabbit cardiac SERCA (SERCA-TG) were generated under the control of the cardiac α-MHC gene promoter in the C57BL/6 background using the same procedure we previously employed to generate mutant SERCA2 (K397/400E)-overexpression mice [10]. Genotyping was performed to identify mice with the transgene using the following primers: 5'- AGG AGA AGG ACG GAC AAG GA -3' and 5'- TGG AGG AGG TGG CAG AAA CA -3'. Non-transgenic littermate (NTG) mice were used as controls. The SERCA-TG mice were born in the expected Mendelian ratios and had a normal life span as wild-type mice.

The generation of mice with a cardiac-specific overexpression of sarcolipin (SLN-TG) has been previously reported [11]. Briefly, flag-tagged SLN was overexpressed under the control of the cardiac β-MLC gene promoter in the FVB background, and genotyping was performed in the mice with flag-tagged SLN using the following primers: 5'-CAG CCT CTG CTA CTC CTC TTC CTG CCT GTT C-3' and 5'-GTA GGA CCT CAC AAG GAG CCA AAT AAG-3'. NTG mice were also used as control. The SLN-TG mice were born in the expected Mendelian ratios and had a normal life span compared with wild-type mice. The left ventricular pressure and heart rate values were also the same between the SLN-TG mice and NTG mice (data not shown). No major Ca^{2+} handling proteins, including SERCA, were altered. The SERCA activity was significantly decreased in the SLN-TG mice hearts, without changes in sensitivity to the Ca^{2+} concentration, as previously described [11].

Preparations

The papillary muscles or trabeculae dissected from the left ventricle of 10- to 12-week-old mice were used for the experiments [5] [12]. The hearts were quickly removed from mice anesthetized with sodium pentobarbital (200-300 mg/kg i.p.). The aorta was cannulated with a blunted 18G needle, and the heart was mounted on a

Langendorff apparatus perfused with Tyrode's solution containing 2 mmol/L of Ca^{2+} at constant pressure for five minutes [5] [12]. The heart beat was completely stopped by changing the solution to a solution containing 20 mmol/L of 2, 3-butanedione monoxime (BDM) (Nacalai Tesque, Inc., Kyoto, Japan). The left ventricle was opened, and the papillary muscles or thin trabeculae were dissected.

Measurement of intracellular Ca^{2+} transient and isometric tension

A Ca^{2+} sensitive photoprotein aequorin was used to measure the intracellular Ca^{2+} transient (CaT) simultaneously with isometric tension in the left ventricular papillary muscle preparations [12]. The aequorin was microinjected into 50-100 superficial cells of each preparation by applying high pressure with nitrogen gas using glass micropipettes. The preparation was electrically stimulated with platinum electrodes (1 mmol/L Ca^{2+}, 0.5 Hz, 30°C) and the evoked aequorin light signal was recorded using a photomultiplier (EMI9789A, Ruislip, UK) simultaneously with measurement of the isometric tension (BG-10, Kulite, NJ, USA). The aequorin light signals were averaged and converted to the intracellular Ca^{2+} concentration using *in vitro* calibration [13].

Measurement of the Ca^{2+} content in the SR

The method used to estimate the SR function has been previously reported (Supplementary Figure I) [5] [14]. Briefly, the papillary muscles or trabeculae were cut along the longitudinal axis (≅200 μm× 2-5 mm) in the experimental solution and both ends of the preparation were tied to a tungsten wire with silk threads. Then, the preparation was permeabilized with saponin (50 μg/ml) in the relaxing solution for 30 minutes and inserted into a glass capillary tube placed on an inverted microscope (Nikon, Tokyo, Japan). An excitation light wavelength of 488 nm and emission wavelength of longer than 510 nm through a cut-off filter (DM-510, Nikon, Tokyo, Japan) were used to monitor the fluorescence signal of fluo-3 (Dojindo Laboratories, Kumamoto, Japan) using a fluorometry system (CAM-230, JASCO, Tokyo, Japan).

In the Ca^{2+} uptake assay (Supplementary Figure IA-D), after the SR was loaded with Ca^{2+} by activating SERCA with adenosine triphosphate (ATP) (4 mmol/L), caffeine (50 mmol/L) was applied to release the accumulated Ca^{2+} from the SR into the experimental solution containing fluo-3 (30 μM). As fluo-3 predominantly binds most of the Ca^{2+} released from the SR, the fluo-3 fluorescence change upon Ca^{2+} binding provides an estimate of the amount of accumulated Ca^{2+} in the SR before caffeine application. In the Ca^{2+} leak assay (Supplementary Figure IE-H), the preparation was

perfused with the experimental solution containing ethylene glycol bis[β-aminoethylether]-N, N, N',N'-tetraacetic acid (EGTA) (1 mmol/L) for various durations following Ca^{2+} loading, and then the remaining Ca^{2+} was measured using the same protocol. We then estimated the maximal Ca^{2+} content, which reflects the level of Ca^{2+} fully-loaded in the SR, by extrapolating of the sampling point of each Ca^{2+} content to reach the Ca^{2+} content at time zero. The amount of Ca^{2+} leak was estimated by subtracting the remaining Ca^{2+} content from the maximal Ca^{2+} content (see the Supplementary Data for details).

Solutions and chemicals

Tyrode's solution buffered with N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (HEPES) was used for Langendorff perfusion of the hearts, dissection of the preparations and the aequorin experiment. The composition of the Tyrode's solution was as follows: 136.9 mmol/L of NaCl, 5.4 mmol/L of KCl, 0.5 mmol/L of $MgCl_2$, 1-2 mmol/L of $CaCl_2$, 0.33 mmol/L of $NaHPO_4$, 5 mmol/L of HEPES and 5 mmol/L of glucose. The pH was adjusted to 7.40±0.05 with NaOH at 30°C, and the solution was equilibrated with 100% O_2.

The solutions used to estimate the SR function was based on potassium

methanesulfonate (KMS) (103–164 mmol/L) to maintain a constant ionic strength under various conditions. The Ca^{2+} concentration was below pCa8, except for the "loading" period (between pCa8 and pCa5.6). The experimental solution contained 20 mmol/L of NaN_3 to block the Ca^{2+} uptake in the mitochondria and 20 mmol/L of piperazine-N-N'-bis[2-ethanesulfonic acid] (PIPES) (ionic strength, 0.2 mol/L; temperature, 22°C; pH adjusted with KOH). ATP (4 mmol/L) was present during the "load" period to activate the SERCA in the loading step. Each assay solution contained 50 mmol/L of caffeine and 25 mmol/L of adenosine-5'-monophosphate (AMP) to open the Ca^{2+} release channel (RyR) of the SR effectively. Fluo-3 was added to the "pre-assay" and "assay" solutions at a final concentration of 30 μM [5] [14] (see Supplementary Table 1 for details).

All reagents were purchased from Sigma (Saint Louis, MO), unless otherwise indicated.

Statistics

All measured data are presented as the means ± standard error of the mean (SEM). Statistical significance was estimated using the unpaired Student's *t*-test for two sets of data and using a one-way analysis of variance (ANOVA) followed by the

Bonferroni post-hoc test for multiple comparisons, with the significance level set at $p<0.05$. All statistical analyses were performed using the SPSS software program version 11.5 (SPSS Inc., Chicago, IL, USA).

Results

Protein expression levels and hemodynamics of the SERCA-TG mice

In the hearts of the SERCA-TG mice, the SERCA protein levels were significantly increased (1.68±0.10-fold increase) compared to those observed in the NTG mice, as demonstrated by Western immunoblotting (n=3 for SERCA-TG, n=3 for NTG) (Supplementary Figure II). No other major Ca^{2+} handling proteins were altered. The left ventricular pressure and heart rate values were the same between the SERCA-TG mice and NTG mice (n=6 for SERCA-TG, n=6 for NTG) (Supplementary Figure III).

Effects of selective upregulation of the SERCA activity on the Ca^{2+} transient and isometric tension in the intact preparations

First, we investigated the Ca^{2+} handling and contractions in the SERCA-TG heart. We evaluated the Ca^{2+} transient and isometric tension under field stimulation at 0.5 Hz, because the contractility of the isolated muscle preparation is stable at a lower stimulation frequency during the long period of the experiments [12]. However, we also evaluated these under a higher stimulation frequency (up to 2 Hz) and found similar

results to those observed at 0.5 Hz (data not shown). The left graphs of Figure 1 present representative traces of the Ca^{2+} transient (A) and isometric tension (B) recorded from the intact papillary muscle preparations of the SERCA-TG and NTG hearts. The peaks of the Ca^{2+} transient and isometric tension were significantly increased in the SERCA-TG hearts compared to that observed in the NTG hearts ($p<0.05$) (n=6 for SERCA-TG, n=8 for NTG). The time to reach the peak of the Ca^{2+} transient (TP) did not change significantly; however, the decay time of the Ca^{2+} transient from the peak to half of the peak (DT) was significantly shortened in the SERCA-TG hearts ($p<0.01$). Both the time to reach the peak of tension (TPT) ($p<0.01$) and the relaxation time from the peak tension to half the peak (RT) ($p<0.01$) were significantly shortened in the SERCA-TG hearts.

Effects of selective upregulation of the SERCA activity on the SR function in the saponin-treated preparations

We then estimated the SR function in the SERCA-TG hearts. Figure 2A shows the time-dependent changes in Ca^{2+} uptake into the SR estimated in the loading solution at pCa7 using the saponin-skinned preparations (n=13 for SERCA-TG, n=8 for NTG). The amount of Ca^{2+} uptake was significantly accelerated in the early phase (less than 10

seconds) of Ca^{2+} loading in the SERCA-TG hearts. Figure 2B shows the Ca^{2+}-dependent changes in the Ca^{2+} uptake estimated at the early phase of Ca^{2+} loading (n=16 for SERCA-TG, n=16 for NTG). At the fixed loading time of 10 seconds, the amount of Ca^{2+} uptake into the SR was significantly increased between pCa7.4 and pCa6.6 in the SERCA-TG hearts. The maximal Ca^{2+} content was estimated in the SERCA-TG and NTG hearts. As shown in Figure 2C, the maximal Ca^{2+} content in the SERCA-TG hearts was not significantly different from that observed in the NTG hearts (n=29 for SERCA-TG, n=24 for NTG).

Finally, we investigated Ca^{2+} leak from the SR under the selective overexpression of SERCA. Figure 2D shows the time-dependent changes in Ca^{2+} leak from the SR (n=29 for SERCA-TG, n=24 for NTG). The time course of Ca^{2+} leak was identical between the SERCA-TG and NTG hearts.

Effects of selective downregulation of the SERCA activity on the Ca^{2+} transient and isometric tension in the intact preparations

In the next series of experiments, we investigated the role of the selective downregulation of SERCA on E-C coupling using SLN-TG hearts. We first estimated the Ca^{2+} transient and isometric tension of the intact papillary muscle preparations

obtained from the SLN-TG and NTG mice. The left graphs of Figure 3 show the representative traces of the Ca^{2+} transient (A) and isometric tension (B) recorded from the preparations of the SLN-TG and NTG hearts. Both the peak of the Ca^{2+} transient ($p<0.01$) and the peak of tension ($p<0.01$) were significantly decreased in the SLN-TG hearts (n=7 for SLN-TG, n=10 for NTG). The TP did not significantly change, whereas the DT was significantly prolonged in the SLN-TG hearts ($p<0.01$). Both the TPT ($p<0.05$) and RT ($p<0.01$) were significantly prolonged in the SLN-TG hearts.

Effects of selective downregulation of the SERCA activity on the SR function in the saponin-treated preparations

We then estimated the SR function in the SLN-TG and NTG hearts using the saponin-skinned preparations. The amount of Ca^{2+} uptake was significantly attenuated in the early phase (less than 15 seconds) of exposure to the loading solution at pCa6.2 in the SLN-TG hearts (Figure 4A) (n=11 for SLN-TG, n=12 for NTG). At the fixed loading time of 15 seconds, the amount of Ca^{2+} uptake was significantly decreased between pCa6.6 and pCa6.2 in the SLN-TG hearts (Figure 4B) (n=18 for SLN-TG, n=16 for NTG). We also estimated the maximal Ca^{2+} content in the SR and found no

significant differences between the SLN-TG and NTG hearts (Figure 4C) (n=29 SLN-TG, n=28 for NTG).

We then estimated the amount of Ca^{2+} leak under the selective downregulation of SERCA. The time course of Ca^{2+} leak from the SR did not differ between the SLN-TG and NTG hearts (Figure 4D) (n=29 for SLN-TG, n=28 for NTG).

Discussion

The primary result of the present study is that the selective modulation of the SERCA activity can produce large changes in the Ca^{2+} transient without changing the amount of Ca^{2+} leak from the SR**.**

In the present study, we used saponin-skinned preparations to estimate the SR function, in which a separate estimation of Ca^{2+} uptake and Ca^{2+} leak can be made using the same preparation [5]. A previous report suggests that a large change in the SERCA activity produces a relatively small change in the Ca^{2+} content in the SR [3] [15]. In this study, in the SERCA-TG mouse heart, the expression level of SERCA proteins was increased 1.68 ± 0.10-fold compared with that observed in the NTG hearts (see Supplementary Figure II), and the amplitude and time course of the Ca^{2+} transient was largely affected (see Figure 1). Although the maximal Ca^{2+} content was unaltered in the SERCA-TG hearts, the Ca^{2+} content of the SR in each beat changed. The Ca^{2+} content of the SR during diastole is determined by the balance between Ca^{2+} uptake and Ca^{2+} leak in the SR. If changes in the SERCA activity directly modulate Ca^{2+} leak, SERCA-dependent changes in the Ca^{2+} content can be off-set by the altered Ca^{2+} leak. However, the lack of significant changes in Ca^{2+} leak in the SERCA-TG heart does not support this possibility. We also showed that a selective decrease in the SERCA activity can produce a large decrease in the amplitude and significant slowing of the time course

of the Ca^{2+} transient in SLN-TG heart preparations. The result showing that the significant attenuation of Ca^{2+} uptake did not change the amount of Ca^{2+} leak from the SR in the SLN-TG hearts also suggests that changes in the Ca^{2+} content upon the selective modulation of the SERCA activity do not have a significant effect on Ca^{2+} leak. Under our experimental conditions in which the SERCA activity was chronically modulated, the maximal Ca^{2+} content of the SR was not affected, although the Ca^{2+} content observed under the beat to beat conditions was altered according to the results of Ca^{2+} transient measurement (see Figures 1 and 3). The unaltered maximal Ca^{2+} content of the SR may explain why the selective modulation of SERCA did not influence Ca^{2+} leak in our system.

Impairment of the SERCA activity is well documented under patho-physiological conditions. Under conditions of heart failure, reduced Ca^{2+} uptake due to inhibition of the SERCA activity has been reported in both animal models and human failing hearts [16-20]. Therefore, the upregulation of the SERCA activity is a promising therapeutic strategy for treating heart failure, and recent observations of gene therapy for heart failure have strongly focused on the SERCA activity as a target of gene transfer in animal models as well as human heart failure [21-23]. However, the increase in the SERCA activity also serves to increase the beat to beat Ca^{2+} content in

the SR, which can cause Ca^{2+} leak through the RyR [24]. Abnormal Ca^{2+} leak through the RyR has also been extensively investigated in various heart failure models. Marks's group originally identified the novel mechanism by which the protein kinase A (PKA)-dependent phosphorylation of the RyR dissociates FKBP12.6, which in turn leads to Ca^{2+} leak through the RyR [4]. The phosphorylation-dependent modulation of RyR has therefore been considered an important mechanism regulating Ca^{2+} leak, especially under pathological conditions such as heart failure and ventricular arrhythmia [25-26]. Three independent Ser residues in cardiac RyR are known to be phosphorylated by protein kinases. PKA mainly phosphorylates Ser2808 whereas Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) mainly phosphorylates Ser2814 [27]. Ser2030 is also a target of PKA-dependent phosphorylation in RyR, however, the importance of this residue is questionable [28]. The first report stated that the PKA-dependent hyperphosphorylation of RyR2 accelerated the dissociation of FKBP12.6 from RyR, which destabilized the Ca^{2+} release channel to increase Ca^{2+} leak [4]. This idea is fits well with the increased activity of the sympathetic nervous system in subjects with heart failure. However, other experimental data suggested that the CaMKII-dependent phosphorylation of RyR is important for pathological Ca^{2+} leak [7] [29-30]. In addition, the identification of an exchange protein that was directly activated

by cAMP (Epac) further supported the importance of CaMKII-dependent phosphorylation through beta-adrenergic receptor stimulation in the heart [31-33]. Very recently, it has been reported that both CaMKII and PKA functionally regulate RyR but have differential roles in human cardiac pathology [34]. Therefore, abnormal Ca^{2+} leak through the RyR may play a significant role in heart failure and may be another promising target of treatment. Our results demonstrating the absence of changes in Ca^{2+} leak upon the selective modulation of the SERCA activity support the utility of gene therapy targeting the SERCA activity. Recent observations in animal models and the findings of a clinical trial support the favorable effects of SERCA-mediated gene therapy [35-37].

Recently, another target of gene therapy for Ca^{2+} handling has been proposed. S100A1 is an EF-hand Ca^{2+} binding protein that exhibits a conformational change upon binding to Ca^{2+} and interacts with various functional proteins [38]. In cardiac muscle, S100A1 has been found to modulate the intracellular Ca^{2+} handling process. For example, S100A1 interacts with SERCA to increase the SERCA activity and with RyR to inhibit Ca^{2+} leak from the SR [39]. S100A1 also modulates the myofilament sensitivity to Ca^{2+}, which can alter cardiac contractility. Therefore, S100A1 may be the ideal therapeutic target for treating heart failure due to both the activation of SERCA

and the inhibition of Ca^{2+} leak. However, elucidating the role of S100A1 in gene therapy for heart failure must wait for the results of pre-clinical and clinical trials [40].

Limitations of this study

1) Although we determined the protein expression levels of various functional modulators in cardiac excitation-contraction coupling, we did not assess the phosphorylation levels of proteins such as phospholamban and RyR. 2) Our measurements of the SR function were obtained under *in vitro* conditions, and the results must be extrapolated into the heart *in vivo*. 3) The SERCA-TG and SLN-TG models are not disease models (the mice did not exhibit heart failure). 4) Both transgenic mice demonstrated cardiac-specific overexpression, but not conditional overexpression. 5) Because we used a high concentration of EGTA (1 mmol/L) in the experimental solution, which might inhibit SERCA activity, the speed of the Ca^{2+} uptake by SERCA under our experimental conditions is much lower than that observed under physiological conditions *in situ*. 6) Because we could not evaluate the detailed effects of ATP (4 mmol/L) on RyR2 due to the nature of our experimental system used for the Ca^{2+} leak assay, we might have underestimated the amount of Ca^{2+} leak under our experimental conditions at (physiological) nmol/L levels of cytosolic Ca^{2+} [41] [42].

Conclusion

In the present study, we found the selective modulation of the SERCA activity to have a profound effect on cardiac excitation-contraction coupling due to alteration of Ca^{2+} uptake into the SR, without significant changes in Ca^{2+} leak from the SR. These results support the utility of gene therapy targeting SERCA and related molecules.

Conflict of interest statement

The authors declare that there are no conflicts of interest associated with this study.

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Figure captions

Figure 1. Ca^{2+} transient and isometric tension in the intact preparations from the SERCA-TG hearts

A. The left graph shows representative traces of the Ca^{2+} transient from the left ventricular papillary muscle in the SERCA-TG (red) and NTG (black) hearts. The bar graphs show the amplitude, time to reach the peak (TP) and decay time from the peak to half of the peak (DT) of the Ca^{2+} transient in the SERCA-TG (red) and NTG (black) hearts.

B. The left graph shows representative traces of isometric tension in the preparations obtained from the SERCA-TG (red) and NTG (black) hearts. The bar graphs show the amplitude, time to reach the peak (TPT) and relaxation time from the peak to half of the peak (DT) of tension in the SERCA-TG (red) and NTG (black) hearts.

Figure 2. Sarcoplasmic reticulum function in the saponin-treated preparations from the SERCA-TG hearts

A. The time-dependent changes in Ca^{2+} uptake into the SR during exposure to the loading solution at pCa 7.

B. The Ca^{2+} uptake estimated at 10 seconds of exposure to the loading solution of various pCa.

C. The maximal Ca^{2+} content of the SR in the SERCA-TG and NTG hearts.

D. The time-dependent changes in Ca^{2+} leak from the SR following Ca^{2+} loading at pCa 6.2 for 120 seconds.

Figure 3. Ca^{2+} transient and isometric tension in the intact preparations from the SLN-TG hearts

A. The left graph shows representative traces of the Ca^{2+} transient from the left ventricular papillary muscle in the SLN-TG (green) and NTG (black) hearts. The bar graphs show the amplitude, time to reach the peak (TP) and decay time from the peak to half of the peak (DT) of the Ca^{2+} transient in the SLN-TG (green) and NTG (black) hearts.

B. The left graph shows representative traces of isometric tension in the preparations obtained from SLN-TG (green) and NTG (black) hearts. The bar graphs show the amplitude, time to reach the peak (TPT) and relaxation time from the peak to half of the peak (DT) of tension in the SLN-TG (green) and NTG (black) hearts.

Figure 4. Sarcoplasmic reticulum function in the saponin-treated preparations from the SLN-TG hearts

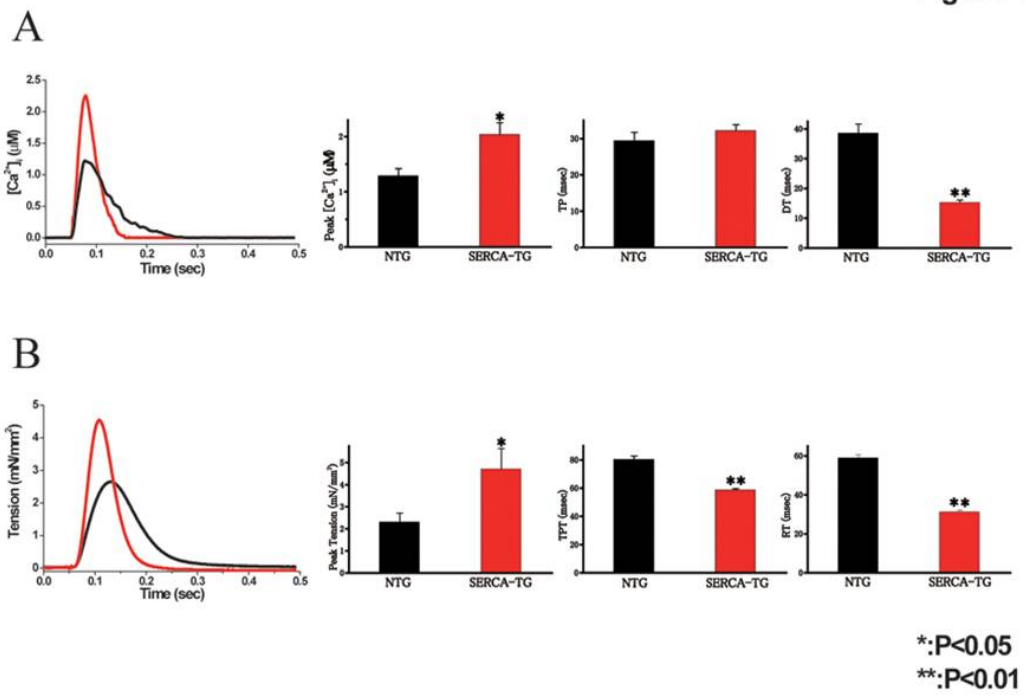
A. The time-dependent changes in Ca^{2+} uptake into the SR during exposure to the loading solution at pCa 6.2.

B. The Ca^{2+} uptake estimated at 15 seconds of exposure to the loading solution of various pCa.

C. The maximal Ca^{2+} content of the SR in the SLN-TG and NTG hearts.

D. The time-dependent changes in Ca^{2+} leak from the SR following Ca^{2+} loading at pCa 6.2 for 120 seconds.

Figure 1



*:P<0.05
**:P<0.01

Figure 2

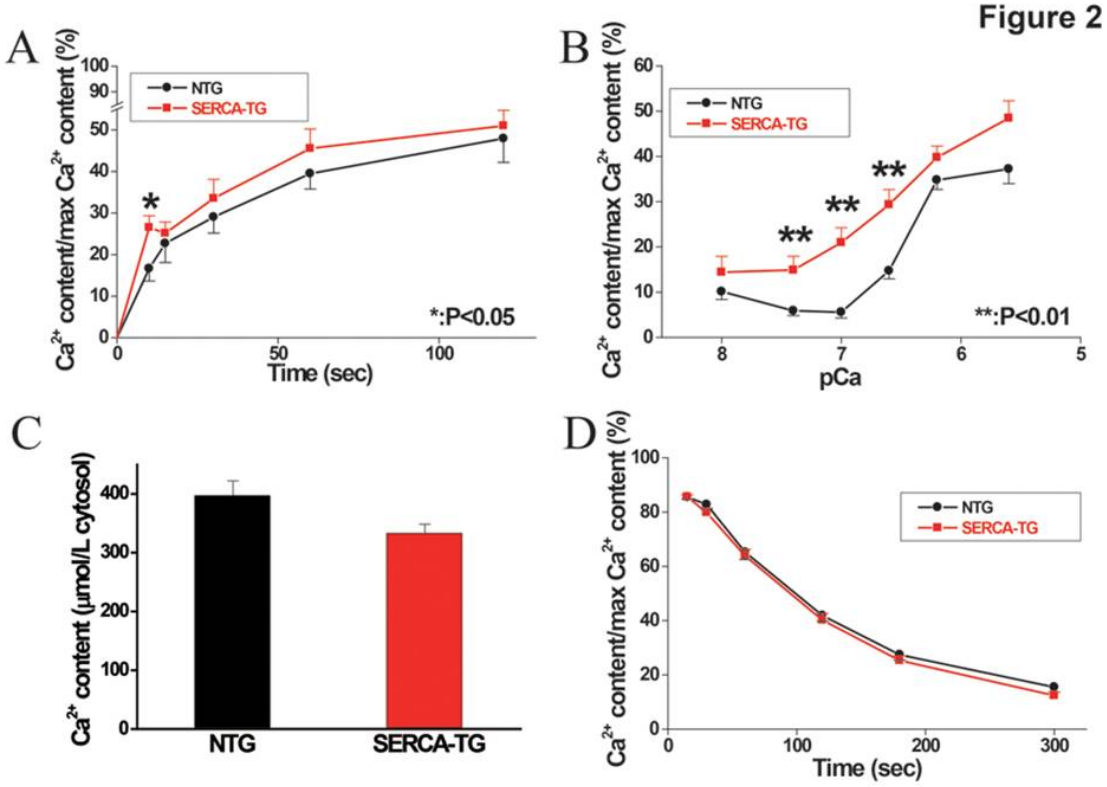
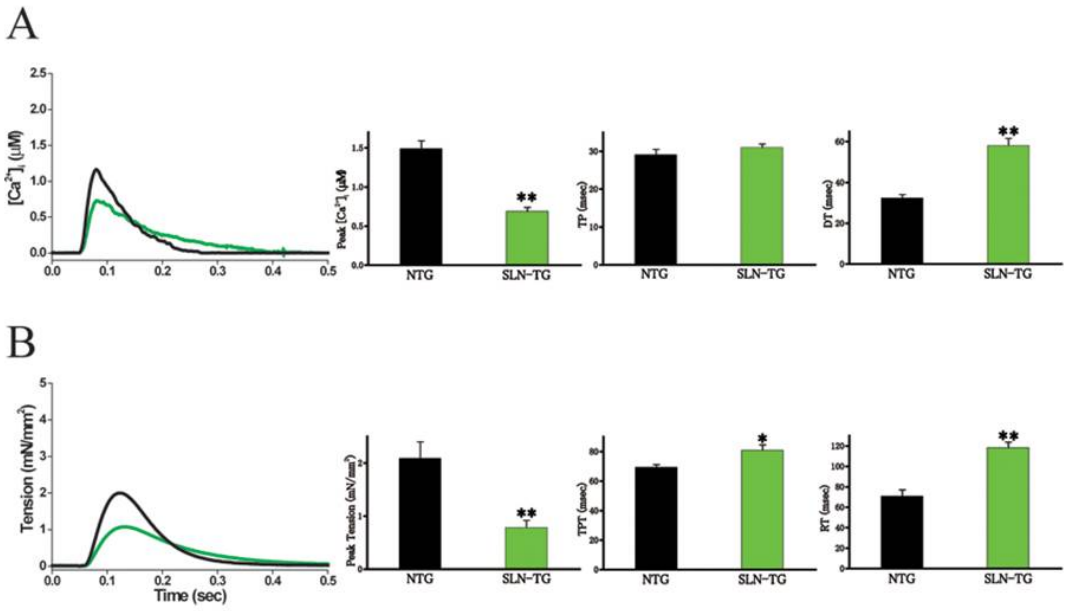
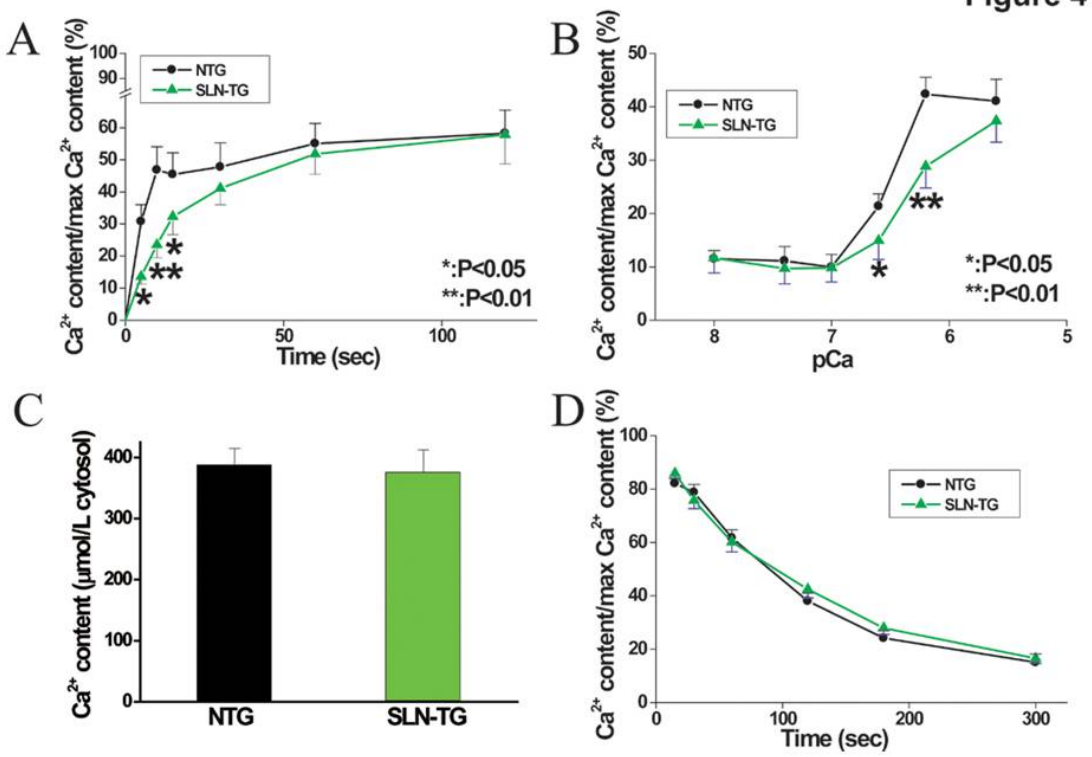

A
B
C
D
NTG
SERCA-TG
Ca2+ content/max Ca2+ content (%)
Time (sec)
*:P<0.05
pCa
**:P<0.01
Ca2+ content (μmol/L cytosol)

Figure 3



*:P<0.05
**:P<0.01

Figure 4


A
B
C
D
NTG
SLN-TG
Time (sec)
pCa
*:P<0.05
**:P<0.01
Ca2+ content/max Ca2+ content (%)
Ca2+ content (μmol/L cytosol)

Supplementary Data

Expanded Materials and Methods

Western immunoblotting

Western immunoblotting was performed to confirm the amount of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and other Ca^{2+}-handling proteins as previously described [1]. Briefly, proteins obtained from myocardial tissue of SERCA-TG (n=3) and NTG (n=3) mice were prepared and subjected to a Western immunoblotting analysis using the following antibodies: SERCA (N-19, Santa Cruz Biotechnology, Santa Cruz, CA), phospholamban (PLN) [2], Na^{+}/Ca^{2+} exchanger (NCX) [3], ryanodine receptor (RyR) [4] and cardiac actin (Sigma, St. Louis, MO). The protein expression level was normalized to that of cardiac actin.

Assessment of the left ventricular pressure and heart rate *in vivo*

Mice were anesthetized via an intra-peritoneal injection of a mixture of ketamine (50-100 mg/kg) and xylazine (3-6 mg/kg). The right carotid artery was

isolated and cannulated with a 1.4 French Millar (Houston, TX) catheter connected to an amplifier (TCP-500, Millar). Then, the catheter was advanced into the left ventricle and the left ventricular pressure was digitized and processed by a computer system (model PE-1000; Nihon Kohden, Tokyo, Japan). The heart rate was monitored simultaneously.

Estimation of the Ca^{2+} uptake and Ca^{2+} leak of the SR

We used a series of solutions to measure the Ca^{2+} content in SR, as shown in Supplementary Table 1. To estimate the Ca^{2+} uptake, Ca^{2+} was loaded into the SR by activating the Ca^{2+} pump in the presence of ATP (4 mmol/L) at various times (10-120 sec) after loading with solutions containing different Ca^{2+} concentrations (pCa 8-5.6) (Supplementary Figure IA). The Ca^{2+} and ATP around the SR were then removed using a washing solution containing O, O'-Bis (2-aminoethyl) ethyleneglycol-N, N, N', N'-tetraacetic acid (EGTA) (1 mmol/L), to terminate the Ca^{2+} uptake of the SR (Supplementary Figure IB). Then, the preparation was perfused with pre-assay solution containing fluo-3 (30 μM) (Supplementary Figure IC). Finally, all Ca^{2+} in the SR was released using a high concentration of caffeine (50 mmol/L) and AMP (25 mmol/L), and

the Ca^{2+} -dependent fluorescence change was measured using fluo-3 (Supplementary Figure ID). The measured change in the fluorescence of fluo-3 was converted to the Ca^{2+} concentration, and the amount of Ca^{2+} in the SR was shown in units of μmol/L cytoplasm.

To estimate the Ca^{2+} leak, the preparation was perfused with the washing solution containing EGTA for various durations of time (15-300 sec) (Supplementary Figure IF) following Ca^{2+} loading (Supplementary Figure IE). Then, the remaining Ca^{2+} in the SR after washing (15-300 sec) was measured usnig the same protocol as was used for the Ca^{2+} uptake assay (Supplementary Figures IG, H). We evaluated the maximal Ca^{2+} content of the SR based on the fitting curve of the Ca^{2+} leak, and the value of the Ca^{2+} content before washing was estimated as the maximal Ca^{2+} content. Finally, the amount of Ca^{2+} leak was estimated by subtracting the remaining Ca^{2+} in the SR from the maximal Ca^{2+} content. We validated the Ca^{2+} leak measurement using ruthenium red to inhibit RyR opening (Supplementary Figure IV).

Use of aequorin to measure the intracellular Ca^{2+} transient

We used aequorin to measure the intracellular Ca^{2+} transient from the intact preparations for the following reasons: 1) Aequorin is sensitive to changes in the Ca^{2+} concentration (one molecule of aequorin can bind three Ca^{2+} ions) [5] and more accurate estimation of the amplitude and the time course of the Ca^{2+} transient can be achieved using aequorin compared with fluorescent dyes such as fura-2 [6]. 2) Upon binding to Ca^{2+}, aequorin does not cause buffering of Ca^{2+} due to the consumptive nature of aeqourin, which overcomes one of the disadvantages of using a fluorescent dye [5]. 3) Using aequorin enabled us to measure the Ca^{2+} transient and isometric tension (not cell shortening) simultaneously [7] [8]. We monitored the membrane potential of cells at the surface of the preparation during pressure injection to check the cell condition to avoid excess damage during injection [7] [8].

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Table 1. Composition of the solutions for sarcoplasmic reticulum function

A. Ca^{2+} uptake assay

period	load	wash	pre-assay	assay
Time (s)	10-120	60	30	60
Ca^{2+} (pCa)	8-5.6	>8	>8	>8
KMs (mM)	126-128	154	162	103
Mg^{2+} (mM)	1.5	1.5	0	0
EGTA (mM)	1	1	0	0
ATP (mM)	4	0	0	0
AMP (mM)	0	0	0	25
caffeine (mM)	0	0	0	50
fluo-3 (μM)	0	0	30	30
PIPES (mM)	20	20	20	20
NaN_3 (mM)	20	20	20	20

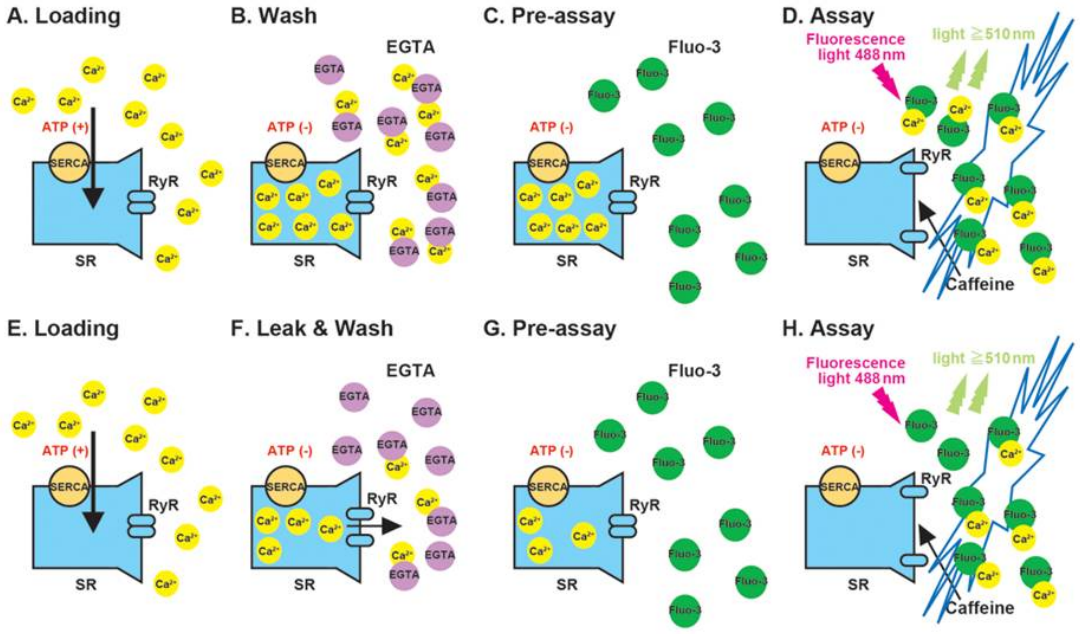
B. Ca^{2+} leak assay

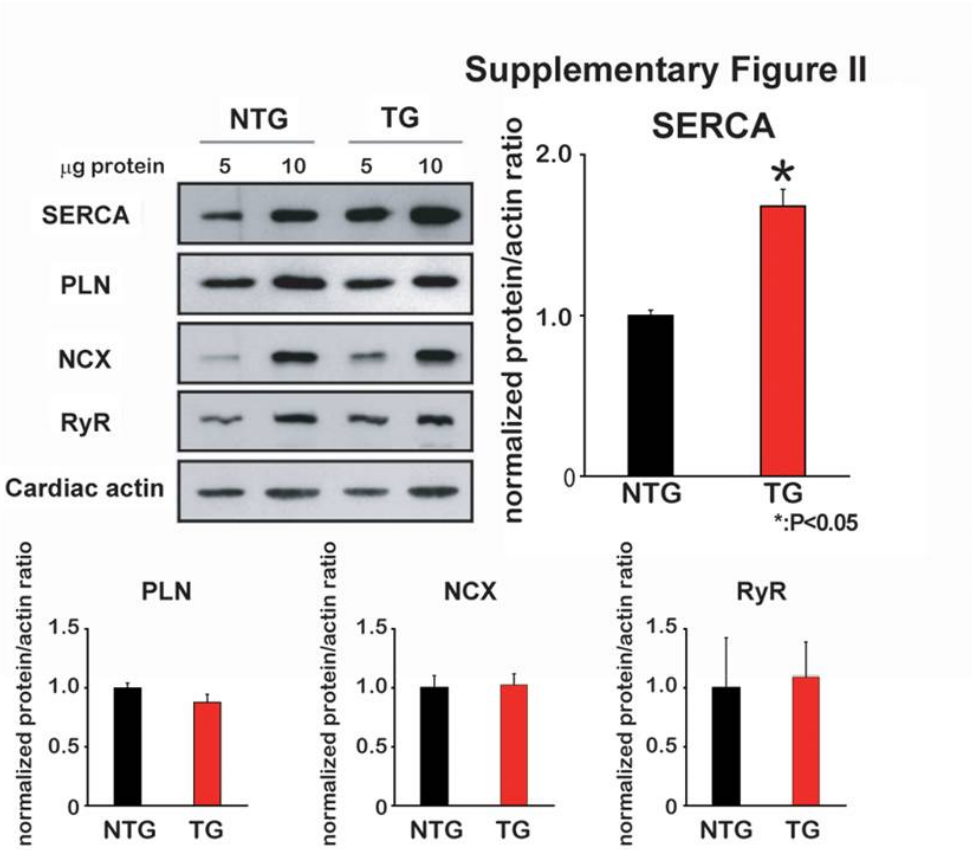
period	load	leak & wash	pre-assay	assay
Time (s)	120	15-300	30	60
Ca^{2+} (pCa)	6.2	>8	>8	>8
KMs (mM)	127	154	162	103
Mg^{2+} (mM)	1.5	1.5	0	0
EGTA (mM)	1	1	0	0
ATP (mM)	4	0	0	0
AMP (mM)	0	0	0	25
caffeine (mM)	0	0	0	50
fluo-3 (μM)	0	0	30	30
PIPES (mM)	20	20	20	20
NaN_3 (mM)	20	20	20	20

MS, methanesulfonate; EGTA, ethylene glycol bis[β-aminoethylether]-N, N,

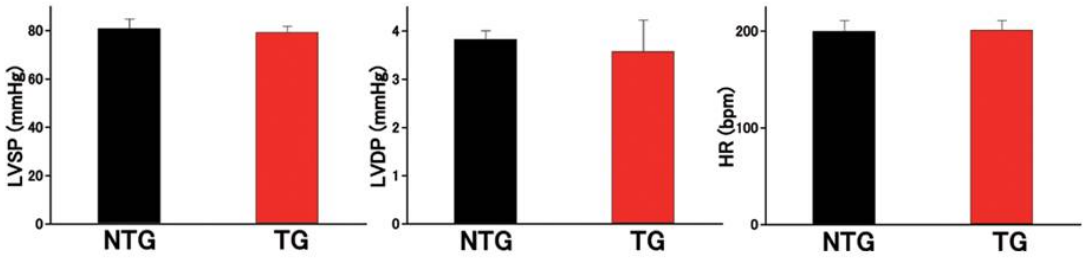
N’,N’-tetraacetic acid; ATP, adenosine triphosphate; AMP adenosine-5’-monophosphate; PIPES, poperazine-N-N’-bis[2-ethanesulfonic acid]; M, mol/L.

Supplementary Figure I




Supplementary Figure II
NTG
TG
μg protein
5
10
5
10
SERCA
PLN
NCX
RyR
Cardiac actin
SERCA
normalized protein/actin ratio
2.0
1.0
0
*
NTG
TG
*:P<0.05
PLN
NCX
RyR
normalized protein/actin ratio
1.5
1.0
0.5
0
NTG
TG

Supplementary Figure III



Supplementary Figure IV