Fig. 1

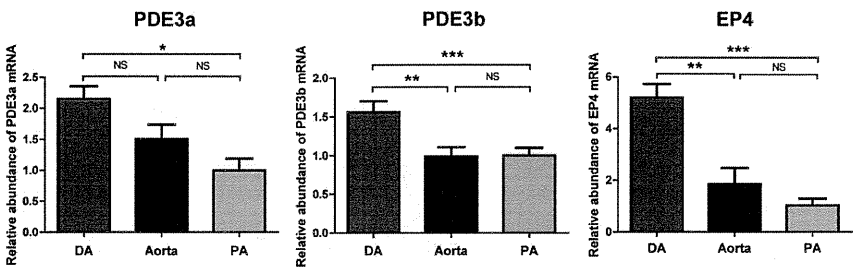

Figure 1. Quantitative RT-PCR analyses of PDE3a, PDE3b, and EP4 in rat e21 DA, aorta, and pulmonary artery (PA) tissue. n = 4–5, *p < 0.05, **p < 0.01, ***p < 0.001, NS indicates not significant.
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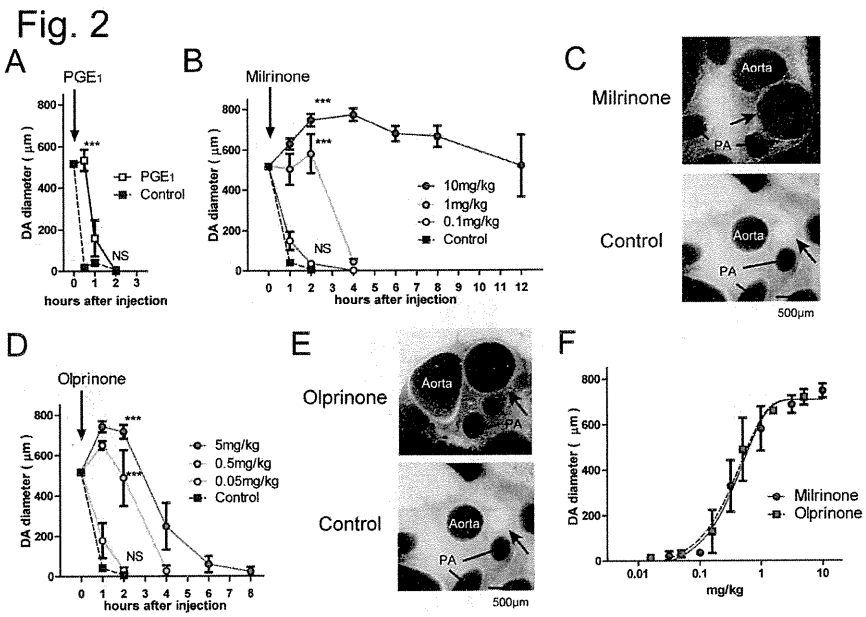

Figure 2. The effects of milrinone and olprinone on vasodilation of the DA as observed by the rapid whole-body freezing method. (A) PGE1 (10 μg/kg)-induced dilation of rat DA (n = 4–6). (B) Vasodilatory effect of milrinone on rat DA. Rat neonates were intraperitoneally injected with milrinone (n = 4–6). (C) Representative images of rat DAs treated with 10 mg/kg of milrinone or saline (control) for 2 h using the whole-body freezing method (arrow). (D) Vasodilatory effect of milrinone on rat DA. Rat neonates were intraperitoneally injected with olprinone (n = 4–6). (E) Representative images of rat DAs treated with 5 mg/kg of olprinone or control for 2 h using the whole-body freezing method (arrow). (F) Milrinone or olprinone dilated DA in a dose-dependent manner. Vasodilatory effects of PDE3 inhibitors were examined 2 h after injection (n = 4–6). *** $p < 0.001$ and NS vs. control. NS indicates not significant.
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Fig. 3

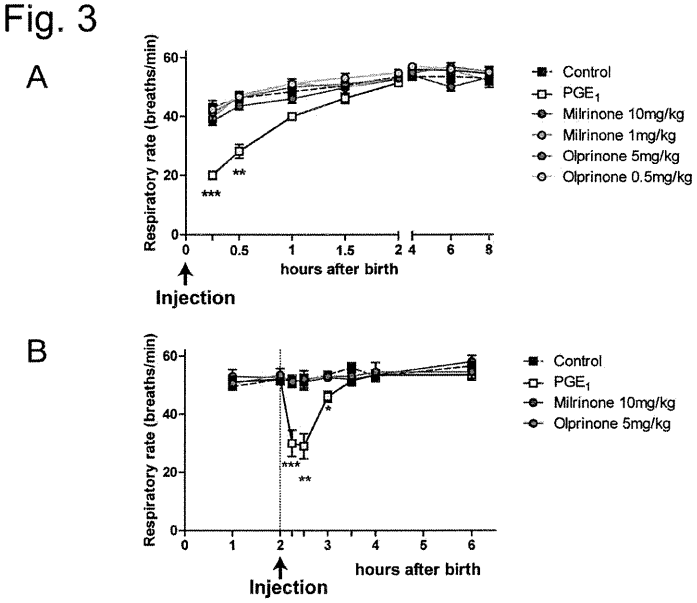

Figure 3. Effects of PDE3 inhibitors and PGE1 on respiratory distress. (A) Respiratory rate of rat neonates administered each drug immediately after birth, the same as in Figure 2 (n = 6–9). (B) Respiratory rate of rat neonates administered each drug 2 h after birth (n = 4). *p < 0.05, **p < 0.01 and ***p < 0.001 vs. control. No mark indicates not significant vs. control.
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Fig. 4

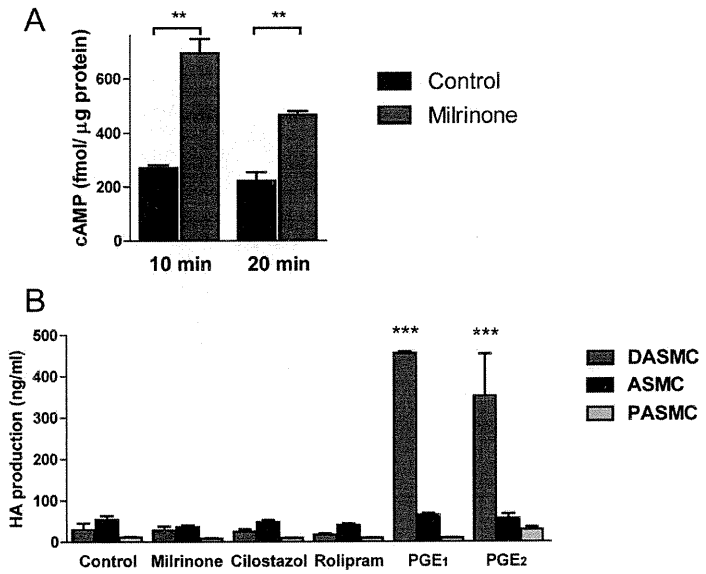

Figure 4. Milrinone increased cAMP production, however, it did not induce HA production. (A) Milrinone (10 μM) significantly increased cAMP accumulation in DASMCs (n = 4). (B) HA production in SMCs treated with milrinone (10 μM), cilostazol (10 μM), rolipram (10 μM), PGE1 (1 μM), or PGE2 (1 μM) (n = 4–6). Cilostazol: PDE3 inhibitor. Rolipram: PDE4 inhibitor. **p < 0.01 and ***p < 0.001 vs. control. No mark indicates not significant vs. control.
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Fig. 5

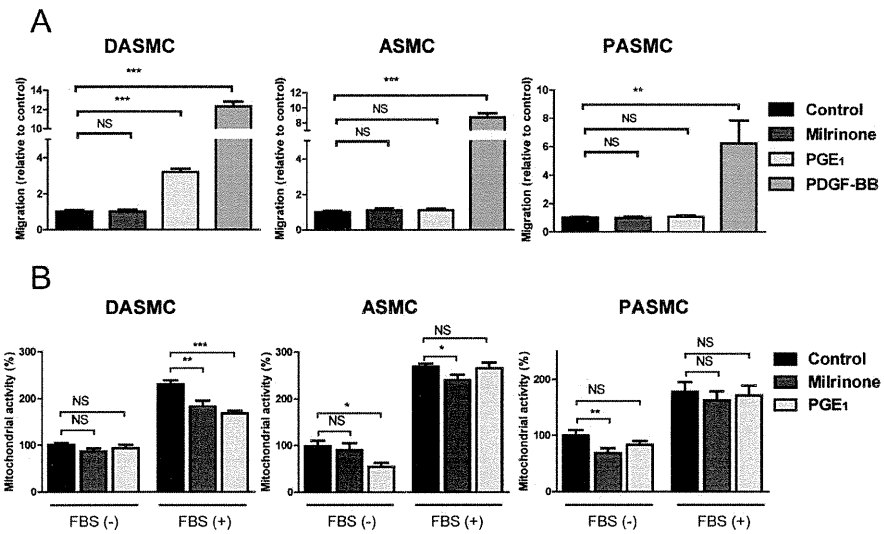

Figure 5. Milrinone did not promote migration and proliferation in SMCs. (A) Migration of SMCs treated with milrinone (10 μM), PGE1 (1 μM), or PDGF-BB (10 ng/ml) using the Boyden chamber method (n = 4–5). (B) Proliferation of SMCs treated with milrinone (10 μM) or PGE1 (1 μM) in the presence of 0 or 10% FBS by an MTT assay (n = 5–9). *p < 0.05, **p < 0.01 and ***p < 0.001. NS indicates not significant.
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Fig. 6

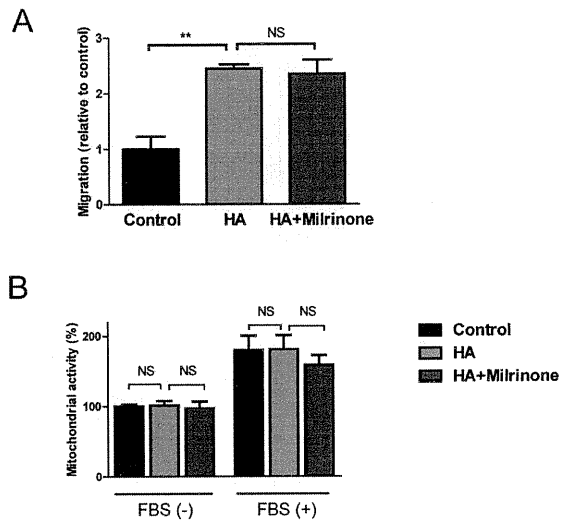

Figure 6. Effect of co-treatment of HA with milrinone on migration and proliferation in DASMCs. (A) Migration of SMCs with co-treatment of HA (200 ng/ml) and milrinone (10 μM) using the Boyden chamber method (n = 4–5). (B) Proliferation of SMCs with co-treatment of HA (200 ng/ml) and milrinone (10 μM) in the presence of 0 or 10% FBS by an MTT assay (n = 8). **p < 0.01, NS indicates not significant.
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Fig. 7

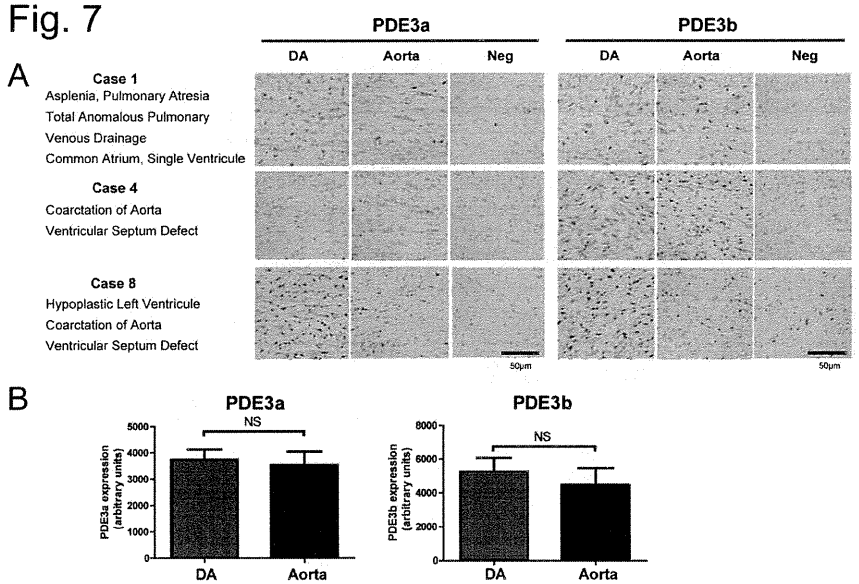

Figure 7. (A) Representative images of immunoreaction to PDE3a and PDE3b in the human DA and aortic smooth muscle layers from various CHDs. No immunoreaction was detected when omitting the primary antibody as in PDE3a Neg and PDE3b Neg. (B) Quantification of PDE3a and PDE3b in the DA and the aorta by a color extraction method (n = 4). NS indicates not significant.
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Table 1.

Summary of patient characteristics

Case No.	Age at Operation	Diagnosis
1	0 days	Asplenia, PA, TAPVD, CA, SV
2	1 day	Asplenia, CoA, CA, SV
3	2 days	IAA, Aorticopulmonary window
4	2 days	CoA, VSD
5	3 days	TGA, CoA
6	4 days	CoA, VSD
7	13 days	CoA, VSD
8	1 month	hypoLV, CoA, VSD

PA: Pulmonary Atresia, TAPVD: Total Anomalous Pulmonary Venous Drainage,
CA: Common Atrium, SV: Single Ventricule,
CoA: Coarctation of Aorta, IAA: Interruption of Aortic Arch,
VSD: Ventricular Septum Defect, TGA: Transposition of the Great Arteries,
hypoLV: Hypoplastic Left Ventricule

Sarcalumenin is essential for maintaining cardiac function during endurance exercise training

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Sarcalumenin is essential for maintaining cardiac function during endurance exercise training

Qibin Jiao,[1] Yunzhe Bai,[1] Toru Akaike,[1] Hiroshi Takeshima,[2] Yoshihiro Ishikawa,[1,3] and Susumu Minamisawa[1,4,5]

[1]*Cardiovascular Research Institute, Yokohama City University Graduate School of Medicine, Yokohama, Japan;* [2]*Department of Medical Chemistry, Kyoto University Graduate School of Pharmaceutical Science, Kyoto, Japan;* [3]*Cardiovascular Research Institute, Departments of Cell Biology and Molecular Medicine and Medicine (Cardiology), New Jersey Medical School, Newark, New Jersey;* [4]*Department of Life Science and Medical Bioscience, and* [5]*Institute for Biomedical Engineering, Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, Tokyo, Japan*

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Jiao Q, Bai Y, Akaike T, Takeshima H, Ishikawa Y, Minamisawa S. Sarcalumenin is essential for maintaining cardiac function during endurance exercise training. *Am J Physiol Heart Circ Physiol* 297: H576–H582, 2009. First published June 5, 2009; doi:10.1152/ajpheart.00946.2008.—Sarcalumenin (SAR), a Ca^{2+}-binding protein located in the longitudinal sarcoplasmic reticulum (SR), regulates Ca^{2+} reuptake into the SR by interacting with cardiac sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a). We have previously demonstrated that SAR deficiency induced progressive heart failure in response to pressure overload, despite mild cardiac dysfunction in sham-operated SAR knockout (SARKO) mice (26). Since responses to physiological stresses often differ from those to pathological stresses, we examined the effects of endurance exercise on cardiac function in SARKO mice. Wild-type (WT) and SARKO mice were subjected to endurance treadmill exercise training (~65% of maximal exercise ability for 60 min/day) for 12 wk. After exercise training, maximal exercise ability was significantly increased by 5% in WT mice ($n = 6$), whereas it was significantly decreased by 37% in SARKO mice ($n = 5$). Cardiac function assessed by echocardiographic examination was significantly decreased in accordance with upregulation of biomarkers of cardiac stress in SARKO mice after training. After training, expression levels of SERCA2a protein were significantly downregulated by 30% in SARKO hearts, whereas they were significantly upregulated by 59% in WT hearts. Consequently, SERCA2 activity was significantly decreased in SARKO hearts after training. Furthermore, the expression levels of other Ca^{2+}-handling proteins, including phospholamban, ryanodine receptor 2, calsequestrin 2, and sodium/calcium exchanger 1, were significantly decreased in SARKO hearts after training. These results indicate that SAR plays a critical role in maintaining cardiac function under physiological stresses, such as endurance exercise, by regulating Ca^{2+} transport activity into the SR. SAR may be a primary target for exercise-related adaptation of the Ca^{2+} storage system in the SR to preserve cardiac function.

treadmill; calcium uptake; heart failure; excitation-contraction coupling

ENDURANCE EXERCISE IS ONE of the most common physiological stresses affecting the homeostasis of the whole body. Adaptations to chronic endurance exercise result in functional and structural changes in the heart (19, 31, 33); for example, after chronic endurance exercise training, it has been shown that resting heart rate is decreased and that maximal stroke volume is increased, since myocardial contractile function is enhanced and left-ventricular cavity dimension is augmented (2, 14, 25). A growing body of evidence has demonstrated that the regulation of intracellular Ca^{2+} through the sarcoplasmic reticulum (SR) plays a critical role in maintaining cardiac function under both physiological and pathological stresses (5, 7, 17). In particular, rapid transport of Ca^{2+} from the cytosol to the SR via the cardiac sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2a (SERCA2a) is a critical determinant for the maintenance of Ca^{2+} storage in the SR. Therefore, it is extremely important for us to understand the effect of endurance exercise training on SERCA2a function and thus on the Ca^{2+} storage system in the heart. In this regard, a considerable number of previous studies on animals have demonstrated that endurance exercise training increases the expression and/or activity of SERCA2a in the heart, resulting in enhanced cardiac function of the healthy (9, 10, 20, 22, 30, 35) or pathological heart (6, 15, 21, 24, 34, 39).

Sarcalumenin (SAR) is an SR luminal glycoprotein responsible for Ca^{2+} buffering in skeletal and cardiac muscles (13, 16). SAR is predominantly found in the longitudinal SR, where SERCA and phospholamban (PLN) are also located. Our laboratory's previous study has demonstrated that SAR interacts with SERCA2 to enhance the protein stability of SERCA2a, and that it facilitates Ca^{2+} sequestration into the cardiac SR (26). Although young sedentary SAR knockout (SARKO) mice exhibit only mild impairments in Ca^{2+} transient and cardiac function (38), we have recently demonstrated that SAR deficiency induced progressive heart failure in response to pressure overload (26), indicating that SAR plays a critical role in adapting to pathological stresses, such as pressure overload in the heart. We found that SAR is essential for maintaining SERCA2a expression and activity in the pressure-overloaded heart. However, it has recently been reported that skeletal muscle from SARKO mice is highly resistant to fatigue compared with that from wild-type (WT) mice (40); this fatigue resistance of SARKO skeletal muscle is likely due to enhanced store-operated Ca^{2+} entry (SOCE) induced by upregulated expression of mitsugumin 29 (MG29), a synaptophysin-related membrane protein that is not expressed in the heart. In addition, it is known that the heart often responds differently to physiological stresses, such as endurance exercise, than to pathological stresses, such as pressure overload. Therefore, it remains unknown whether SAR also plays a role in maintaining cardiac function when the heart is exposed to physiological stresses, such as endurance exercise. To clarify the mode of action of SAR in the heart under a physiological stress, such as endurance exercise training, we investigated the

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impact of SAR deficiency on the expression and activity of SERCA2a in the heart and on cardiac function after endurance exercise training.

MATERIALS AND METHODS

Animal preparation. Generation of SARKO mice has been described previously (38). SARKO and C57BL/6J WT mice (8–10 wk of age) were bred at Yokohama City University. All mice used in the present study came from the same genetic background. All animal care and study protocols were approved by the Animal Ethics Committees of Yokohama City University School of Medicine and Waseda University, and the investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (National Institutes of Health Publication No. 85–23, revised 1996).

Maximal exercise ability and treadmill endurance exercise training. Mice were randomized into four groups: sedentary WT (SED-WT) and sedentary SARKO (SED-SARKO) mice, and WT (ET-WT) and SARKO (ET-SARKO) mice subjected to endurance exercise training.

Animals ran on a rodent motor-driven treadmill (MANUAL, LE 8700 series, Panlab, Barcelona, Spain) with adjustable belt speed (0–150 cm/s). The treadmill apparatus was equipped with adjustable-amperage (0–2 mA) shock bars at the rear of the belt, through which mild electrical stimulation (grid shock <1 mA) was applied to encourage the mice to run. A detector located above the shock grid measured the number of shock stimuli received by each mouse.

First, mice were acclimated to the treadmill via three 15-min running sessions with mild shock stimulation and a belt speed of 30 cm/s. After acclimation, all mice underwent a treadmill exercise test to determine their exercise ability before the endurance exercise training described below; a similar assessment was made during and after training for comparison purposes. The belt speed of the treadmill was set to 30 cm/s at the beginning of each test. It was then increased linearly by 2 cm/s every 30 s until the mice could not continue to run regularly on the treadmill, or until they had rested on the shock grid more than three times. The final belt speed achieved by each mouse was considered to be that mouse's maximal exercise ability. Maximal exercise ability was determined by averaging the maximal belt speeds of at least three measurements for each mouse; there was an intermission of at least 1 h between each measurement. Workloads of endurance exercise training were then adjusted for each mouse in accordance with its maximal exercise ability.

Before the start of each exercise training session, each mouse performed a 5-min warm-up at 40% of its maximal speed. ET-WT and ET-SARKO mice then ran on the treadmill (at 0° inclination) at 65% of their maximal speeds for 60 min/day, 5 days/wk, for 12 wk. Each mouse's maximal exercise ability was reevaluated every 4 wk, and each mouse's workload was adjusted again based on its current maximal speed (Supplemental Fig. 1). (The online version of this article contains supplemental data.) For sedentary mice, running skill was maintained by treadmill running for 15 min at 0° inclination at a belt speed of 30 cm/s, 3 days/wk.

Citrate synthase activity. As a marker for endurance training, the myocardial citrate synthase (CS) activity was measured at 37°C in the presence of 0.2% Triton X-100 with 20 μg protein sample, as previously described (27, 32). CS activity was also measured in soleus muscle homogenates to assess the efficacy of endurance exercise training.

Cardiac function assessed by echocardiography. Mice were anesthetized with an intraperitoneal injection of Avertin (250 μg/g) and subjected to echocardiography, as described in our laboratory's previous publications (28, 38). Since we have observed that the heart rates of mice decrease after intraperitoneal injection of Avertin, reaching stable minimal levels around 15–20 min after injection (Supplemental Fig. 2), we obtained the echocardiographic data around 15–20 min after injection of Avertin. After the final assessment of cardiac function after endurance training, heart and skeletal (soleus) 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 stored at −80°C.

Quantitative RT-PCR analysis. Total RNA was isolated from various tissues using TRIzol reagent (Invitrogen, Carlsbad, CA), as recommended by the manufacturer. Generation of cDNA and RT-PCR analysis was performed as described previously (36, 37). 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.

Immunoblot analysis. We prepared protein samples from the left ventricular tissues of the sedentary and trained mice, which had been immediately frozen and stored at −80°C after death of the animals. Immunoblot analyses were performed as described previously (26, 36). 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), and BSA (0.1–1 mg/ml) was used as a standard. The protein samples (20 μg) were separated in the same gel by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). When the molecular size of target proteins was different, polyvinylidene difluoride 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]. Antibodies used in the present study are shown in Supplemental Table 1. 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 three times with 0.1% Tris buffered saline-Tween 20 buffer.

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 5 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, with or without 0.5 $CaCl_2$]. The reaction medium was preincubated 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), as described previously (8). 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 $CaCl_2$.

Statistical analysis. All values are expressed as means ± SE. Comparisons of data from multiple groups were performed by unpaired ANOVA followed by the Student Newman-Keuls post hoc test. Statistical significance was defined as $P < 0.05$.

RESULTS

Effects of endurance exercise training on exercise ability in SARKO mice. Before the start of endurance exercise training, exercise ability was examined in WT and SARKO mice by a treadmill-based exercise stress test, described above. Maximal exercise ability, as evaluated by maximal belt speed, was lower in SARKO mice (n = 16, 65.0 ± 3.6 cm/s) than in WT mice

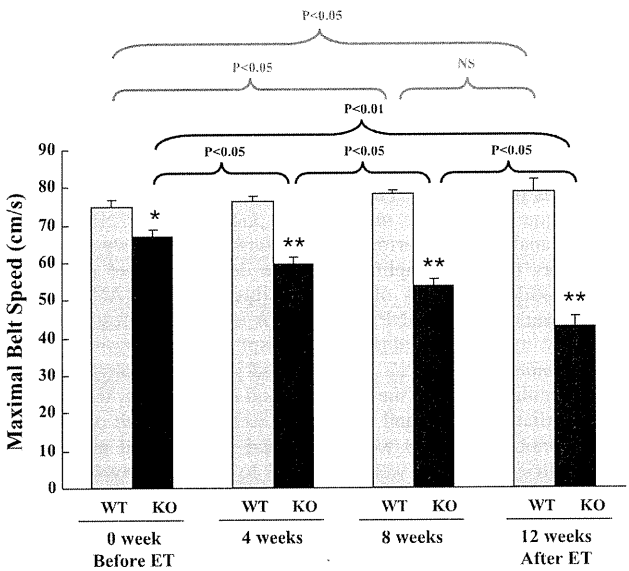

Fig. 1. The effect of endurance exercise training (ET) on maximal exercise ability in sarcalumenin (SAR) knockout (SARKO) mice. Maximal exercise ability, as evaluated by maximal belt speed, is already lower in SARKO mice than in wild-type (WT) mice before ET. During ET, maximal exercise ability gradually increased in WT mice, whereas it actually decreased in SARKO mice in a time-dependent manner. Maximal exercise ability after ET was significantly increased in WT mice, whereas it was actually decreased in SARKO mice compared with their maximal exercise ability before the training. Values are means ± SE; n = 6 and 5 for WT and knockout (KO), respectively.

(n = 16, 74.1 ± 2.1 cm/s), although it did not reach a statistical significance (P = 0.059). As expected, maximal exercise ability in sedentary animals (SED-WT and SED-SARKO mice) did not significantly change during the 12-wk training period (data not shown). In ET-WT mice, maximal exercise ability gradually increased during endurance exercise training, whereas, in ET-SARKO mice, it gradually decreased (Fig. 1). Whenever a change in a mouse's maximal exercise ability was detected by a regular treadmill test, that mouse's training workload was adjusted based on its current maximal speed (Supplemental Fig. 1). Maximal exercise ability after endurance exercise training significantly increased by 5% in ET-WT mice, whereas it actually decreased by 37% in ET-SARKO mice compared with their ability measured before the training regime began (Fig. 1).

Exercise training did not improve CS activity in SARKO mice. We observed no difference between WT and SARKO mice in terms of CS activity of skeletal or cardiac muscle at a basal condition. After the endurance exercise training, ET-WT mice exhibited increased CS activity of soleus muscle (Fig. 2*A*), indicating an appropriate effect of the training program on working muscles. In accordance, they also exhibited increased CS activity of cardiac muscle (Fig. 2*B*), which is consistent with several previous studies (1, 20), although most of previous studies have demonstrated that CS activity is not increased or little increased by endurance exercise in rodent hearts (4, 19). In ET-SARKO mice, on the other hand, CS activity was not increased in either soleus or cardiac muscle (Fig. 2).

Endurance exercise training resulted in cardiac dysfunction in SARKO mice. To examine the effect of endurance exercise training on cardiac function, we investigated it using transthoracic echocardiography. Before endurance exercise training, all parameters listed in Table 1 were similar between WT and SARKO mice, including body weight, heart rate, left ventricular fractional shortening, thickness of myocardial walls, and ejection time. After endurance exercise training, left ventricular fractional shortening was significantly decreased in ET-SARKO mice, whereas it was not changed in ET-WT mice (Table 1). As we expected, the diameter of the end-diastolic left ventricular chamber was significantly increased in ET-SARKO mice. Furthermore, ejection time was significantly prolonged in ET-SARKO mice, and their heart rate corrected velocity of circumferential fiber shortening was significantly lower (Table 1).

Biomarkers of cardiac stress were increased in ET-SARKO hearts. To examine the effect of endurance exercise training on the myocardium itself, we measured molecular markers of

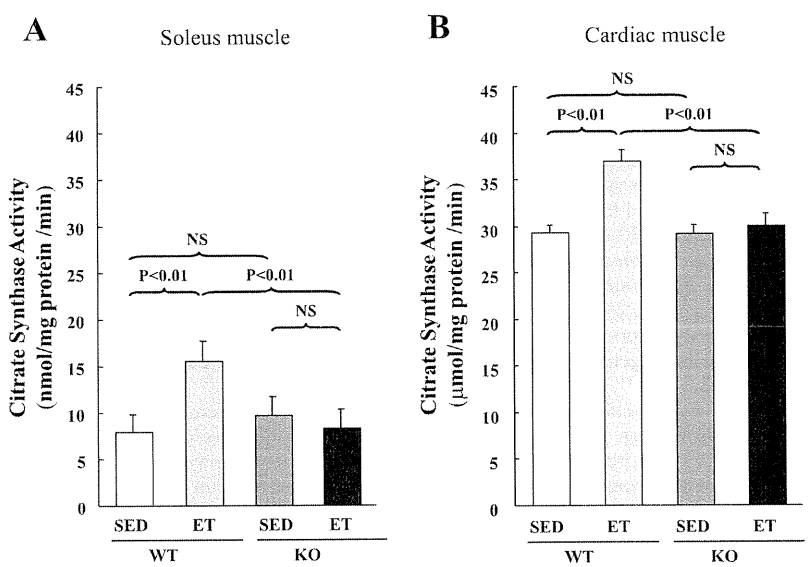

Fig. 2. Citrate synthase (CS) activity after ET. After ET, CS activity of soleus muscle (*A*) and cardiac muscle (*B*) was increased in ET-WT mice, but not in ET-SARKO mice. Values are means ± SE; n = 5 for each group. SED, sedentary; NS, not significant.

Table 1. *Cardiac function after endurance exercise training*

	SED-WT		ET-WT		SED-SARKO		ET-SARKO	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
n	10	10	6	6	10	10	6	5
BW, g	24.2±1.3	32.6±1.9	26.3±1.7	31.1±1.3	23.3±1.0	27.3±1.1	22.6±1.2	26.3±1.8
HR, beats/min	464±15	474±15	438±18	436±18	430±12	409±14	449±24	425±14
LV weight, mg		115±9		111±7		93±6		86±7
LV-to-BW weight ratio, mg/g		3.55±0.25		3.58±0.15		3.38±0.12		3.26±0.15
LV FS, %	35.6±1.1	35.5±1.0	34.7±1.2	34.9±2.6	37.5±1.3	34.0±1.2	38.6±2.7	28.4±1.1[a,c,d]
LVIDd, mm	4.11±0.08	4.12±0.11	4.03±0.06	4.25±0.12	3.88±0.10	4.02±0.09	3.84±0.12	4.14±0.19[a]
IVSTd, mm	0.77±0.03	0.79±0.05	0.77±0.02	0.71±0.05	0.76±0.02	0.67±0.01[b]	0.76±0.02	0.66±0.02[b]
LVPWTd, mm	0.76±0.03	0.75±0.04	0.76±0.04	0.76±0.06	0.72±0.07	0.65±0.04[a]	0.69±0.02	0.67±0.02[a,c]
Ejection time, ms	60±1	60±1	64±2	66±3	64±2	65±2	58±2	69±3[a]
Vcfc, circumferences/s	2.14±0.06	2.13±0.07	2.12±0.10	2.14±0.17	2.21±0.10	2.04±0.14	2.44±0.18	1.56±0.05[b,c,d]

Values are means ± SE; *n*, no. of mice. SED, sedentary; WT, wild-type mice; ET, endurance exercise training; SARKO, sarcalumenin-knockout mice; Pre, before ET; Post, after ET; BW, body weight; HR, heart rate; LV, left ventricle; FS, fractional shortening; LVIDd, LV internal dimensions at end diastole; IVSTd, interventricular septum thickness at end diastole; LVPWTd, LV posterior wall thickness at end diastole; Vcfc, corrected velocity of circumferential fiber shortening. Significant difference vs. Pre: [a]$P < 0.05$ and [b]$P < 0.01$; vs. WT: [c]$P < 0.05$; and vs. SED: [d]$P < 0.05$.

cardiac stress, such as ANF and BNP mRNAs. These were significantly upregulated in ET-SARKO mice (Fig. 3). Endurance training did not affect the expression of ANF and BNP mRNAs in ET-WT mice.

Significant reductions in the expression of Ca^{2+} handling proteins in ET-SARKO mice. Since the expression levels of SERCA2a and other Ca^{2+} handling proteins are critical for the regulation of cardiac function, we examined them by Western blot analyses (Fig. 4, Table 2). Consistent with our laboratory's previous report (26, 38), the expression levels of SERCA2a and total PLN were significantly downregulated in SED-SARKO mice compared with those in SED-WT mice. After endurance exercise training, the expression level of SERCA2a protein was significantly increased by 59% in ET-WT mice, whereas it was reduced by 30% in ET-SARKO mice compared with sedentary mice of each group's respective genotype. Endurance exercise training also resulted in a further significant downregulation of both total and phosphorylated PLN proteins in ET-SARKO mice, but not in ET-WT mice. The SERCA2a-to-PLN protein ratio was significantly decreased in ventricular muscles of ET-SARKO mice (Table 2). The ratio of phosphorylated threonine 17 PLN to total PLN protein was significantly lower in ET-SARKO than in ET-WT, but that of serine 16 to total PLN protein was not (Table 2). It should be noted that intraperitoneal injection of Avertin did not affect the phosphorylation status of serine 16 and threonine 17 in PLN (Supplemental Fig. 2).

The expression levels of calsequestrin 2 (CSQ2) and ryanodine receptor type 2 (RyR2) proteins in SED-SARKO mice were comparable to those in SED-WT mice, while those of sodium/calcium exchanger 1 (NCX1) protein were even higher in SED-SARKO mice than in SED-WT mice. After the endurance exercise training, all of these proteins were significantly downregulated in ET-SARKO mice, but not in ET-WT mice (Fig. 4, Table 2). Overall, in addition to SERCA2, all other Ca^{2+} handling proteins that we examined were downregulated in ET-SARKO mice after endurance exercise training.

Significant reduction in SERCA2a activity in ET-SARKO mice. As measured in myocardial homogenates, maximal Ca^{2+}-ATPase activity was lower in SED-SARKO mice than in SED-WT mice (Fig. 5). After the endurance exercise training, maximal Ca^{2+}-ATPase activity was further significantly decreased in ET-SARKO mice, whereas it was significantly increased in ET-WT mice. This result was consistent with the change in the ratio of SERCA2a to PLN protein expression shown in Table 2.

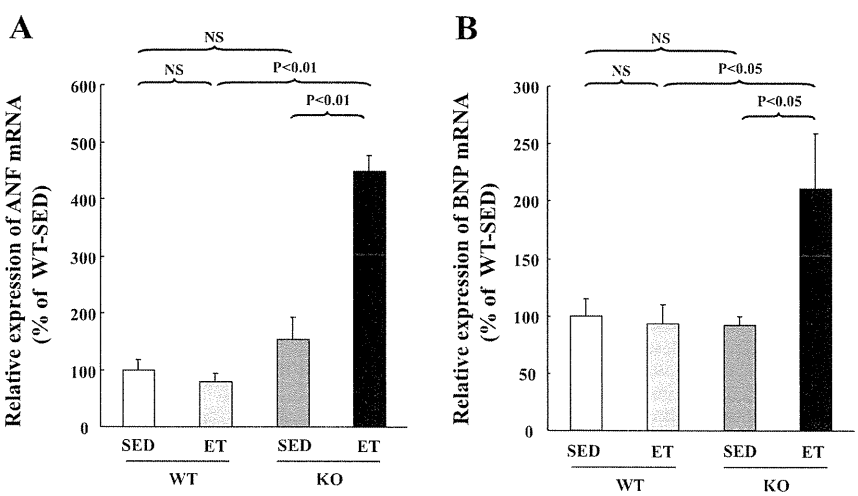

Fig. 3. Upregulation of atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) mRNAs in ET-SARKO mice. Quantitative RT-PCR analyses revealed that the expression levels of ANF (*A*) and BNP (*B*) mRNAs were significantly upregulated in the ventricles of ET-SARKO mice. The expression levels observed in SED-WT mice were set as 100% as a control. mRNA expression was normalized by GAPDH. Values are means ± SE; $n = 5$ for each group.

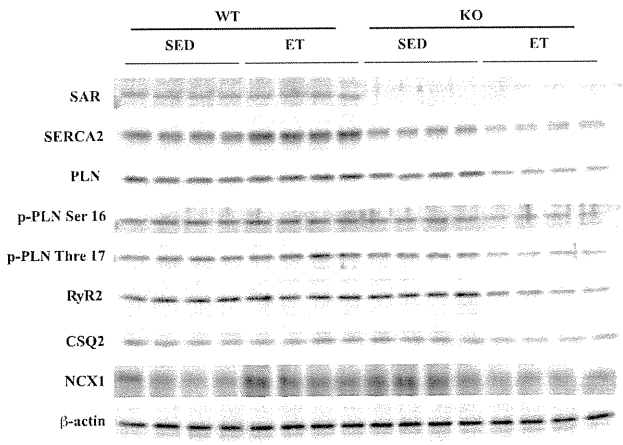

Fig. 4. The expression of Ca^{2+} handling proteins after ET. The expression levels of sarcalumenin (SAR), sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2 (SERCA2), phospholamban (PLN), phosphorylated PLN (p-PLN), ryanodine receptor 2 (RyR2), calsequestrin 2 (CSQ2), and sodium/calcium exchanger 1 (NCX1) proteins were quantified in hearts isolated from SED and ET mice. Protein expression was normalized by β-actin.

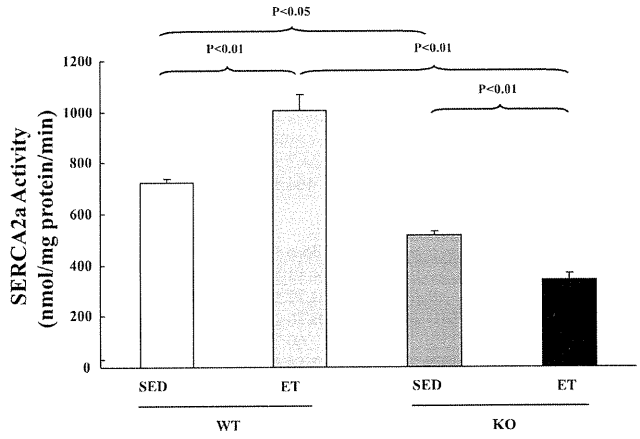

Fig. 5. SERCA2a activity after ET. SERCA2a activity was increased in ET-WT, whereas it was decreased in ET-SARKO after ET. Values are means ± SE; $n = 5$ for each group.

DISCUSSION

The most striking finding in the present study is that long-term (12 wk) endurance exercise training induced a significant cardiac dysfunction in mice that harbor systemic ablation of the SAR gene. Along the same lines, we have recently demonstrated that SARKO mice failed to adapt to pressure-overloaded stress induced by transverse aortic constriction (26), whereas sedentary young SARKO mice exhibit mild cardiac dysfunction (38). Since exercise is one of the most common physiological stresses, the present data indicate that SAR plays an important role in preserving cardiac function during adaptation to not only pathological, but also physiological, stresses.

It should be noted that the absolute training intensity undertaken by SARKO mice was significantly lower than that undertaken by WT mice (Supplemental Fig. 1), because the intensity of each mouse's exercise regime was determined on the basis of that mouse' maximal exercise ability. Accordingly,

Table 2. *The expression of calcium handling proteins after endurance exercise training*

	SED-WT	ET-WT	SED-SARKO	ET-SARKO
SAR	100±5	108±9		
SERCA2	100±10	159±13§	74±4*	52±6†‡
PLN	100±6	123±8	83±2*	71±2†§
p-PLN Ser 16	100±3	120±11	95±5	82±3†‡
p-PLN Thre 17	100±4	112±7	92±6	78±4†‡
SERCA2/PLN	100±5	132±12‡	94±4	75±10†‡
p-PLN Ser 16/PLN	100±4	98±8	113±4	116±8
p-PLN Thre 17/PLN	100±1	98±4	93±2	88±2*
RyR2	100±5	100±10	97±6	68±8*‡
CSQ2	100±7	101±4	99±3	83±6*‡
NCX1	100±10	139±11‡	124±3*	92±11*‡

Values are means ± SE; $n = 5$ mice for each group. The expression level in SED-WT mice was referred to 100% as a control. Protein expression was normalized by β-actin. SAR, sarcalumenin; SERCA2, sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2; PLN, phospholamban; p-PLN: phosphorylated phospholamban; RyR2, ryanodine receptor 2; CSQ2, calsequestrin 2; NCX1, sodium/calcium exchanger 1. Significant difference vs. WT: *$P < 0.05$ and †$P < 0.01$; vs. SED: ‡$P < 0.05$ and §$P < 0.01$.

CS activity in soleus muscle after endurance exercise training was significantly lower in ET-SARKO mice than in ET-WT mice (Fig. 2). Since skeletal muscle CS activity is a marker for mitochondrial content (a hallmark of endurance exercise) and muscle oxidative capacity, this result indicates that our exercise training program is sufficient to enhance the exercise ability of WT mice, but insufficient to enhance that of SARKO mice. Although this may explain a number of the negative effects on SARKO mice that were caused by exercise training in the present study, it is, nevertheless, very difficult to explain why ET-SARKO mice exhibited progressive cardiac dysfunction. We assume that inadequate adaptation to endurance exercise in ET-SARKO mice caused impaired cardiac function, the primary insult, which, secondarily, resulted in a number of negative effects on SARKO mice caused by training.

The mechanism by which endurance exercise induced progressive cardiac dysfunction in SARKO mice is a critical question. One observation that may be relevant to this question is the significant decrease in the expression and activity of SERCA2a in ET-SARKO mice. A number of previous studies have reported that endurance exercise training increased the expression and/or activity of SERCA2a in healthy (9, 10, 20, 22, 30, 35) or diseased rodents (6, 21, 24, 34, 39); similarly, we found that the expression and activity of SERCA2a increased after endurance exercise training in control mice. Yet other studies have demonstrated that endurance exercise training does not change the expression and/or activity of SERCA2a (3, 4) or Ca^{2+} transients (12) in rodents. It is worth noting that these conflicting results may have their origins in such factors as differences in species, exercise protocols, and/or condition of the subjects; few studies, however, have shown that endurance exercise decreases the expression and/or activity of SERCA2a. Therefore, our results found in ET-SARKO mice were so remarkable that it is very important to investigate why SAR deficiency caused the significant reduction in the expression and activity of SERCA2a under endurance exercise training.

Our laboratory's recent study has demonstrated that SAR interacts with SERCA2 to enhance the protein stability of SERCA2a (26). Since exercise training usually increases pro-

tein synthesis and degradation in muscle (11, 23), we assume that endurance exercise training also increased the turnover rate of SERCA2a protein. Then we postulate that SAR deficiency induced a progressive degradation of SERCA2a protein due to impaired protein stabilization under endurance exercise training and resulted in the significant decrease in the expression of SERCA2a in ET-SARKO mice. Importantly, the present study demonstrated that endurance exercise training slightly increased the expression levels of SAR protein in WT hearts, in accordance with a significant increase in the expression of SERCA2a protein. To our knowledge, this is the first report to show the effect of endurance exercise training on the expression of SAR protein. These data suggest that SAR is a key regulatory protein to maintain the expression level of SERCA2a protein under pathophysiological stresses. In addition, the ratios of SERCA2a to PLN protein and phosphorylated threonine 17 PLN to total PLN protein were significantly decreased in the ventricular muscles of ET-SARKO mice, indicating that SERCA2a activity was inhibited by PLN more in ET-SARKO mice than in other groups. Taken together, this evidence shows that SAR deficiency induced a significant reduction in SERCA2a activity and deterioration of the Ca^{2+} storage system in the SR under endurance exercise stress, which is very likely to play a primary role in the exercise-induced cardiac dysfunction exhibited by ET-SARKO mice.

Interestingly, in addition to the decreases in the SERCA2a and PLN proteins that interact with SAR in the longitudinal SR, other Ca^{2+} handling proteins, such as RyR2, CSQ2, and NCX1, were also significantly downregulated in ET-SARKO mice, which has not been investigated in pressure-overloaded SARKO hearts (26). These abnormalities probably contribute to the further impairment of cardiac function during endurance exercise training. We assume that the downregulation of RyR2, CSQ2, and NCX1 could be a secondary phenomenon that occurs under physiological stress conditions, as SAR does not directly interact with these proteins. The mechanism of these discrepant responses to different stresses in SARKO mice is currently not clear; it is an important question that should be addressed in future studies.

In one way, the results of the present study somehow contradict those of a recent report by Zhao et al. (40), which showed that skeletal muscles from SARKO mice are highly resistant to fatigue compared with those from WT mice. The same authors have also demonstrated that SOCE was promoted in SARKO skeletal muscle by the upregulation of MG29 (40). They proposed that the promotion of SOCE played a role in making skeletal muscle more fatigue resistant (40). In the present study, however, we did not detect any expression of MG29 protein in either WT or SARKO hearts, before or after exercise training, although we used the same membranes for our Western blot analyses (data not shown). This observation is consistent with a previous study (29). Currently, we cannot explain the exact reason for the disagreement between the results of Zhao et al. (40) and our own. A possible explanation is the difference in the exercise programs our two groups used to evaluate the exercise performance of SARKO mice. Further investigation is needed to clarify whether a defect of MG29 may cause the negative responses to exercise in SARKO cardiac muscle cells.

In conclusion, we found that cardiac function and maximal exercise ability were significantly impaired in SARKO mice after endurance treadmill exercise training. These impairments were due, at least in part, to a significant downregulation of SERCA2a and other Ca^{2+} handling proteins and to a deterioration of the Ca^{2+} storage system in the SARKO heart under endurance exercise. Thus present study indicates that SAR plays a critical role in maintaining cardiac function under physiological stresses, such as endurance exercise, by regulating Ca^{2+} transport activity into the SR. SAR may be a primary target for exercise-related adaptation of the Ca^{2+} storage system in the SR to preserve cardiac function.

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ORIGINAL PAPER

Effect of ascorbic acid on reactive oxygen species production in chemotherapy and hyperthermia in prostate cancer cells

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Abstract Cellular reactive oxygen species (ROS) production is increased by both temperature and anticancer drugs. Antioxidants are known to suppress ROS production while cancer patients may take them as dietary supplement during chemotherapy and hyperthermic therapy. We examined changes in ROS production in prostate cancer cells in the presence of various anticancer drugs and antioxidants at different temperatures. ROS production was increased with temperature in cancer cells, but not in normal cells; this increase was potently inhibited by ascorbic acid. ROS production was also increased in the presence of some anticancer drugs, such as vinblastine, but not by others. Dietary antioxidant supplements, such as β-carotene, showed variable effects. Ascorbic acid potently inhibited ROS production, even in the presence of anticancer drugs, while β-carotene showed no inhibition. Accordingly, our results suggest that cancer patients should carefully choose antioxidants during their cancer chemotherapy and/or hyperthermic therapy.

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Introduction

Physiology of cancer cells has been extensively studied, and the understanding of mechanisms for their rapid growth and proliferation has been advanced in the past decade [1–3]. Accordingly, various therapeutic strategies in cancer treatment have been developed [1, 4]. Although surgical removal of the cancer tissue is still the golden standard for complete cure, it is not always feasible in cases with advanced or metastatic cancer. Surgical stress may be too large for geriatric and/or exhausted patients. In such cases, combination of various therapeutic strategies has been recommended. Among such strategies, hyperthermic therapy may be applied on the top of the conventional cancer chemotherapy or radiation therapy [5, 6]. Although it may not achieve complete remission of cancer by itself, clinical studies have demonstrated that the survival and quality of life may be significantly improved [3, 7].

Molecular mechanism of hyperthermic therapy includes the overstimulation metabolism of rapidly proliferating cancer cells, leading to the induction of apoptosis [8]. Increased production of reactive oxygen species (ROS) from mitochondria may also be involved [9]. Because ROS production may be increased in the presence of anticancer drugs on their own, the combination of chemotherapy and hyperthermic therapy will synergistically increase ROS production, leading to effective cancer cell death [6]. However, ROS production is inhibited in the presence of various antioxidants [10]. In this regard, various antioxidants, which are also used as dietary supplements, may interfere with the

efficacy of such chemotherapy and/or hyperthermic therapy. Unfortunately, however, evaluation of the effect of such antioxidants in the combination of cancer chemotherapy has not been well performed [11, 12]. Ascorbic acid, for example, is often used as a dietary supplement. Because ascorbic acid may improve immunity or peripheral circulation [13], people, including cancer patients, take this antioxidant. However, the use of ascorbic acid in cancer patients remains controversial; ascorbic acid may enhance [10] or suppress [13] the efficacy of chemotherapy.

In this study, we examined the effect of temperature, anticancer drugs, and antioxidants on ROS production. We used MAT-Lu prostate cancer cells since hyperthermia therapy has often been applied to prostatic cancer patients [14, 15], and thus it is necessary to evaluate the effect of hyperthermia on this cancer cell type. We demonstrated their effect on ROS production, and make potential suggestions for future use of antioxidants in cancer patients.

Materials and methods

Materials

We used the following anticancer drugs; vinblastine (VBL) (Nihon Kayaku, Japan), cisplatin (CIS), (Pfizer, Japan), adriamycin (ADR), (Wako, Japan), docetaxel (DTX), (Sanofi Aventis, Japan). Similarly, as antioxidants, we used *N*-acetyl-cysteine (NAC), (Sigma, Japan), retinoic acid (Sigma), quercetin (Sigma), catechin (Wako), lutein (Sigma), β-carotene (Sigma), and ascorbic acid (Wako).

Cell culture

Rat prostatic adenocarcinoma cells (R3327-MAT-Lu) were cultured in RPMI-1640 medium supplemented with 10% FBS and 250 nM dexamethasone, which were kindly provided by Dr. J. T. Isaacs (Johns Hopkins University, MD, USA). Cells were incubated at 37°C in 5% CO_2. In some experiments, cells were incubated at 42°C as hyperthermic treatment (see below). Rat cardiac fibroblasts were isolated from adult rats (250–300 g, male) by using a modification of published methods [16]. Fibroblasts were separated from cardiac myocytes by gravity separation and grown to confluence on 10-cm cell culture dishes at 37°C with 90% air with 10% CO_2 in growth media (DMEM with 10% FBS, 1% penicillin, and 1% streptomycin).

Hyperthermic stress and measurement of reactive oxygen species

Cells were plated in 24-well culture plates (5.0×10^4 cells/well) overnight. Cells were then treated with various agents, including anticancer drugs, at 37°C for 3 h. For hyperthermic treatment, cells were further incubated in the presence or absence of various reagents at 42°C for 1 h. The intracellular ROS level was then measured using a fluorescent dye 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Life technologies, Japan) as previously described [17]. In the presence of oxidant, DCFH is converted into the highly fluorescent 2′,7′-dichlorofluorescein. Cells were first washed with PBS, and serum-free DMEM containing 10 μM DCFH-DA was added to each well. Cells were then incubated at 37°C for 45 min. ROS production was measured using a microplate reader equipped with a spectrofluorometer (PerkinElmer ARVO MX, Japan) at an emission wavelength of 538 nm and extinction wavelength of 485 nm.

Statistical analysis

Data are expressed as means ± SEM. Data was analyzed by one-way ANOVA followed by Tukey post hoc using Graph-pad Prism software. Statistical significance was set at $p < 0.05$.

Results

Effect of temperature on ROS generation

It is known that cancer cells exhibit higher metabolism than normal cells. High metabolic rate may be reflected by increased ROS generation, in particular, upon hyperthermia. Accordingly, we compared the effect of temperature on ROS production between MAT-Lu prostate cancer cells and normal fibroblasts obtained from the cardiac tissue. It is known that fibroblasts grow rapidly and thus possess high metabolic rate in comparison to other normal cell types.

As shown in Fig. 1a, ROS production was lower at 32°C than at 37°C while it was higher at 42°C. Thus, ROS production was increased in a temperature-dependent manner, at least in prostate cancer cells. In contrast, ROS production in cardiac fibroblasts was not increased at 42°C in comparison to that at 37°C (Fig. 1b). Thus, ROS production by hyperthermia was increased only in cancer cells.

Effect of ascorbic acid on ROS production

We then examined the effect of ascorbic acid, which has been used in cancer treatment as part of chemotherapy, but is also known as a major antioxidant. In the presence of an increasing concentration of ascorbic acid (10 μM–100 mM), ROS production was decreased in a concentration-dependent manner at 37°C (Fig. 1c). Similar inhibition was observed at 42°C. Thus, ascorbic acid potently inhibited the production of ROS.

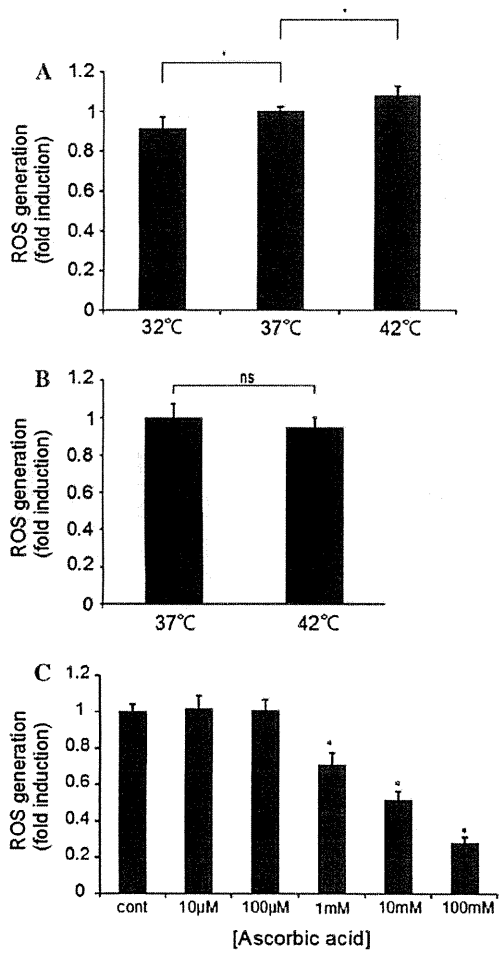

Fig. 1 ROS production in cancer cells and normal cells at different temperatures. a ROS production in cancer cells at 32, 37, and 42°C. Prostate cancer cells were incubated at different temperatures, followed by determination of ROS production (mean ± SEM; $n = 4$, $^*p < 0.05$). b ROS production in cardiac fibroblasts at 37 and 42°C. Cardiac fibroblasts were incubated at different temperatures similarly, followed by determination of ROS production (mean ± SEM; $n = 4$, $^*p < 0.05$). c ROS production was determined with cancer cells in the presence of an increasing concentration of ascorbic acid (10 μM–100 mM). Prostate cancer cells were incubated at 37°C, followed by determination of ROS production (mean ± SEM; $n = 4$, $^*p < 0.05$)

Effect of anticancer drugs on ROS production

Anticancer drugs may induce cytotoxicity through various mechanisms. We examined the effect of these anticancer drugs, which have been widely used in many cancer cell types, including prostate cancer, on ROS production. We first determined the EC_{50} values of these drugs in prostate cancer cells, which were 200 nM for VBL, 15 μM for CIS, 7.5 μM for ADR, and 1 mM for DTX. When prostate cancer cells were incubated with these drugs at the EC_{50} value concentration, ROS production was slightly, but significantly, increased with VBL and CIS, but not with DTX and ADR at 37°C (Fig. 2a). When hyperthermic treatment at 42°C was added, ROS production by VBL and CIS became even greater (Fig. 2a). Thus, hyperthermia by itself can increase ROS production, which is further enhanced in the presence of certain anticancer drugs.

We then examined the effect of ascorbic acid in the presence of anticancer drugs. ROS production was potently inhibited by 1 mM ascorbic acid in the presence of any anticancer drugs (Fig. 2b). ROS production at 37°C was similar among these anticancer drugs. However, when hyperthermic treatment at 42°C was added, ROS production was significantly greater with VBL (Fig. 2b). Thus, ascorbic acid may negate ROS production induced by certain anticancer drugs at 37°C; however, it cannot negate ROS production of VBL at 42°C. Accordingly, anticancer drug-induced ROS enhancement may be retained in hyperthermia for VBL, but not others.

Effect of ascorbic acid on ROS production by Resovist

Resovist is super-paramagnetic iron oxide nanoparticle that has been used as MRI contrast agent. Because of its magnetic property, similar compounds have been used as source of heat production in hyperthermic therapy. We found that the ROS production was increased in the presence of 10 μM Resovist at 37°C, suggesting that Resovist can produce ROS with cancer cells. When ascorbic acid was added, ROS production was negated or instead decreased (Fig. 3). Thus, ascorbic acid could potently inhibit ROS production induced by Resovist.

Effect of various antioxidants on ROS production

Patients may take various dietary supplements during cancer chemotherapy. In some cases, patients may take supplementary antioxidants on the top of anticancer drugs. We thus examined the effect of these antioxidants and related drugs, namely, *N*-acetyl cysteine (NAC), retinoic acid, quercetin, catechin, lutein, and β-carotene, on ROS production. We used these antioxidants at concentrations as previously demonstrated to be effective in various assays [11, 18, 19]. We examined their effect on VBL and CIS, which increased ROS production in the above assays.

As shown in Fig. 4a–f, these antioxidative compounds exhibited various degrees of antioxidative effects. NAC showed the most potent inhibition on ROS production; ROS production was decreased by a quarter in prostate cancer cells. VBL or CIS did not further increase ROS production in the presence of NAC at either 37 or 42°C, suggesting the ROS production by these anticancer drugs was completely suppressed by NAC. Thus, NAC showed