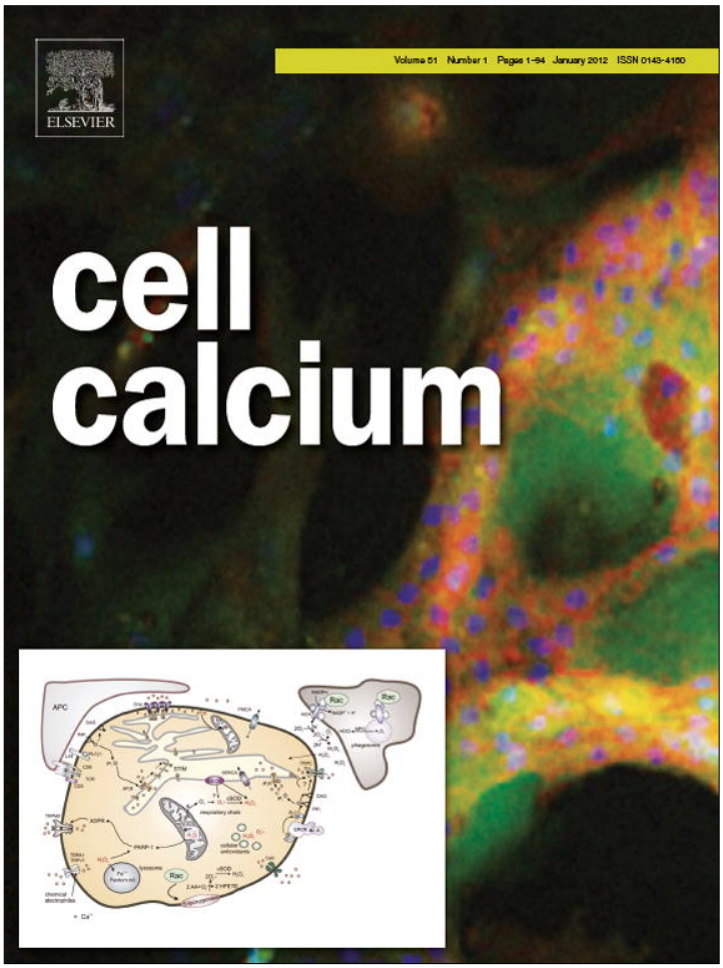

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Sarcalumenin plays a critical role in age-related cardiac dysfunction due to decreases in SERCA2a expression and activity

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ABSTRACT

Impaired Ca^{2+} reuptake into the sarcoplasmic reticulum (SR) underlies a primary pathogenesis of heart failure in the aging heart. Sarcalumenin (SAR), a Ca^{2+}-binding glycoprotein located in the longitudinal SR, regulates Ca^{2+} reuptake by interacting with SR Ca^{2+}-ATPase (SERCA). Here we found that the expression levels of both SAR and SERCA2 proteins were significantly downregulated in senescent wild-type mice (18-month old) and that downregulation of SAR protein preceded downregulation of SERCA2 protein. The downregulation of SERCA2 protein was greater in senescent SARKO mice than in age-matched senescent wild-type mice, which was at least in part due to progressive degradation of SERCA2 protein in SARKO mice. Senescent SARKO mice exhibited typical findings of heart failure such as increased sympathetic activity, impaired exercise tolerance, and upregulation of biomarkers of cardiac stress. Consequently, cardiac function was progressively decreased in senescent SARKO. We also found that the expression levels of endoplasmic reticulum (ER) stress-related genes such as x-box binding protein 1 (XBP1) were significantly increased in senescent SARKO mice, indicating that senescent SARKO mice exhibited ER stress. Thus we uncovered the important role of SAR in maintaining Ca^{2+} transport activity of SERCA2a and cardiac function in the senescent population.

1. Introduction

Heart failure due to cardiac dysfunction represents one of the major causes of morbidity and mortality in the senescent population [1,2]. Diastolic dysfunction in particular is a hallmark of the aging heart [3,4]. A growing body of evidence has demonstrated that impaired Ca^{2+} reuptake into the sarcoplasmic reticulum (SR) underlies the pathogenesis of diastolic dysfunction in the aging heart [5–8]. Since the SR Ca^{2+}-ATPase type 2a (SERCA2a) plays a primary role in Ca^{2+} sequestration into the cardiac SR, a decrease in the expression and/or activity of SERCA2a directly relates to diastolic dysfunction. Therefore, exploring the molecular mechanism for the regulation of SERCA2a expression and activity is extremely important to understand the pathogenesis of diastolic dysfunction in the aging heart.

Sarcalumenin (SAR) is an SR luminal glycoprotein responsible for Ca^{2+} buffering in skeletal and cardiac muscles [9,10]. SAR is predominantly found in the longitudinal SR, where SERCA and phospholamban (PLN) are also located [11]. We have recently demonstrated that the gene disruption of SAR resulted in mild cardiac dysfunction in young mice at the age of 2–4-month old [12]. This impairment is likely due to a decrease in the expression of SERCA2 protein [12]. In addition, cardiac dysfunction was further progressed under a pressure-overloaded stress induced by transverse aortic constriction [13] and endurance exercise training [14]. However, it is not yet widely known that SAR plays a role in the impairment of SR function and in cardiac dysfunction in the aging hearts. In this study, therefore, we investigated the impact of SAR deficiency on the expression and activity of SERCA2a and cardiac function in the aging heart.

2. Materials and methods

2.1. Animal preparation

All animal care and study protocols were approved by the Animal Ethics Committee of Yokohama City University School of Medicine, and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). SAR knockout (SARKO) mice and C57BL/6J wild-type controls from the same genetic backgrounds were bred in an animal facility of the

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University of Waseda. All animals were housed under standard day–night rhythm and ad libitum conditions until beginning of the experiments (4 and 18 months of age).

2.2. Immunoblot analysis

We prepared protein samples from the left ventricular tissues of the young and senescent mice. Mice were anesthetized with an intraperitoneal injection of Avertin (250 μg/g) to avoid adding extra stresses to the animals. Heart muscles were immediately placed in chilled phosphate-buffered saline to remove all residual blood. Hearts were then weighed, and left ventricles were immediately frozen in liquid nitrogen and then stored at −80 °C. Immunoblot analyses were performed as described previously [13,14]. Briefly, tissues were defrosted to 0 °C and homogenized in a chilled homogenization buffer [in mM: 50 Tris (pH 8.0), 1 EDTA, 1 EGTA, 1 dithiothreitol, and 200 sucrose] with protease inhibitors (Complete Mini, Roche, Basel, Switzerland). Protein content was determined using the Coomassie Plus protein assay (Pierce Chemical, Rockford, IL, USA) and ovalbumin was used as a standard. The protein samples (16 μg) were warmed to 95 °C for 5 min and separated in the same gel by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes (Bio-Rad, Hercules, CA, USA). When the molecular size of target proteins was different, PVDF membranes were cut in accordance with their size. When the molecular size of target proteins was similar, we reused the same membrane for a different antibody after washing the membrane with a stripping buffer [in mM: 62.5 Tris (pH 8.0), 100 2-mercaptoethanol, and 2% SDS]. Primary antibodies used in the present study were: anti-SAR (Sigma, Japan), anti-SERCA2a (Sigma, Japan), anti-PLN (Badrilla, United Kingdom), anti-phosphorylated PLN (serine 16 or threonine 17) (Badrilla, United Kingdom), anti-RyR2 (kindly provided by Dr. Andrew Marks at Columbia University, America), anti-CSQ2 (Affinity BioReagents, USA), anti-NCX1 (kindly provided by Dr.Takahiro Iwamoto at Fukuoka University, Japan), and anti-β-actin (Sigma, Japan). After application of a secondary antibody, quantification of the target signals was performed using the LAS-3000 imaging system (FUJIFILM, Tokyo, Japan). The protein levels of interest were normalized to rat β-actin. For reuse, a membrane was washed with a stripping buffer at 55 °C for 10 min, and was washed 3 times with 0.1%TBS-Tween 20 buffer.

2.3. Quantitative RT-PCR analysis

Total RNA was isolated from various tissues using TRIzol reagent (Invitrogen, La Jolla, CA) as recommended by the manufacturer. Generation of cDNA and RT-PCR analysis were performed as described previously [15,16]. The primers for PCR amplification were designed based on the mouse nucleotide sequences of atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP). The mRNA levels of interest were normalized to mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.4. Analysis of SERCA2 protein stability in in vivo mice

Protein stability was analyzed as described previously with some modification [17]. WT or SARKO mice were intraperitoneally injected with cycloheximide (0.1 mg/kg body weight). Animals were scarified 48 h after injection. The hearts were immediately removed and frozen in liquid nitrogen and stored at −80 °C. Homogenized tissue lysates were subjected to SDS–PAGE and immunoblot analysis. Assays were performed in triplicate.

2.5. SR Ca^{2+}-ATPase assay

SR Ca^{2+}-ATPase activity was measured in triplicate spectrophotometrically at 37 °C as described previously with some modifications [18]. Briefly, using 4 μg of SR protein from mice heart tissues, the reaction was carried out at 37 °C in a reaction medium [in mM: 30 TES, 100 KCl, 5 NaN_3, 5 $MgCl_2$, 0.5 EGTA, and 4 ATP (pH 7.2) with or without 0.5 $CaCl_2$]. The reaction medium was pre-incubated at 37 °C for 5 min. The reaction was started at 37 °C by adding SR protein to the medium. After 5 min the reaction was stopped by adding 0.5 ml of ice cold 10% trichloroacetic acid solution and the mixture was placed on ice. Inorganic phosphate was measured by using U2001 (HITACHI, Japan) as described previously [19]. Ca^{2+}-ATPase activity was calculated by subtracting the ATPase activity in the presence of 0.5 mM EGTA (no added Ca^{2+}) from the activity in the presence of 0.5 mM $CaCl_2$. In-house tests showed that the calculated Ca^{2+}-ATPase activity was assumed to be the SR Ca^{2+}-ATPase activity at the approximately V_{max} levels from in-house tests.

2.6. In vivo hemodynamic study

Mice were anesthetized with intraperitoneal injection of Avertin (250 μg/g) and subjected to *in vivo* hemodynamic study as described in our previous publication [12]. After the hemodynamic study, mice were weighed and killed. Hearts were placed in phosphate-buffered saline to remove all residual blood. Hearts were then weighed, and left ventricles were immediately frozen in liquid nitrogen and stored at −80 °C.

2.7. Treadmill exercise test

SARKO and WT mice were subjected to a graded exercise stress test [14]. Mice were placed on a rodent treadmill equipped with an electric grid at the rear. The test consisted of an incremental protocol with increasing workloads and is commonly used to screen for cardiovascular disease and evaluate cardiovascular fitness. This test began with a 7° incline and 20 m/min belt speed. Belt speed was then increased linearly by 1.5 m/min every 2 min. "Failure" was defined as the inability to continue regular treadmill running. Each mouse's maximal exercise speed was used as the measure of its exercise capacity.

2.8. Heart rate variability

Senescent WT and SARKO mice were anesthetized with 2,2,2-tribrimoethanol (1.25%) and then the 3.5-g wireless radio frequency transmitter was placed on each mouse's back. Mice were allowed to recover for 72 h after surgery. ECG recordings (24 h) were obtained in an unrestrained, temperature-controlled environment with the mice housed in separate, isolated cages far away from the stimulation of other animals and with normal light-dark cycles. Mice were free to eat and drink during the recording. ECG signals were recorded from the telemetric unit with the use of an under-cage receiver (Data Sciences International), digitized at a sampling rate of 2 kHz, and fed into a microcomputer-based data acquisition system (Power Lab System, AD Instruments, Milford, MA). ECG signal processing was performed with the software program Chart v5.0 and heart rate variability (HRV) analysis with the HRV plug-in for Chart v5.0 (AD Instruments). HRV was quantified with the use of frequency-domain techniques as described previously with some modifications [20]. In the frequency domain, power spectral density of the beat interval time series was computed by use of a modified averaged periodogram method. The squared magnitudes of the discrete Fourier transform of the segments were averaged

to form the power spectral density. Three different frequency-domain measures of HRV were computed. Standard deviation of all normal R-R intervals (SDNN) was calculated directly from the sequence of interevent times. Cut-off frequencies for power in the low-frequency range (LF) and high-frequency range (HF) were defined as 0.4–1.5 Hz and 1.5–4 Hz, respectively; total power (TP) was defined as 0.00–4 Hz. LF and HF were also measured in normalized units, which represent the relative value of each power component in proportion to the sum of the HF and LF components.

2.9. Proteasome activity assay

We examined proteasome activity of the left ventricular tissues by using Cyclex Proteasome activity assay kit (Cyclex, Japan) at 37 °C as described previously [21,22]. Fluorescence intensity was measured using a 380/460 nm filter set in a fluorometer for 1–2 h at every 5 min.

2.10. Statistical analysis

All values are expressed as mean ± SEM. The group data were compared using one-way or two-way ANOVA followed by multiple comparisons testing by Turkey–Kramer's post hoc test. Statistical significance was defined as $p < 0.05$.

3. Results

3.1. Downregulation of SAR and SERCA2 in senescent mice

The young and senescent SAR knockout (SARKO) mice and C57BL/6J wild-type controls with the same genetic backgrounds were obtained from and animal facility of Waseda University. They were housed until beginning of the experiment (5, 8, 13 and 18 months of age). We attempted to survey the relationship between age and expression levels of SAR and SERCA2 proteins in normal ventricular muscles. Western blotting analyses demonstrated that both SAR and SERCA2 protein levels were significantly downregulated in senescent ventricular muscles at the age of 18-month old (Fig. 1). It should be noted that downregulation of SAR protein preceded downregulation of SERCA2 protein and was already found in ventricular muscles at the age of 8-month old.

3.2. Significant reductions in the expression of Ca^{2+} handling proteins in senescent SARKO mice

Our previous study demonstrated that the expression of SERCA protein, although not that of mRNA, was downregulated in the ventricles of young SARKO mice [12]. As we reported in that study, SERCA2a protein was significantly downregulated in the ventricular muscles of young SARKO mice than in those of young WT mice (Fig. 2A). In the present study, we investigated the effect of the disruption of SAR on age-related downregulation of SERCA2a protein. Remarkably, the expression level of SERCA2 protein in senescent SARKO mice was further decreased to 44% and 37% of that found in young SARKO and senescent WT mice, respectively. This data indicated that SAR deficiency resulted in a significant decline in SERCA2a protein during aging. Furthermore, the SERCA2a/PLN protein ratio was significantly decreased in ventricular muscles of senescent SARKO mice than those of young SARKO and senescent WT mice, although the expression level of PLN protein was also lower in ventricular muscles of senescent SARKO mice than in those of young SARKO and senescent WT (Fig. 2B). Importantly, the lowest expression level of phosphorylated PLN protein (serine 16 and threonine 17) among the groups was in ventricular muscles of senescent SARKO (Fig. 2C and D). It should be noted that intraperitoneal injection of avertin did not affect the phosphorylation status of serine 16 and threonine 17 in PLN as described previously [14].

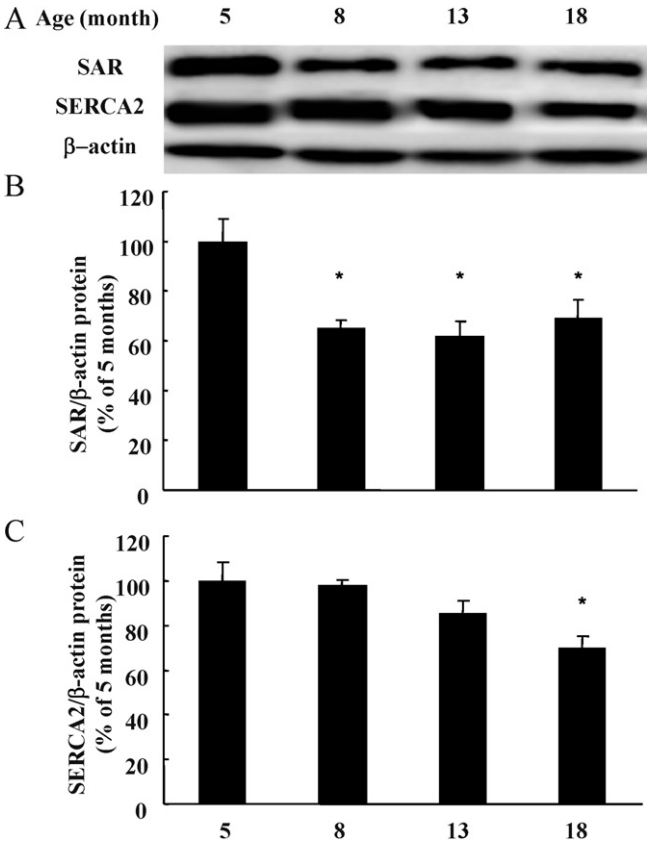

Fig. 1. Expression of SAR and SERCA2 protein in aging mouse cardiac muscle. The upper panel shows a representative western blot analysis (A). The relationship between aging and the expression levels of SERCA2 (B) and SAR (C) proteins were surveyed in cardiac muscles of wild-type mice. $n = 4$ for each group. The expression level at 5 months of age was set to 100% as a control. Protein expression was normalized by β-actin. $^{*}p < 0.05$.

We also examined other proteins related to Ca^{2+} cycling in the heart. The expression levels of calsequestrin 2 (Fig. 2E), sodium-Ca^{2+} exchanger 1 (NCX1) (Fig. 2F), and RyR2 (data not shown) proteins were significantly downregulated in senescent SARKO mice. Overall, all Ca^{2+} handling proteins we examined were downregulated in senescent SARKO mice.

3.3. Senescent SARKO mice exhibited significant reduction in SERCA2a activity and cardiac dysfunction

We also examined SERCA2a activity in SR vesicles prepared from WT and SARKO hearts (Table 1). The result in Table 1 indicated that SERCA2 activity at pH 7.2 was the lowest in senescent SARKO mice; this finding is consistent with the decrease in SERCA2a protein, SERCA2a/PLN protein ratio, and the levels of phosphorylated status of PLN protein.

Since we found that the expression and activity of SERCA2 were significantly decreased in senescent SARKO mice, we investigated the impact of the SERCA2 impairment on *in vivo* phenotypes of senescent SARKO mice. First, we examined the effects of aging on cardiac function in WT and SARKO mice (Table 1). Both systolic and diastolic functions were impaired in senescent WT and SARKO mice when compared with those in young WT mice. Both systolic and diastolic functions were decreased to a greater degree in senescent SARKO mice than in senescent WT mice. Heart rate was also the lowest in senescent SARKO mice.

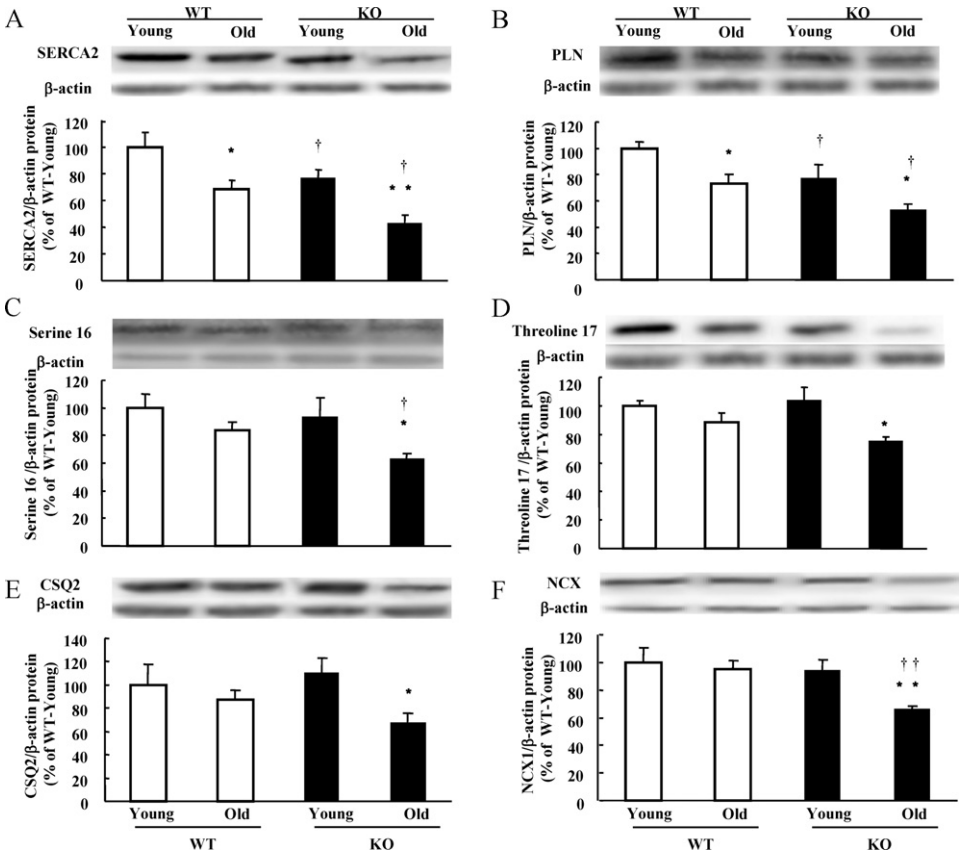

Fig. 2. Dramatic declines in SERCA2a and other Ca^{2+} handling proteins in senescent SARKO mice. Quantification of SERCA2 (A), PLN (B), and serine 16 phosphorylated PLN (C) threonine 17 phosphorylated PLN (D) levels in hearts isolated from young and senescent mice ($n = 10$, 10, and 5, respectively). (D) The SERCA2/PLN ratios in young and senescent mice. Quantification of the expression levels of CSQ2 (E) and NCX1 (F) proteins. $n = 5$ for each group. The expression level in young WT mice was set to 100% as a control. Protein expression was normalized by β-actin. * vs. young; † vs. WT; *,†$p < 0.05$; **,††$p < 0.01$.

3.4. Senescent SARKO mice exhibited higher sympathetic activity

Since abnormality of autonomic nervous tone is often associated with heart failure [23], and since we found that heart rate was lower in sedated senescent SARKO mice, we examined heart rate variability to determine the status of the autonomic nervous tone in non-sedated senescent SARKO and WT mice, using telemetric electrocardiography. Non-sedated mice exhibited a heart rate approximately 1.3 times that of sedated mice. Heart rate was lower in sedated senescent SARKO mice than in sedated senescent WT mice; likewise, heart rate was lower in non-sedated senescent SARKO mice than in non-sedated senescent WT mice (Fig. 3). Interestingly, normalized low frequency (nLF), an indicator of both sympathetic and parasympathetic tone, was higher in senescent SARKO mice than in senescent WT mice. Normalized high frequency (nHF), an indicator of parasympathetic tone, was lower

Table 1
Effects of aging on cardiac function.

	WT		SARKO	
	Young	Senescent	Young	Senescent
Number	11	12	12	13
Age, months	5.1 ± 0.1	21.2 ± 0.1	4.6 ± 0.6	19.3 ± 0.3
HR, bpm	458 ± 8	452 ± 7	438 ± 8	390 ± 18**,††
LVP, mmHg	98 ± 5	85 ± 4**	89 ± 2	67 ± 2**,††
LV d*p*/d*t* max, mmHg/s	9314 ± 364	7091 ± 657**	6836 ± 298††	3897 ± 134**,††
LV d*p*/d*t* min, mmHg/s	−5839 ± 282	−4527 ± 478**	−4504 ± 282†	−2860 ± 239**,††
EDP, mmHg	2.3 ± 0.7	2.5 ± 0.7	2.3 ± 0.3	2.3 ± 0.7
Tau, ms	6.2 ± 0.4	13.4 ± 1.1*	8.3 ± 0.4†	20.3 ± 2.8**,††
BW, g	31.7 ± 1.5	35.1 ± 1.6	27.7 ± 0.9†	26.3 ± 1.0††
atria, mg	9.3 ± 0.8	11.1 ± 0.7	8.5 ± 0.6	9.5 ± 0.5
RV, mg	24.7 ± 1.3	29.5 ± 1.2*	22.3 ± 1.3	25.9 ± 1.3†
LV, mg	107.3 ± 6.6	116.2 ± 4.1	92.0 ± 4.7	99.6 ± 4.0††
SERCA2a activity, nmol/mg/min	1133 ± 84	863 ± 69*	835 ± 63†	628 ± 50*,†

All data are presented as means ± SEM; *$p < 0.05$, **$p < 0.01$ vs. young, †$p < 0.05$, ††$p < 0.01$ vs. WT.
WT: wild-type mice; SARKO: sarcalumenin-knockout mice; HR: heart rate; bpm: beats/min; LVP: maximal left ventricular systolic pressure; LV d*p*/d*t* max or min: maximal or minimal first derivate of left ventricular pressure; EDP: end-diastolic pressure; Tau: the time course of pressure decline; RV: right ventricular; LV: left ventricular; SERCA2a: sarcoplasmic reticulum CA2+-ATPase type 2a.

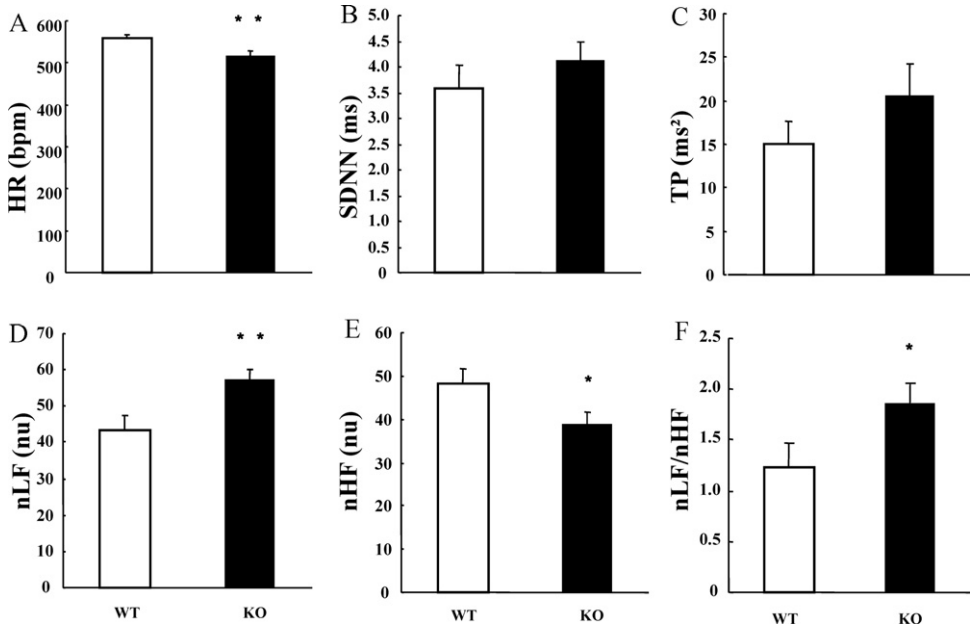

Fig. 3. Heart rate variability in senescent SARKO mice. The heart rate variability (HRV) was compared between senescent WT ($n = 6$) and senescent SARKO mice ($n = 8$) using telemetric ECG. (A) Mean heart rate (HR), (B) standard deviation of normal R-R intervals (SDNN), (C) total power (TP), (D) normalized low frequency power (LF), (E) normalized high frequency power (HF), (F) LF-to-HF ratio. * vs. WT; *$p < 0.05$; **$p < 0.01$.

in senescent SARKO mice than in senescent WT mice. The ratio of nLF to nHF was therefore higher in senescent SARKO mice than in senescent WT mice, suggesting that senescent SARKO mice exhibited higher sympathetic activity than WT mice despite their lower heart rate.

3.5. Senescent SARKO mice exhibited impaired exercise capacity

Since exercise intolerance is one of the symptoms of heart failure and often linked to the condition of cardiac dysfunction, we next examined exercise capacity in WT and SARKO mice by a treadmill-based exercise stress test (Fig. 4A). The maximal speed was slightly lower in young SARKO mice than in young WT mice, although the difference did not reach statistical significance ($p = 0.06$). Aging significantly reduced the maximal speed in both WT and SARKO mice, but the difference between young mice and senescent mice was greater among the SARKO mice. The observation that senescent SARKO mice exhibited poor exercise capacity is consistent with the results of the hemodynamic portion of this study. We also examined the effect of maximal exercise on heart rate in senescent WT and SARKO mice. Although heart rate at rest was lower in senescent SARKO mice than in senescent WT mice, peak heart rate just after exercise was the same for both genotypes (Fig. 4B), suggesting that the response to sympathetic activity was preserved in senescent SARKO mice.

3.6. Biomarkers of cardiac stress were increased in senescent SARKO hearts

The findings outlined above indicated that asymptomatic heart failure was developing in senescent SARKO mice, although no obvious symptom of heart failure such as accelerated breathing and ruffled fur was observed. We then tested for molecular markers of cardiac stress such as atrial natriuretic factor (ANF) and natriuretic factor type B (BNP). We found that the expression levels of ANF and BNP mRNAs were significantly upregulated in senescent SARKO mice (Fig. 4C and D).

3.7. Progressive degradation of SERCA2a protein in cycloheximide-treated SARKO mice

To investigate why the expression of SERCA protein was decreased in SARKO mice, we investigated whether SAR deficiency resulted in progressive degradation of SERCA2 protein and other Ca^{2+} handling proteins using SARKO mice, because our previous study demonstrated that SAR significantly prolonged the half-life of SERCA2a protein in HEK293T cells that were transfected with both SERCA2a and SAR when compared with those transfected with SERCA2a alone [13]. The treatment of cycloheximide for 48 h significantly decreased the expression of SERCA2 protein in the hearts of SARKO mice when compared with the control treatment of PBS (Fig. 5A). The effect of cycloheximide on the decreased expression of SERCA2 protein was greater in SARKO mice than in WT mice, suggesting SAR deficiency caused progressive degradation of SERCA2 protein in the heart. We also examined other Ca^{2+} handling proteins such as PLN, calsequestrin 2, and NCX1 proteins. As found in senescent SARKO mice, the expression levels of PLN, calsequestrin 2, and NCX1 proteins were significantly downregulated in cycloheximide-treated SARKO mice (Fig. 5B–D).

3.8. Senescent SARKO mice exhibited ER stress

We further attempted to examine whether endoplasmic reticulum (ER) stress involved critical degradation of SERCA. There are three response pathways (PERK, ATF6, and IRE1 pathways) that regulate the mammalian ER stress response [24]. We found that the expression levels of Bip, IRE1, EDEM, and XBP1 mRNAs were significantly increased in senescent SARKO mice whereas those of PERK and ATF6 mRNAs were not increased in senescent SARKO mice (Fig. 6), We also found that the expression of XBP1 protein was significantly increased in senescent SARKO mice (Supplemental Figure 1). Furthermore, we observed proteasomal activity in SARKO mice. Interestingly, proteasomal activity was higher in young mice than old mice. However, proteasomal activity was not different between WT and SARKO mice (Supplemental Figure 2A). We also investigated the SERCA2a protein expression in senescent ventricular muscles by using proteasome inhibitors. Even in the presence

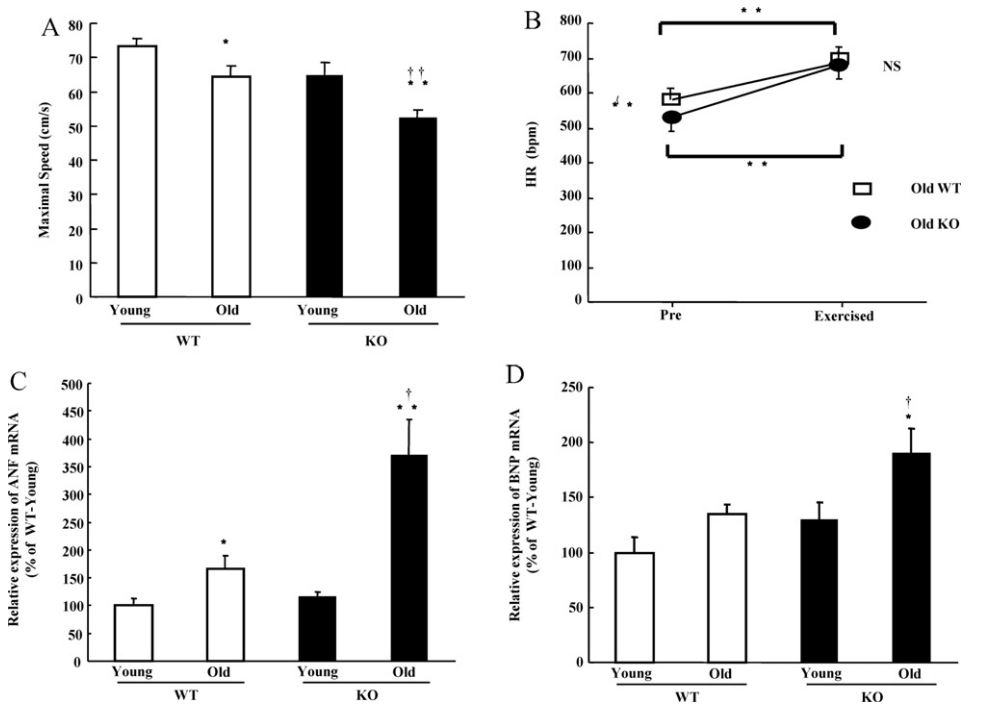

Fig. 4. Senescent SARKO mice exhibited impaired exercise capacity, and upregulation of ANF and BNP mRNAs. (A) Exercise capacity was examined by a treadmill-based exercise stress test in young WT ($n = 16$), senescent WT ($n = 12$), young SARKO ($n = 16$), and senescent SARKO mice ($n = 17$). * vs. young; † vs. WT; *,†$p < 0.05$; **,††$p < 0.01$; (B) The effect of maximal exercise on heart rate in senescent WT ($n = 8$) and SARKO ($n = 10$) mice. Although heart rate at rest was lower in senescent SARKO mice than in senescent WT mice, peak heart rate just after exercise was the same in SARKO and WT mice. Quantitative RT-PCR analyses revealed that the expression levels of ANP (C) and BNP (D) mRNAs were significantly upregulated in the ventricles of senescent SARKO mice. Young WT mice were set to 100% as a control. $n = 5$ for each group. mRNA expression was normalized by GAPDH. * vs. young; † vs. WT; *,†$p < 0.05$; **,††$p < 0.01$.

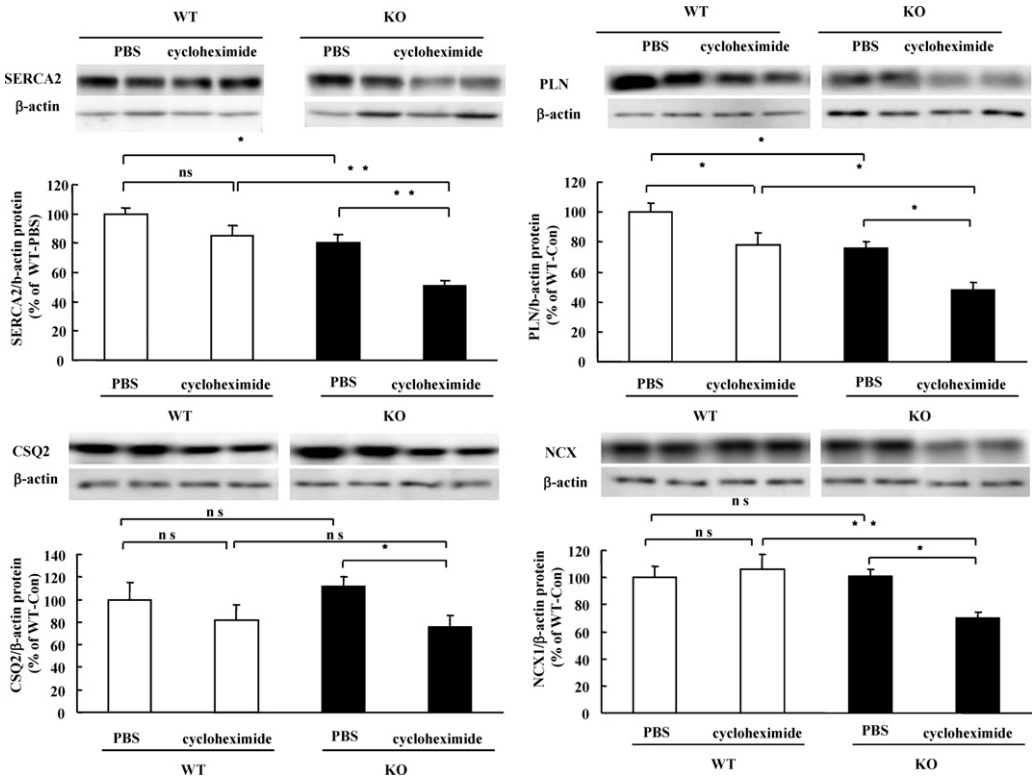

Fig. 5. Progressive decreases in SERCA2 and other Ca^{2+} handling proteins after cycloheximide treatment in senescent SARKO mice. Quantification of the expression levels of SERCA2 (A), PLN (B), CSQ2 (C), and NCX1 (D) proteins. $n = 4$–6 for each group. Protein expression was normalized by β-actin. $n = 5$ for each group. The expression level in PBS-treated WT mice was set to 100% as a control. * and ** indicate $p < 0.05$ and $p < 0.01$, respectively. PBS: physiological saline; ns: not significant.

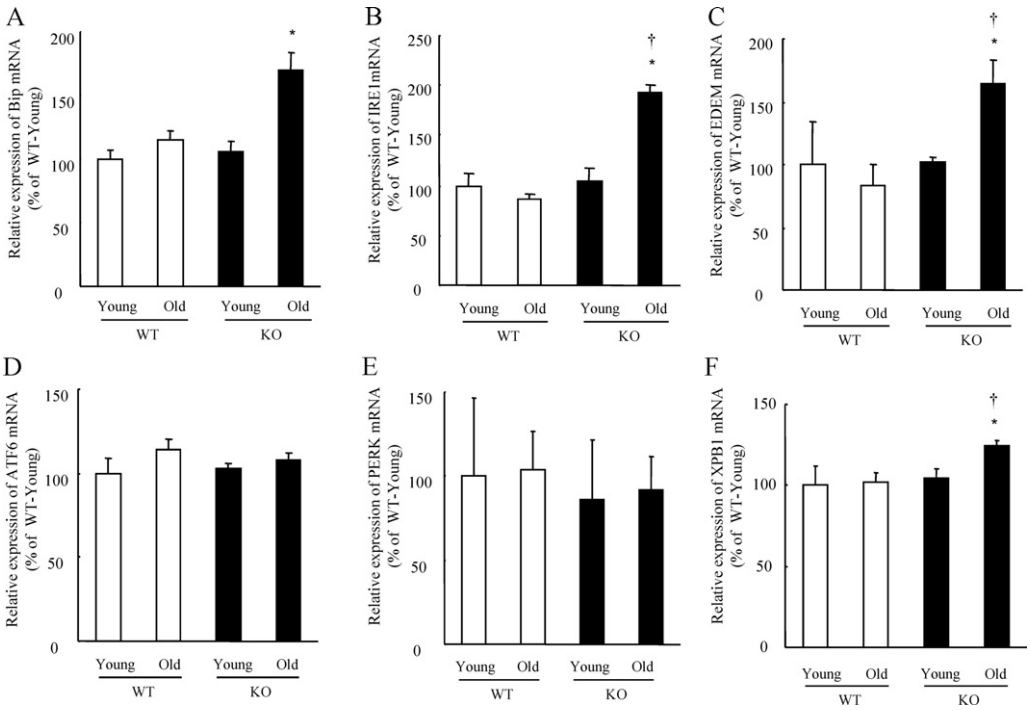

Fig. 6. A significant increase in activation of the ER stress pathway in senescent SARKO mice. Quantitative RT-PCR analyses revealed that the expression levels of Bip (A), IRE1 (B), EDEM (C), and XBP1 (F) mRNAs were significantly increased in the ventricles of senescent SARKO mice. Young WT mice were set to 100% as a control. $n = 4$ for each group. mRNA expression was normalized by GAPDH. * vs. young; † vs. WT; *,†$p < 0.05$.

of proteasome inhibitors, the expression level of SERCA2a protein in senescent SARKO mice was decreased to 64% of that found in senescent WT mice (Supplemental Figure 2B). These data provided evidence that senescent SARKO mice exhibited ER stress without an increase in proteasomal activity.

4. Discussion

Several studies have demonstrated that SERCA2a is one of the most important determinants of cardiac function. The expression levels and/or activity of SERCA2a are often decreased in the failing heart. While our previous study revealed a modest decrease in the expression of SERCA2 and cardiac function in young SARKO mice [12], a major finding in the present study is a marked decrease in cardiac function and the expression and activity of SERCA2 in senescent SARKO mice. Furthermore, we have recently reported that pressure overload stress induced by transverse aortic constriction significantly decreased the expression levels of SERCA2 protein and cardiac function in young SARKO mice [13]. In addition, our previous study have reported that SAR is a primary target for exercise-related adaptation of the Ca^{2+} storage system in the SR to preserve cardiac function [14]. These studies indicate that SAR plays an important role in preserving the expression levels of SERCA2 protein and cardiac function under biological and mechanical stresses such as aging and pressure overload.

Significantly, downregulation of SAR protein preceded that of SERCA2 as the mice aged. Downregulation of SAR also preceded that of SERCA2 when pressure-overload was introduced into the heart [13]. These observations suggest that downregulation of SAR triggers a further reduction in the expression level of SERCA2a protein, especially in the stressed hearts. Accordingly, in SERCA2a-transformed HEK293 cells, we found that the stability of SERCA2a protein was significantly lower in the absence of co-transformation of SAR than in the presence of co-transformation of SAR [13]. Consistent with the *in vitro* study, an *in vivo* experiment in the present study demonstrated that SAR deficiency caused progressive degradation of SERCA2 protein in the heart (Fig. 4). Therefore, we postulate that the loss of SAR promotes a subsequent degradation of SERCA2a protein and decreased SERCA2a activity in the process of aging. It also should be noted that the ratio of phosphorylated PLN to total PLN protein was significantly lower in senescent SARKO mice than in young SARKO mice. These eventually resulted in advancing progressive cardiac dysfunction in senescent SARKO mice. In addition to a decrease in the expression level of sarcalumenin, the status of phosphorylation of sarcalumenin may be important for SERCA2a activity like PLN, because cardiac sarcalumenin has been shown to undergo phosphorylation as described previously [25]. It is intriguing to investigate whether the phosphorylation level of SAR is changed in mice under pathophysiological stresses. The important question should be addressed in a future study.

In addition to a decrease in SERCA2 protein, the present study demonstrated that other Ca^{2+} handling proteins were downregulated in senescent SARKO mice, although the expression levels of these proteins were the same in young and senescent WT mice. This result indicated that Ca^{2+} cycling in senescent SARKO mice was largely deteriorated, which also contributed to progressive cardiac dysfunction in these animals. It is well documented that NCX1 is often upregulated to compensate for the dysfunction of Ca^{2+} reuptake into the SR [26], but this mechanism does not work in senescent SARKO mice, at least at the protein expression level. Although the mechanism by which SAR deficiency induces a significant reduction in other Ca^{2+} handling proteins remains unclear, the *in vivo* experiment using cycloheximide suggested that SAR played an important role in stability of Ca^{2+} handling proteins such as SERCA2, PLN, CSQ2, and NCX1 (Fig. 5). SAR deficiency may result in degradation of Ca^{2+} handling proteins in the aging heart through the deterioration of Ca^{2+} homeostasis in the SR or due to progressive ER stress (Fig. 6). Further study is required to prove this assumption.

Although senescent SARKO mice exhibited no obvious symptom of heart failure such as accelerated breathing and ruffled fur,

several typical clinical findings of heart failure such as increased sympathetic activity (Fig. 3), impaired exercise tolerance (Fig. 4A and B), and upregulation of ANF and BNP mRNAs (Fig. 4C and D) were observed. Since heart failure is a syndrome with many symptoms and findings from clinical examinations, these evidences indicated that asymptomatic heart failure was developing in senescent SARKO mice. In this regard, the findings observed in senescent SARKO mice were similar with those seen in senescent human population. We also found a significant reduction in heart rate in senescent SARKO mice. The reduction in heart rate could be caused by downregulation of the β-adrenergic signaling pathway in the sinus node; this explanation is supported by our observation of a sustained increase in sympathetic activity in senescent SARKO mice. On the other hand, heart rate increased normally in response to exercise and infusion of isoproterenol (data not shown) in senescent SARKO mice, indicating that the responsiveness of the sinus node to a β-adrenergic signal remains normal and the decrease in heart rate was not due to sinus node dysfunction.

As malfolded proteins and protein aggregates can evoke ER stress, it has been speculated that ER stress is involved in most conformational diseases, particularly Alzheimer's disease and Parkinson's disease, although it is still heavily disputed whether ER stress (or protein aggregates) is a major cause of these diseases. Our data suggested that SAR deficiency is susceptible to ER stress. These findings support that the presence of SAR is necessary for proper protein folding in aged cardiac cells. While specific role of SAR in this process remains to be elucidated in further study, it is possible that SAR could directly act as a molecular chaperone in the SR.

On the other hand, it has been reported that skeletal muscle from SARKO mice is highly resistance to fatigue when compared with wild-type muscle, likely due to enhanced store-operated Ca^{2+} entry (SOCE) induced by upregulated expression of mitsugumin 29 (MG29), a synaptophysin-related membrane protein located in the triad junction [27]. The improved performance in SARKO skeletal muscle might contradict our present observations that cardiac function is clearly worsened in SARKO mice. However, it should be noted that MG29 is not expressed in the heart and that its expression did not become detectable in both WT and SARKO hearts (data not shown). It appears that SAR effects in striated muscle may be dependent on the expression of MG29, with knockout of SAR producing fatigue resistance in skeletal muscle where MG29 is expressed and reduced SAR expression producing detrimental effects in aged cardiac muscle where MG29 in not expressed. Therefore, the impaired exercise capacity in the aged SARKO mice would probably result from the compromised cardiac function rather than a change in skeletal muscle.

In conclusion, the present study suggested that SAR is essential for maintaining the Ca^{2+} transport activity of SERCA2a. Since impaired SERCA2a activity is a hallmark of cardiac dysfunction in the senescent population, this study uncovered the important role of SAR in SERCA2a activity and thus in cardiac function, especially in the aging heart.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ceca.2011.10.003.

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