Sarcalumenin is essential for maintaining cardiac function during endurance exercise training

Qibin Jiao, Yunzhe Bai, Toru Akaike, Hiroshi Takeshima, Yoshihiro Ishikawa and Susumu Minamisawa

Am J Physiol Heart Circ Physiol 297:H576-H582, 2009. First published 5 June 2009;
doi:10.1152/ajpheart.00946.2008

AJP - Heart and Circulatory Physiology publishes original investigations on the physiology of the heart, blood vessels, and lymphatics, including experimental and theoretical studies of cardiovascular function at all levels of organization ranging from the intact animal to the cellular, subcellular, and molecular levels. It is published 12 times a year (monthly) by the American Physiological Society, 9650 Rockville Pike, Bethesda MD 20814-3991. ISSN: 0363-6135, ESSN: 1522-1539. Visit our website at http://www.the-aps.org/.

Am J Physiol Heart Circ Physiol 297: H576–H582, 2009.
First published June 5, 2009; doi:10.1152/ajpheart.00946.2008.

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*

Submitted 22 August 2008; accepted in final form 2 June 2009

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

Address for reprint requests and other correspondence: S. Minamisawa, Dept. of Life Science and Medical Bioscience, Waseda Univ., 2-2 Wakamatsu-cho, Shinjuku-ku, Tokyo 162-8480, Japan (E-mail: sminamis@waseda.jp).

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

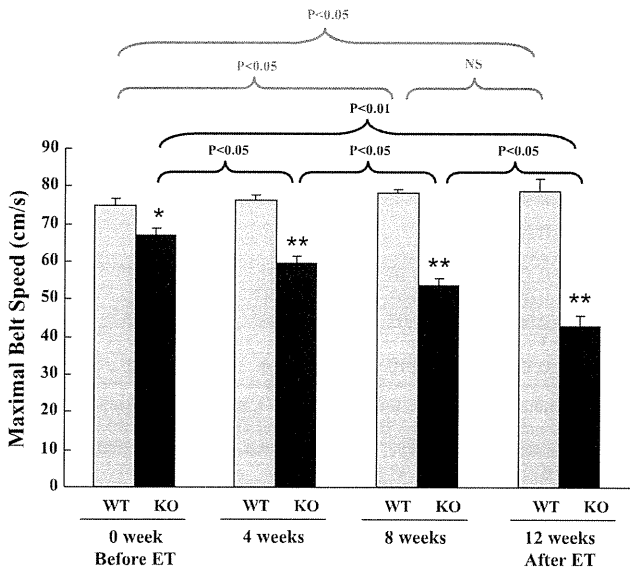


Fig. 1. The effect of endurance exercise training (ET) on maximal exercise ability in sarcalumenin (SAR) knockout (SARKO) mice. Maximal exercise ability, as evaluated by maximal belt speed, is already lower in SARKO mice than in wild-type (WT) mice before ET. During ET, maximal exercise ability gradually 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

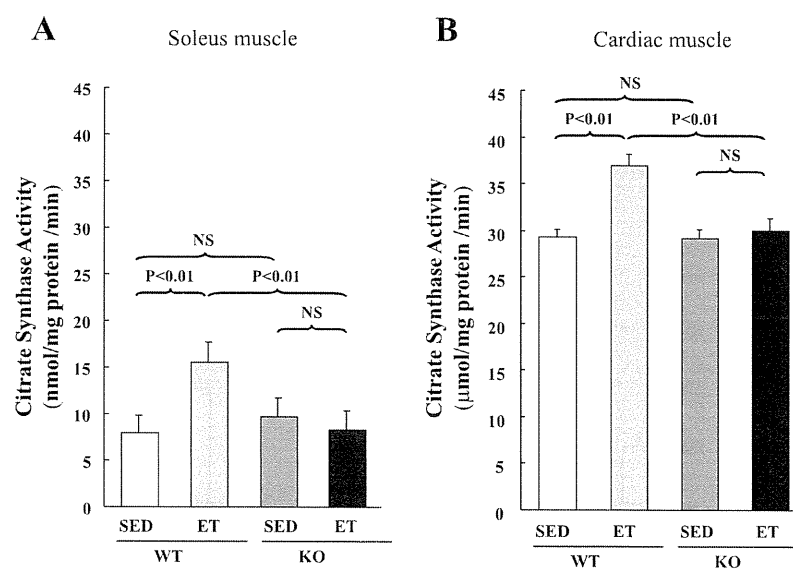


Fig. 2. Citrate synthase (CS) activity after ET. After ET, CS activity of soleus muscle (*A*) and cardiac muscle (*B*) was increased in ET-WT mice, but not in ET-SARKO mice. Values are means ± SE; n = 5 for each group. SED, sedentary; NS, not significant.

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.

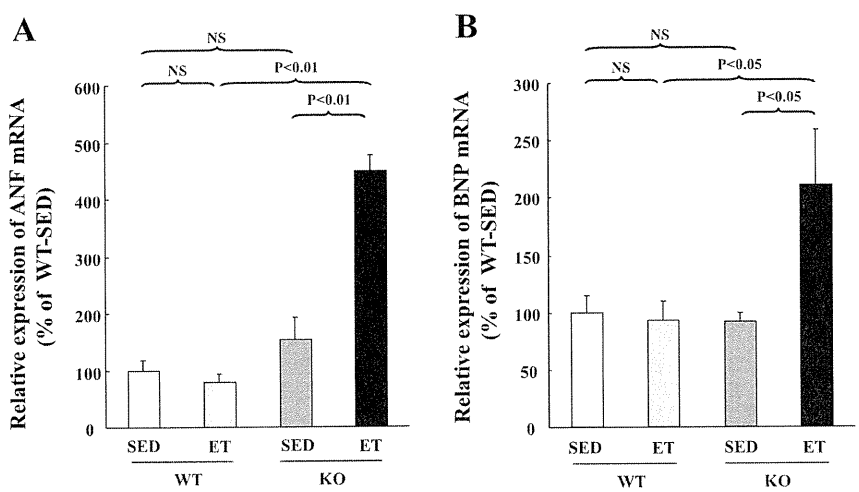


Fig. 3. Upregulation of atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) mRNAs in ET-SARKO mice. Quantitative RT-PCR analyses revealed that the expression levels of ANF (*A*) and BNP (*B*) mRNAs were significantly upregulated in the ventricles of ET-SARKO mice. The expression levels 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.

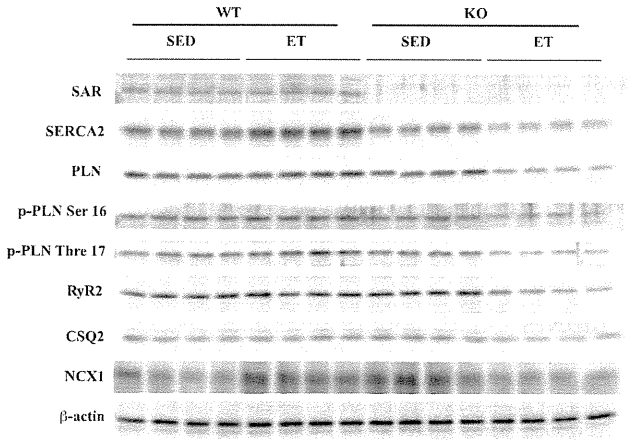


Fig. 4. The expression of Ca^{2+} handling proteins after ET. The expression levels of sarcalumenin (SAR), sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2 (SERCA2), phospholamban (PLN), phosphorylated PLN (p-PLN), ryanodine receptor 2 (RyR2), calsequestrin 2 (CSQ2), and sodium/calcium exchanger 1 (NCX1)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

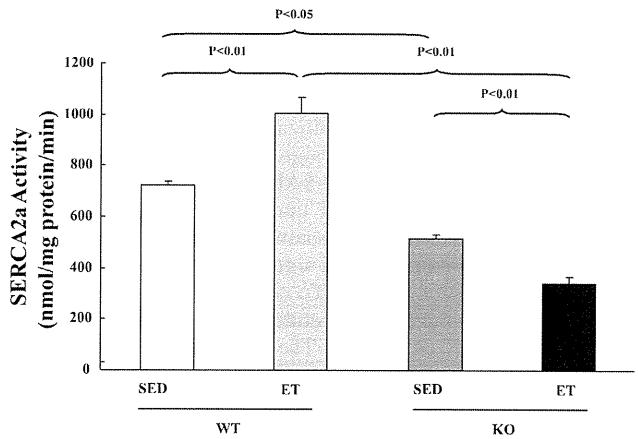


Fig. 5. SERCA2a activity after ET. SERCA2a activity was increased in ET-WT, whereas it was decreased in ET-SARKO after ET. Values are means ± SE; $n = 5$ for each group.

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, 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.

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 $\dagger P < 0.01$; vs. SED: $\ddagger P < 0.05$ and $\S P < 0.01$.

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.

GRANTS

This work was partly supported by grants from the Honjo International Scholarship Foundation (Q. Jiao), the Yokohama Foundation for Advanced Medical Science (S. Minamisawa, T. Akaike, Y. Ishikawa), the Ministry of Education, Science, Sports and Culture of Japan (S. Minamisawa, Y. Ishikawa), the Special Coordination Funds for Promoting Science and Technology, MEXT (S. Minamisawa), the "High-Tech Research Center" Project for Private Universities: matching fund subsidy from MEXT (S. Minamisawa), a Waseda University Grant for Special Research Projects (S. Minamisawa), the Mother and Child Health Foundation (S. Minamisawa), the Miyata Cardiology Research Promotion Funds (S. Minamisawa), the Takeda Science Foundation (S. Minamisawa), the Foundation for Growth Science (S. Minamisawa), the Japan Cardiovascular Research Foundation (S. Minamisawa), the Mitsubishi Pharma Research Foundation (S. Minamisawa), the Yokohama Academic Foundation (T. Akaike), the Inoue Foundation for Science (T. Akaike), the Naito Foundation (T. Akaike), the Japan Space Forum (Y. Ishikawa), and the National Institute of General Medical Sciences (RO1 GM067773) (Y. Ishikawa).

REFERENCES

1. **Call JA, Voelker KA, Wolff AV, McMillan RP, Evans NP, Hulver MW, Talmadge RJ, Grange RW.** Endurance capacity in maturing mdx mice is markedly enhanced by combined voluntary wheel running and green tea extract. *J Appl Physiol* 105: 923–932, 2008.
2. **Charlton GA, Crawford MH.** Physiologic consequences of training. *Cardiol Clin* 15: 345–354, 1997.
3. **de Waard MC, van der Velden J, Bito V, Ozdemir S, Biesmans L, Boontje NM, Dekkers DH, Schoonderwoerd K, Schuurbiers HC, de Crom R, Stienen GJ, Sipido KR, Lamers JM, Duncker DJ.** Early exercise training normalizes myofilament function and attenuates left ventricular pump dysfunction in mice with a large myocardial infarction. *Circ Res* 100: 1079–1088, 2007.
4. **Delgado J, Saborido A, Moran M, Megias A.** Chronic and acute exercise do not alter Ca^{2+} regulatory systems and ectonucleotidase activities in rat heart. *J Appl Physiol* 87: 152–160, 1999.
5. **Frank KF, Bolck B, Erdmann E, Schwinger RH.** Sarcoplasmic reticulum Ca^{2+}-ATPase modulates cardiac contraction and relaxation. *Cardiovasc Res* 57: 20–27, 2003.
6. **French JP, Quindry JC, Falk DJ, Staib JL, Lee Y, Wang KK, Powers SK.** Ischemia-reperfusion-induced calpain activation and SERCA2a degradation are attenuated by exercise training and calpain inhibition. *Am J Physiol Heart Circ Physiol* 290: H128–H136, 2006.
7. **Houser SR, Piacentino V 3rd, Weisser J.** Abnormalities of calcium cycling in the hypertrophied and failing heart. *J Mol Cell Cardiol* 32: 1595–1607, 2000.
8. **Hwang KJ.** Interference of ATP and acidity in the determination of inorganic phosphate by the Fiske and Subbarow method. *Anal Biochem* 75: 40–44, 1976.
9. **Iemitsu M, Miyauchi T, Maeda S, Tanabe T, Takanashi M, Matsuda M, Yamaguchi I.** Exercise training improves cardiac function-related gene levels through thyroid hormone receptor signaling in aged rats. *Am J Physiol Heart Circ Physiol* 286: H1696–H1705, 2004.
10. **Kemi OJ, Ceci M, Condorelli G, Smith GL, Wisloff U.** Myocardial sarcoplasmic reticulum Ca^{2+} ATPase function is increased by aerobic interval training. *Eur J Cardiovasc Prev Rehabil* 15: 145–148, 2008.
11. **Kumar V, Atherton P, Smith K, Rennie MJ.** Human muscle protein synthesis and breakdown during and after exercise. *J Appl Physiol* 106: 2026–2039, 2009.

12. **Laughlin MH, Schaefer ME, Sturek M.** Effect of exercise training on intracellular free Ca^{2+} transients in ventricular myocytes of rats. *J Appl Physiol* 73: 1441–1448, 1992.
13. **Leberer E, Charuk JH, Green NM, MacLennan DH.** Molecular cloning and expression of cDNA encoding a lumenal calcium binding glycoprotein from sarcoplasmic reticulum. *Proc Natl Acad Sci USA* 86: 6047–6051, 1989.
14. **Levy WC, Cerqueira MD, Abrass IB, Schwartz RS, Stratton JR.** Endurance exercise training augments diastolic filling at rest and during exercise in healthy young and older men. *Circulation* 88: 116–126, 1993.
15. **Lu L, Mei DF, Gu AG, Wang S, Lentzner B, Gutstein DE, Zwas D, Homma S, Yi GH, Wang J.** Exercise training normalizes altered calcium-handling proteins during development of heart failure. *J Appl Physiol* 92: 1524–1530, 2002.
16. **MacLennan DH, Wong PT.** Isolation of a calcium-sequestering protein from sarcoplasmic reticulum. *Proc Natl Acad Sci USA* 68: 1231–1235, 1971.
17. **Minamisawa S, Sato Y, Cho MC.** Calcium cycling proteins in heart failure, cardiomyopathy and arrhythmias. *Exp Mol Med* 36: 193–203, 2004.
18. **Minamisawa S, Wang Y, Chen J, Ishikawa Y, Chien KR, Matsuoka R.** Atrial chamber-specific expression of sarcolipin is regulated during development and hypertrophic remodeling. *J Biol Chem* 278: 9570–9575, 2003.
19. **Moore RL.** Cellular adaptations of the heart muscle to exercise training. *Ann Med* 30, *Suppl* 1: 46–53, 1998.
20. **Moran M, Saborido A, Megias A.** Ca^{2+} regulatory systems in rat myocardium are altered by 24 weeks treadmill training. *Pflügers Arch* 446: 161–168, 2003.
21. **Mou YA, Reboul C, Andre L, Lacampagne A, Cazorla O.** Late exercise training improves non-uniformity of transmural myocardial function in rats with ischaemic heart failure. *Cardiovasc Res* 81: 555–564, 2009.
22. **Pierce GN, Sekhon PS, Meng HP, Maddaford TG.** Effects of chronic swimming training on cardiac sarcolemmal function and composition. *J Appl Physiol* 66: 1715–1721, 1989.
23. **Pikosky MA, Gaine PC, Martin WF, Grabarz KC, Ferrando AA, Wolfe RR, Rodriguez NR.** Aerobic exercise training increases skeletal muscle protein turnover in healthy adults at rest. *J Nutr* 136: 379–383, 2006.
24. **Rolim NP, Medeiros A, Rosa KT, Mattos KC, Irigoyen MC, Krieger EM, Krieger JE, Negrao CE, Brum PC.** Exercise training improves the net balance of cardiac Ca^{2+} handling protein expression in heart failure. *Physiol Genomics* 29: 246–252, 2007.
25. **Seals DR, Hagberg JM, Spina RJ, Rogers MA, Schechtman KB, Ehsani AA.** Enhanced left ventricular performance in endurance trained older men. *Circulation* 89: 198–205, 1994.
26. **Shimura M, Minamisawa S, Takeshima H, Jiao Q, Bai Y, Umemura S, Ishikawa Y.** Sarcalumenin alleviates stress-induced cardiac dysfunction by improving Ca^{2+} handling of the sarcoplasmic reticulum. *Cardiovasc Res* 77: 362–370, 2008.
27. **Singh M, Brooks GC, Srere PA.** Subunit structure and chemical characteristics of pig heart citrate synthase. *J Biol Chem* 245: 4636–4640, 1970.
28. **Tadano M, Edamatsu H, Minamisawa S, Yokoyama U, Ishikawa Y, Suzuki N, Saito H, Wu D, Masago-Toda M, Yamawaki-Kataoka Y, Setsu T, Terashima T, Maeda S, Satoh T, Kataoka T.** Congenital semilunar valvulogenesis defect in mice deficient in phospholipase C epsilon. *Mol Cell Biol* 25: 2191–2199, 2005.
29. **Takeshima H, Shimuta M, Komazaki S, Ohmi K, Nishi M, Iino M, Miyata A, Kangawa K.** Mitsugumin29, a novel synaptophysin family member from the triad junction in skeletal muscle. *Biochem J* 331: 317–322, 1998.
30. **Tate CA, Helgason T, Hyek MF, McBride RP, Chen M, Richardson MA, Taffet GE.** SERCA2a and mitochondrial cytochrome oxidase expression are increased in hearts of exercise-trained old rats. *Am J Physiol Heart Circ Physiol* 271: H68–H72, 1996.
31. **Thomas DP.** Effects of acute and chronic exercise on myocardial ultrastructure. *Med Sci Sports Exerc* 17: 546–553, 1985.
32. **Trounce IA, Kim YL, Jun AS, Wallace DC.** Assessment of mitochondrial oxidative phosphorylation in patient muscle biopsies, lymphoblasts, and transmitochondrial cell lines. *Methods Enzymol* 264: 484–509, 1996.
33. **Ventura-Clapier R, Mettauer B, Bigard X.** Beneficial effects of endurance training on cardiac and skeletal muscle energy metabolism in heart failure. *Cardiovasc Res* 73: 10–18, 2007.
34. **Wisloff U, Loennechen JP, Currie S, Smith GL, Ellingsen O.** Aerobic exercise reduces cardiomyocyte hypertrophy and increases contractility, Ca^{2+} sensitivity and SERCA-2 in rat after myocardial infarction. *Cardiovasc Res* 54: 162–174, 2002.
35. **Wisloff U, Loennechen JP, Falck G, Beisvag V, Currie S, Smith G, Ellingsen O.** Increased contractility and calcium sensitivity in cardiac myocytes isolated from endurance trained rats. *Cardiovasc Res* 50: 495–508, 2001.
36. **Yokoyama U, Minamisawa S, Adachi-Akahane S, Akaike T, Naguro I, Funakoshi K, Iwamoto M, Nakagome M, Uemura N, Hori H, Yokota S, Ishikawa Y.** Multiple transcripts of Ca^{2+} channel α1-subunits and a novel spliced variant of the α1C-subunit in rat ductus arteriosus. *Am J Physiol Heart Circ Physiol* 290: H1660–H1670, 2006.
37. **Yokoyama U, Minamisawa S, Quan H, Ghatak S, Akaike T, Segi-Nishida E, Iwasaki S, Iwamoto M, Misra S, Tamura K, Hori H, Yokota S, Toole BP, Sugimoto Y, Ishikawa Y.** Chronic activation of the prostaglandin receptor EP4 promotes hyaluronan-mediated neointimal formation in the ductus arteriosus. *J Clin Invest* 116: 3026–3034, 2006.
38. **Yoshida M, Minamisawa S, Shimura M, Komazaki S, Kume H, Zhang M, Matsumura K, Nishi M, Saito M, Saeki Y, Ishikawa Y, Yanagisawa T, Takeshima H.** Impaired Ca^{2+} store functions in skeletal and cardiac muscle cells from sarcalumenin-deficient mice. *J Biol Chem* 280: 3500–3506, 2005.
39. **Zhang LQ, Zhang XQ, Ng YC, Rothblum LI, Musch TI, Moore RL, Cheung JY.** Sprint training normalizes Ca^{2+} transients and SR function in postinfarction rat myocytes. *J Appl Physiol* 89: 38–46, 2000.
40. **Zhao X, Yoshida M, Brotto L, Takeshima H, Weisleder N, Hirata Y, Nosek TM, Ma J, Brotto M.** Enhanced resistance to fatigue and altered calcium handling properties of sarcalumenin knockout mice. *Physiol Genomics* 23: 72–78, 2005.

J Physiol Sci (2012) 62:251–257
DOI 10.1007/s12576-012-0204-0

ORIGINAL PAPER

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

Hidenobu Fukumura · Motohiko Sato · Kyouhei Kezuka · Itaru Sato · Xianfeng Feng · Satoshi Okumura · Takayuki Fujita · Utako Yokoyama · Haruki Eguchi · Yoshihiro Ishikawa · Tomoyuki Saito

Received: 14 February 2012 / Accepted: 14 February 2012 / Published online: 6 March 2012

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.

H. Fukumura · T. Saito
Department of Orthopaedic Surgery, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawaku, Yokohama 236-0004, Japan

M. Sato · K. Kezuka · I. Sato · X. Feng · S. Okumura · T. Fujita · U. Yokoyama · Y. Ishikawa (✉)
Cardiovascular Research Institute, Yokohama City University School of Medicine, 3-9 Fukuura, Kanazawaku, Yokohama 236-0004, Japan
e-mail: yishikaw@med.yokohama-cu.ac.jp

H. Eguchi
IHI Corporation, 1, Shin-nakahara, Isogoku, Yokohama 235-8501, Japan

Keywords Reactive oxygen species · Prostate cancer cells · Hyperthermia · Ascorbic acid · Anti-oxidants · Anti-cancer drugs

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.

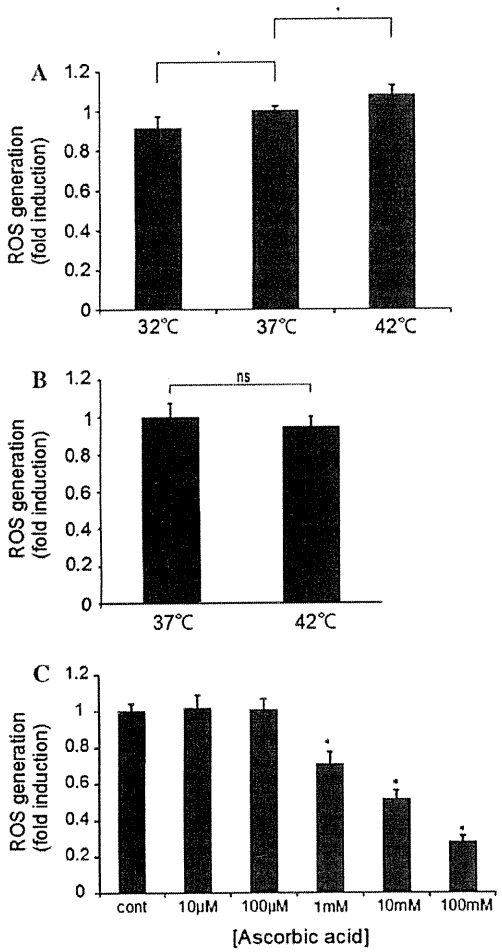


Fig. 1 ROS production in cancer cells and normal cells at different temperatures. **a** ROS production in cancer cells at 32, 37, and 42°C. Prostate cancer cells were incubated at different temperatures, followed by determination of ROS production (mean ± SEM; $n = 4$, $^*p < 0.05$). **b** ROS production 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

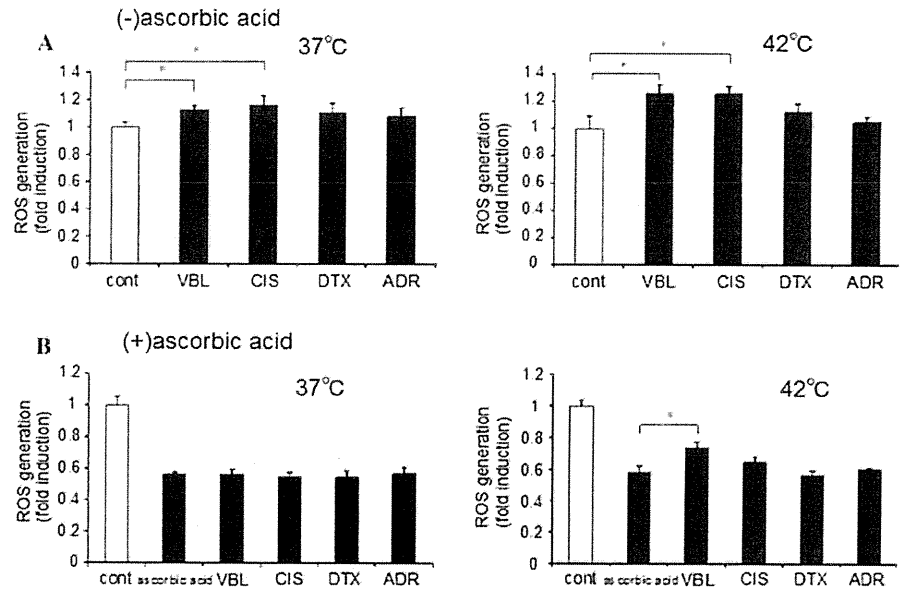


Fig. 2 Effect of anticancer drugs and ascorbic acid on ROS production. **a** ROS production was determined at 37 or 42°C in the presence of 200 nM VBL, 15 μM CIS, 7.5 μM DTX or 1 μM ADR (mean ± SEM; $n = 4$, $^*p < 0.05$). **b** ROS production was similarly determined in the presence of 1 mM ascorbic acid at 37 or 42°C (mean ± SEM; $n = 4$, $^*p < 0.05$)

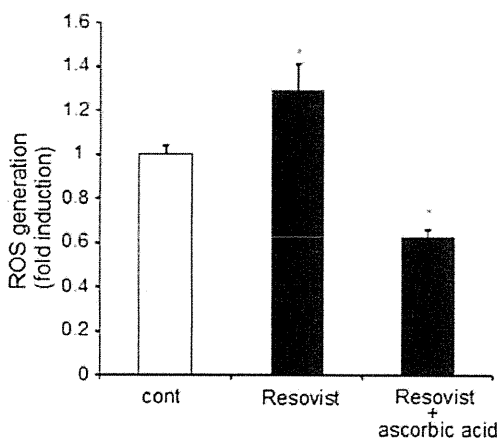


Fig. 3 Effect of Resovist on ROS production. ROS production was determined in the presence of 10 μM Resovist and/or 1 mM ascorbic acid at 37°C. Prostate cancer cells were incubated for 45 min, followed by ROS production assays (mean ± SEM; $n = 4$, $^*p < 0.05$)

similar, but perhaps greater, antioxidative effect compared to ascorbic acid. Retinoic acid, quercetin, and lutein showed comparable results to each other. They inhibited ROS production at both 37 and 42°C. However, both VBL and CIS could increase ROS production in the presence of these antioxidants, suggesting that these antioxidants could not inhibit anticancer drug-mediated ROS production. Catechin and β-carotene are best known as general antioxidants. However, they did not inhibit ROS production, at either 37 or 42°C, in the absence or presence of anticancer drugs. Thus, the effects of many antioxidants are not always the same.

Discussion

The current study has demonstrated that ROS production was higher in cancer cells than in normal cells, and was further increased with temperature. Ascorbic acid exhibited the potent inhibition of ROS production regardless of temperature. ROS production was also increased in the presence of anticancer drugs, such as VBL and CIS, but not by DTX or ADR. Importantly, ROS production of these anticancer drugs was inhibited in the presence of ascorbic acid regardless of temperature. In contrast, antioxidants, some of which have been used as dietary supplements among the general population, showed variable effects. NAC inhibited ROS production regardless of the presence of anticancer drugs, while catechin or β-carotene did not inhibit ROS production. Lutein, quercetin, and retinoic acid inhibited ROS production in the absence of anticancer drugs, while they did not inhibit the ROS production as induced by anticancer drug. Thus, these antioxidants should be taken carefully by patients since they may variably affect the effect of anticancer drugs, at least in their ROS production.

ROS as a cause of cytotoxicity of anticancer drugs has been extensively studied in the past [20, 21]. CIS may interfere with mitochondrial membrane function and thus increases ROS production. Paclitaxel, which is comparable to DTX, may regulate membrane NOX release, and increases ROS production [22–25]. We found that both CIS and VBL increased ROS production in prostate cancer cells. Hyperthermic therapy potentiates ROS production, leading to enhanced cytotoxicity [26]. We also found that increased temperature enhanced ROS production by CIS

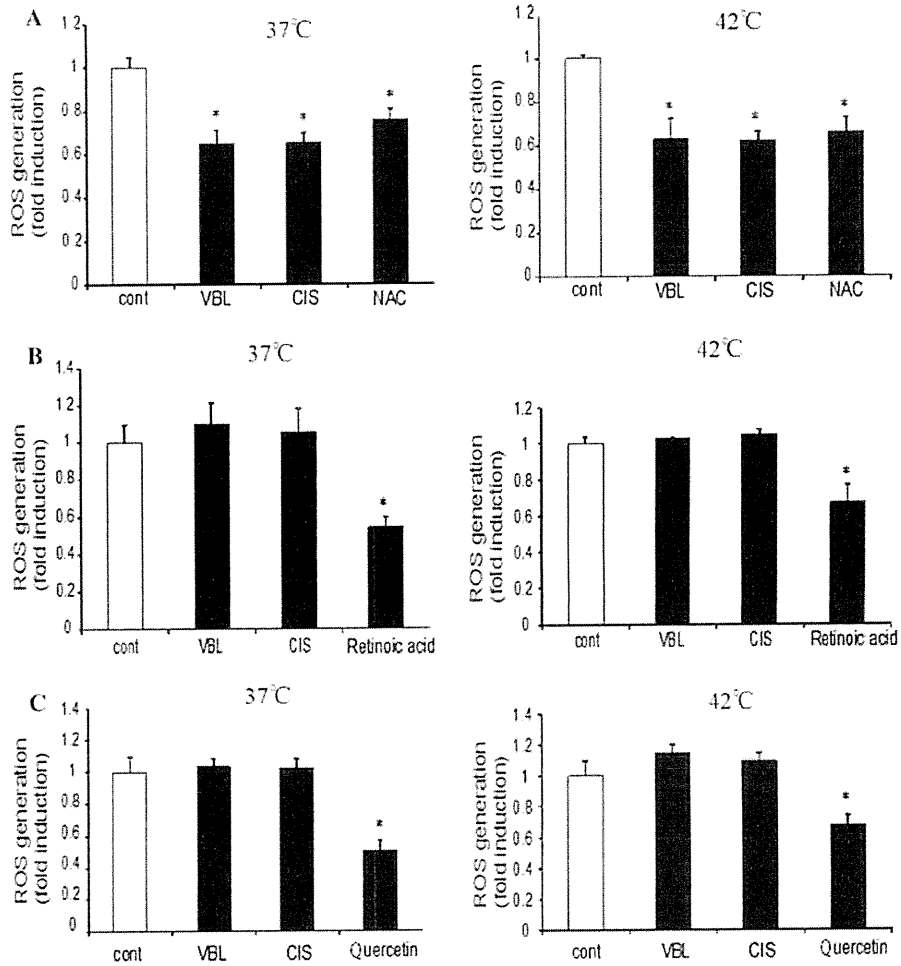


Fig. 4 Effect of various antioxidants on ROS production. ROS production was determined in the presence of 200 nM VBL or 15 μM CIS at 37 or 42°C. Various antioxidants, i.e., 10 mM NAC (*N*-acetyl-cysteine), 50 nM retinoic acid, 100 nM quercetin, 50 μM catechin, 100 nM lutein, and 20 μM, β-carotene, were added. Cells were incubated for 45 min, followed by determination of ROS production (mean ± SEM; $n = 4$, $^*p < 0.05$)

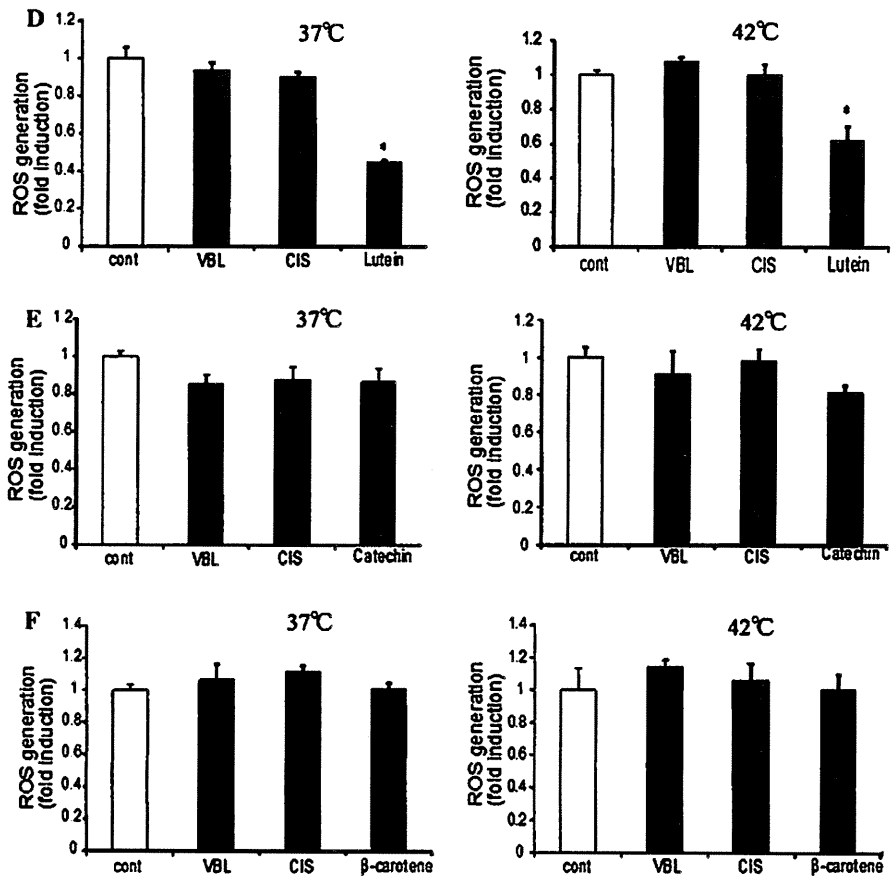
and VBL. Thus, both cancer chemotherapy and hyperthermic treatment enhanced ROS production, at least in prostate cancer cells.

With increasing public interest in antioxidant therapy, many nutritional supplements have been taken by the general public including cancer patients. There have been multiple studies that have examined the interaction between anticancer drugs and antioxidants. However, the results of these studies are not in agreement with each other. Anticancer drugs may produce ROS, which may damage cancer cells [27, 28]. Thereby, some studies demonstrated that antioxidants reduced the effect of these anticancer drugs [29]. In contrast, others demonstrated that ROS production was enhanced by antioxidants [30]. More specifically, ascorbic acid can quench ROS within the cell, and thus stabilize mitochondrial membrane, leading to protection of the cell [13, 26]. Although previous studies have demonstrated that ascorbic acid increased the effect of anticancer drugs, more recently attenuation of anticancer drug effect has also been reported [26].

We found that antioxidants indeed exhibited various effects on ROS production. NAC, which by itself scavenges ROS [18], potently decreased ROS production, and ROS production by anticancer drugs was also negated. Thus, the use of NAC may hamper the effect of anticancer drugs. In contrast, lutein, quercetin, and retinoic acid, which are also known as ROS scavengers, decreased ROS production. However, they were not potent enough to inhibit the ROS-producing effect of anticancer compounds. Thus, these antioxidants may be taken safely by cancer patients during chemotherapy and hyperthermic therapy. Catechin and β-carotene are known as antioxidants and are contained in various kinds of foods, such as green tea or carrot [11, 12]. However, they did not exhibit inhibitory effect on ROS production regardless of the presence of anticancer drugs, suggesting that they do not interfere with such drug effects. Thus, cancer patients may take these antioxidants as well as foods containing these antioxidants.

Putting it together, administration of NAC and ascorbic acid may need caution while other antioxidants may not

Fig. 4 continued



require major attention, at least in terms of ROS production in cancer patients. In particular, ascorbic acid is widely used for multiple purposes, including for viral infection. Accordingly, the current study has suggested that the use of ascorbic acid may be considered carefully by both cancer patients and oncologists. Further, with our findings, the effects of ascorbic acid and its related antioxidants need to be clinically examined in future in cancer patients who are to be treated with chemotherapy and/or hyperthermic therapy.

References

1. Johnson KA, Brown PH (2010) Drug development for cancer chemoprevention: focus on molecular targets. Semin Oncol 37(4):345–358
2. Zhang H, Wang G, Yang H (2011) Drug delivery systems for differential release in combination therapy. Expert Opin Drug Deliv 8:171–190
3. Suit HD, Shwayder M (1974) Hyperthermia: potential as an anti-tumor agent. Cancer 34:122–129
4. Li LF, Wang HQ, Liu XM, Zhang HL, Qiu LH, Qian ZZ, Li W (2011) Nimotuzumab in combination with chemotherapy in patients with advanced non-small cell lung cancer. Zhonghua Zhong Liu Za Zhi 33:626–628
5. Rodriguez-Luccioni HL, Latorre-Esteves M, Mendez-Vega J, Soto O, Rodriguez AR, Rinaldi C, Torres-Lugo M (2011) Enhanced reduction in cell viability by hyperthermia induced by magnetic nanoparticles. Int J Nanomed 6:373–380
6. Chen F, Wang CC, Kim E, Harrison LE (2008) Hyperthermia in combination with oxidative stress induces autophagic cell death in HT-29 colon cancer cells. Cell Biol Int 32:715–723
7. Hurwitz MD, Hansen JL, Prokopios-Davos S, Manola J, Wang Q, Bornstein BA, Hynynen K, Kaplan ID (2011) Hyperthermia combined with radiation for the treatment of locally advanced prostate cancer: long-term results from Dana–Farber Cancer Institute study 94–153. Cancer 117:510–516
8. Arai Y, Kondo T, Tanabe K, Zhao QL, Li FJ, Ogawa R, Li M, Kasuya M (2002) Enhancement of hyperthermia-induced apoptosis by local anesthetics on human histiocytic lymphoma U937 cells. J Biol Chem 277:18986–18993
9. Chan SW, Nguyen PN, Ayele D, Chevalier S, Aprikian A, Chen JZ (2011) Mitochondrial DNA damage is sensitive to exogenous H(2)O(2) but independent of cellular ROS production in prostate cancer cells. Mutat Res 716:40–50
10. Kurbacher CM, Wagner U, Kolster B, Andreotti PE, Krebs D, Bruckner HW (1996) Ascorbic acid (vitamin C) improves the

antineoplastic activity of doxorubicin, cisplatin, and paclitaxel in human breast carcinoma cells in vitro. Cancer Lett 103:183–189
11. Kim IS, Jin JY, Lee IH, Park SJ (2004) Auranofin induces apoptosis and when combined with retinoic acid enhances differentiation of acute promyelocytic leukaemia cells in vitro. Br J Pharmacol 142:749–755
12. Yeh SL, Wang WY, Huang CS, Hu ML (2006) Flavonoids suppresses the enhancing effect of beta-carotene on DNA damage induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in A549 cells. Chem Biol Interact 160:175–182
13. Heaney ML, Gardner JR, Karasavvas N, Golde DW, Scheinberg DA, Smith EA, O'Connor OA (2008) Vitamin C antagonizes the cytotoxic effects of antineoplastic drugs. Cancer Res 68:8031–8038
14. Maluta S, Dall'oglio S, Nadalini L (2010) Treatment for intermediate and high-risk prostate cancer: controversial issues and the role of hyperthermia. Int J Hyperthermia 26:765–774
15. Venkataraman S, Wagner BA, Jiang X, Wang HP, Schafer FQ, Ritchie JM, Patrick BC, Oberley LW, Buettner GR (2004) Overexpression of manganese superoxide dismutase promotes the survival of prostate cancer cells exposed to hyperthermia. Free Radic Res 38:1119–1132
16. Swaney JS, Roth DM, Olson ER, Naugle JE, Meszaros JG, Insel PA (2005) Inhibition of cardiac myofibroblast formation and collagen synthesis by activation and overexpression of adenylyl cyclase. Proc Natl Acad Sci USA 102:437–442
17. Kuznetsov AV, Kehrer I, Kozlov AV, Haller M, Redl H, Hermann M, Grimm M, Troppmair J (2011) Mitochondrial ROS production under cellular stress: comparison of different detection methods. Anal Bioanal Chem 400:2383–2390
18. Supabphol A, Muangman V, Chavasiri W, Supabphol R, Gritsanapan W (2009) *N*-acetylcysteine inhibits proliferation, adhesion, migration and invasion of human bladder cancer cells. J Med Assoc Thai 92:1171–1177
19. Jimenez-Aliaga K, Bermejo-Bescos P, Benedi J, Martin-Aragon S (2011) Quercetin and rutin exhibit antiamyloidogenic and fibril-disaggregating effects in vitro and potent antioxidant activity in APPswe cells. Life Sci 89:939–945
20. Sinha BK, Mimnaugh EG (1990) Free radicals and anticancer drug resistance: oxygen free radicals in the mechanisms of drug cytotoxicity and resistance by certain tumors. Free Radic Biol Med 8:567–581
21. Simon HU, Haj-Yehia A, Levi-Schaffer F (2000) Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis 5:415–418
22. Alexandre J, Hu Y, Lu W, Pelicano H, Huang P (2007) Novel action of paclitaxel against cancer cells: bystander effect mediated by reactive oxygen species. Cancer Res 67:3512–3517
23. Fukui M, Yamabe N, Zhu BT (2010) Resveratrol attenuates the anticancer efficacy of paclitaxel in human breast cancer cells in vitro and in vivo. Eur J Cancer 46:1882–1891
24. Narayana K (2010) Cisplatin induces duplex 3′ overhangs and 5′ blunt ends in epididymal epithelium in a Bax-dependent manner without any protection from L-ascorbic acid. Eur J Pharmacol 641:238–245
25. Kim HJ, Lee JH, Kim SJ, Oh GS, Moon HD, Kwon KB, Park C, Park BH, Lee HK, Chung SY et al (2010) Roles of NADPH oxidases in cisplatin-induced reactive oxygen species generation and ototoxicity. J Neurosci 30:3933–3946
26. Verrax J, Calderon PB (2008) The controversial place of vitamin C in cancer treatment. Biochem Pharmacol 76:1644–1652
27. Jackson IL, Batinic-Haberle I, Sonveaux P, Dewhirst MW, Vujaskovic Z (2006) ROS production and angiogenic regulation by macrophages in response to heat therapy. Int J Hyperthermia 22:263–273
28. Manda G, Nechifor MT, Neagu TM (2009) Reactive oxygen species, cancer and anti-cancer therapies. Curr Chem Biol 3:342–366
29. Labriola D, Livingston R (1999) Possible interactions between dietary antioxidants and chemotherapy. Oncology (Williston Park) 13:1003–1008 (discussion 1008, 1011-1002)
30. Block KI, Koch AC, Mead MN, Tothy PK, Newman RA, Gyllenhaal C (2007) Impact of antioxidant supplementation on chemotherapeutic efficacy: a systematic review of the evidence from randomized controlled trials. Cancer Treat Rev 33:407–418

Hypertension
Journal of the American Heart Association

Mice Lacking Hypertension Candidate Gene ATP2B1 in Vascular Smooth Muscle Cells Show Significant Blood Pressure Elevation

Yusuke Kobayashi, Nobuhito Hirawa, Yasuharu Tabara, Hidenori Muraoka, Megumi Fujita, Nobuko Miyazaki, Akira Fujiwara, Yasuhiro Ichikawa, Yuichiro Yamamoto, Naoaki Ichihara, Sanae Saka, Hiromichi Wakui, Shin-ichiro Yoshida, Keisuke Yatsu, Yoshiyuki Toya, Gen Yasuda, Katsuhiko Kohara, Yoshikuni Kita, Kohtaro Takei, Yoshio Goshima, Yoshihiro Ishikawa, Hirotsugu Ueshima, Tetsuro Miki and Satoshi Umemura

Hypertension. 2012;59:854-860; originally published online February 6, 2012;
doi: 10.1161/HYPERTENSIONAHA.110.165068
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0194-911X. Online ISSN: 1524-4563

Mice Lacking Hypertension Candidate Gene ATP2B1 in Vascular Smooth Muscle Cells Show Significant Blood Pressure Elevation

Yusuke Kobayashi, Nobuhito Hirawa, Yasuharu Tabara, Hidenori Muraoka, Megumi Fujita, Nobuko Miyazaki, Akira Fujiwara, Yasuhiro Ichikawa, Yuichiro Yamamoto, Naoaki Ichihara, Sanae Saka, Hiromichi Wakui, Shin-ichiro Yoshida, Keisuke Yatsu, Yoshiyuki Toya, Gen Yasuda, Katsuhiko Kohara, Yoshikuni Kita, Kohtaro Takei, Yoshio Goshima, Yoshihiro Ishikawa, Hirotsugu Ueshima, Tetsuro Miki, Satoshi Umemura

Abstract—We reported previously that ATP2B1 was one of the genes for hypertension receptivity in a large-scale Japanese population, which has been replicated recently in Europeans and Koreans. ATP2B1 encodes the plasma membrane calcium ATPase isoform 1, which plays a critical role in intracellular calcium homeostasis. In addition, it is suggested that ATP2B1 plays a major role in vascular smooth muscle contraction. Because the ATP2B1 knockout (KO) mouse is embryo-lethal, we generated mice with vascular smooth muscle cell-specific KO of ATP2B1 using the Cre-loxP system to clarify the relationship between ATP2B1 and hypertension. The KO mice expressed significantly lower levels of ATP2B1 mRNA and protein in the aorta compared with control mice. KO mice showed significantly higher systolic blood pressure as measured by tail-cuff method and radiotelemetric method. Similar to ATP2B1, the expression of the Na^+-Ca^{2+} exchanger isoform 1 mRNA was decreased in vascular smooth muscle cells of KO mice. However, ATP2B4 expression was increased in KO mice. The cultured vascular smooth muscle cells of KO mice showed increased intracellular calcium concentration not only in basal condition but also in phenylephrine-stimulated condition. Furthermore, phenylephrine-induced vasoconstriction was significantly increased in vascular rings of the femoral artery of KO mice. These results suggest that ATP2B1 plays important roles in the regulation of blood pressure through alteration of calcium handling and vasoconstriction in vascular smooth muscle cells. (***Hypertension*. 2012;59:854-860.**) • **Online Data Supplement**

Key Words: hypertension ■ ATP2B1 ■ Cre-loxP system ■ blood pressure ■ Millennium Genome Project ■ Global Blood Pressure Genetics

Numerous studies have attempted to identify genetic markers for hypertension over the past 2 decades, but no cross-validated loci in different ethnic groups have thus far been identified except for the mendelian forms of hypertension.[1] In the Millennium Genome Project[2] we identified single nucleotide polymorphisms located upstream or within the ATP2B1 gene as strong susceptible polymorphisms for hypertension in Japanese. Some of these findings have been replicated in individuals of European descent in the Global Blood Pressure Genetics sample and have also been validated in other studies in individuals of European descent,[3] Koreans,[4–6] and Japanese.[7] The single nucleotide polymorphisms of ATP2B1 identified in these studies showed a significant association with hypertension in various large-scale study populations with different methods, genome-wide association study in the Cohorts for Heart and Aging Research in Genomic Epidemiology Consortium and the Korean study and candidate gene analysis in our previous study. However, the functional roles of ATP2B1 in blood pressure control have not yet been proven in vivo. The ATP2B1-null mutant mouse has been reported to be embryolethal[8]; thus, we need to make a conditional knockout (KO) mouse model of ATP2B1 using the Cre-loxP system to reveal the function of the gene. Because the ATP2B1 gene encodes one of the calcium pumps and plays an important role in contraction of bladder smooth muscle,[9] we selected vascular smooth

Received October 25, 2010; first decision November 17, 2010; revision accepted January 15, 2012.

From the Departments of Medical Science and Cardiorenal Medicine (Y.K., M.F., N.M., A.F., N.I., S.S., H.W., S.Y., Y.T., S.U.) and Molecular Pharmacology and Neurobiology (H.M., K.T., Y.G.) and Cardiovascular Research Institute (Y.Ic., Y.Is.), Yokohama City University Graduate School of Medicine, Yokohama, Japan; Division of Nephrology and Hypertension (N.H., Y.Y., K.Y., G.Y.), Yokohama City University Medical Center, Yokohama, Japan; Department of Geriatric Medicine (Y.T., K.K., T.M.), Ehime University Graduate School of Medicine, Toon, Japan; Department of Health Science (Y.K., H.U.), Shiga University of Medical Science, Otsu, Japan.

The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.110.165068/-/DC1.

Correspondence to Nobuhito Hirawa, Division of Nephrology and Hypertension, Yokohama City University Medical Center, 45-7 Urafune-cho, Minami-ku, Yokohama 232-0024, Japan. E-mail hirawa@yokohama-cu.ac.jp

Hypertension **is available at http://hyper.ahajournals.org** **DOI: 10.1161/HYPERTENSIONAHA.110.165068**

muscle cells (VSMCs) as target tissue of KO. Because we have already demonstrated that ATP2B1 mRNA expression in human umbilical artery smooth muscle cells was significantly lower in those having the risk allele for hypertension than in those with no risk allele,[10] we hypothesized that VSMC ATP2B1 KO mice would exhibit high blood pressure. In the present study, we made the VSMC-specific ATP2B1 KO mice and evaluated their blood pressure and related mechanisms.

Materials and Methods

Animal Care

Animals were housed under a 12-hour light-dark cycle at a temperature of 25°C. Tap water was provided ad libitum. Experiments were conducted under the guidelines for animal experiments set by the animal experiment committee of Yokohama City University School of Medicine.

Creation of VSMC-Targeted ATP2B1 KO Mice

$ATP2B1^{loxP/loxP}$ mice were generated using the Cre-loxP and flippase recombination enzyme-flippase recognition target (FLP-FRT) recombination system. ATP2B1 is encoded by 21 exons on chromosome 10, and mice systemically deficient in exon 10 are reported to be embryolethal.[8] We, therefore, designed a vector to KO exon 10 of the ATP2B1 gene. The detailed technical strategy for conditional KO mouse generation is described in the Methods section of the online-only Data Supplement. To target inactivation of the ATP2B1 gene to VSMCs, $ATP2B1^{loxP/loxP}$ mice were intercrossed with SM22-Cre transgenic mice (see details in the online-only Data Supplement) expressing Cre recombinase under control of the mouse transgelin (smooth muscle protein 22-α) promoter. The resulting $ATP2B1^{loxP/-}$/SM22-Cre animals were further mated with $ATP2B1^{loxP/loxP}$ mice to generate $ATP2B1^{loxP/loxP}$/SM22-Cre (VSMC ATP2B1 KO) mice and $ATP2B1^{loxP/loxP}$ mice without SM22-Cre (control mice). Animals used for experiments were backcrossed ≥6 times.

BP Measurement by Tail-Cuff Method and Radiotelemetric Method

Systolic blood pressure was measured by the tail-cuff method (BP-monitor MK-2000; Muromachi Kikai Co) at the age of 8 weeks and 22 weeks, as described previously.[11,12] Furthermore, direct blood pressure measurement was performed by a radiotelemetric method in which a blood pressure transducer (PA-C10, Data Sciences International) was inserted into the left carotid artery. Ten days after transplantation, each mouse was housed individually in a standard cage on a receiver under a 12-hour light-dark cycle. Direct blood pressure was recorded every minute by radiotelemetry, as described previously.[13]

Real-Time Quantitative RT-PCR Analysis

Total RNA was extracted from the aorta or cultured VSMCs with ISOGEN (Nippon Gene), and cDNA was synthesized using the SuperScript III First Strand System (Invitrogen). Real-time quantitative RT-PCR was performed by incubating the reverse-transcription product with TaqMan PCR Master Mix and a designed TaqMan probe (Applied Biosystems).[12] RNA quantities were expressed relative to the 18S mRNA control.

Western Blot Analysis of ATP2B1

Western blot analysis was performed as described previously.[11] Further details of the Western blot analysis are described in the online-only Data Supplement.

Cell Culture of Mouse VSMCs and Measurement of Intracellular Calcium Concentration

VSMCs were aseptically isolated from thoracic aortic explants of an 8-week–old ATP2B1 KO mouse and its wild-type littermate, as described previously.[14] Further details of the cell culture of mouse VSMCs are described in the online-only Data Supplement. Measurement of basal condition and phenylephrine-stimulated changes in intracellular calcium concentration were assessed by Fura-2 fluorescence ratio imaging using a microscopic digital imaging system (IX71, Olympus), as described previously.[15] Briefly, ATP2B1 KO or control VSMCs grown on 25-mm coverslips were loaded with the calcium-specific dye Fura-2-acetoxymethyl ester (2.5 μmol/L, Invitrogen) and 0.01% Pluronic acid (Invitrogen) for 30 minutes at 37°C. After washing with the Hank balanced salt solution, cells were incubated for 20 minutes at 37°C in the Hank balanced salt solution to allow complete hydrolysis of Fura-2-acetoxymethyl ester to Fura-2. Emissions fluorescence was measured with a CCD camera (U-PMTV1X, Olympus) at a wavelength of 510 nm. Real-time shifts in Fura-2 ratio fluorescence (ratio of emissions: F340:F380), indicating changes in intracellular calcium concentration, were recorded before, during, and after stimulating VSMCs with 10^{-6} M phenylephrine (Sigma Aldrich), and we used calcium ionophore A23187 (Calbiochem) as positive control for the accuracy of the intracellular calcium concentrations. Summary data represent the average difference in basal condition and the peak increase in phenylephrine-induced intracellular calcium concentration.

Isometric Tension of Vascular Rings of Femoral Artery

We measured isometric tension of femoral artery vascular rings from KO mice and control mice, as described previously.[16] Phenylephrine and potassium-enriched solution were added to stimulate vasoconstriction. Further details of the vasoconstriction assay are described in the online-only Data Supplement.

Statistical Analysis

For statistical analysis of differences between groups, Mann-Whitney U test or ANOVA followed by Bonferroni method was used. All of the quantitative data are expressed as mean±SE. Values of $P<0.05$ were considered statistically significant.

Results

High Efficiency, VSMC-Selective Deletion of ATP2B1 Gene

Figure 1A shows the Southern blot analysis of tail DNA obtained from VSMCs. ATP2B1 KO mice demonstrated a deletion event occurring in VSMCs within the vascular bed of the tail. Quantitative RT-PCR analysis demonstrated that expression of ATP2B1 mRNA in isolated aorta of VSMC ATP2B1 KO mice was reduced by 80% to 90% compared with that in control mice (Figure 1B). Similarly, Western blot analysis showed that ATP2B1 protein in isolated aorta of VSMC ATP2B1 KO mice was reduced by 80% compared with that in control mice (Figure 1C and 1D).

VSMC ATP2B1 KO and Control Mice Were Both Born at Expected Mendelian Ratio

As shown in Figure 1E, both VSMC ATP2B1 KO mice and control mice, male and female, were born at the expected mendelian ratio and could not be distinguished from one another at birth.

No Difference in Growth Between VSMC ATP2B1 KO Mice and Control Mice

Body weight was measured at 8, 12, 22, and 36 weeks after birth. As seen in Figure 1F, there was no difference in

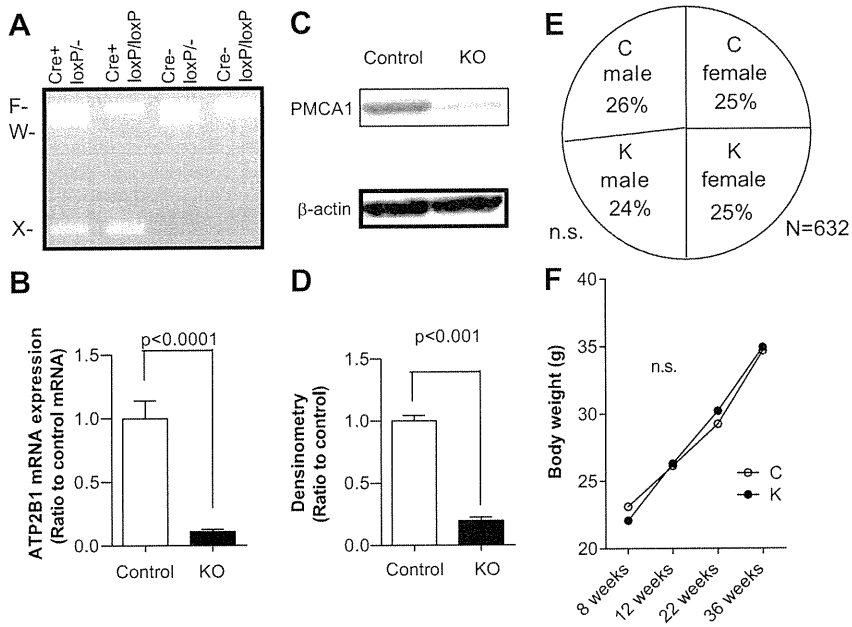


Figure 1. High efficiency, vascular smooth muscle cell (VSMC)-selective deletion of ATP2B1 gene. **A**, Tail DNA was prepared by standard methods, and the ATP2B1 gene was amplified by PCR using the forward primer 5′-CATCCTCTTTAGTTATTAAGGAAGCAGT-3′ (located in the intron before the first loxP site) and reverse primer 5′-GCCTTTTACAGCATGAACATAGCGA-3′ (located in the intron after the second loxP site). The presence of wild-type ATP2B1 (W), floxed ATP2B1 (F), and recombined ATP2B1 (X) was determined using the forward primer and reverse primer, generating products of 1282 bp for W, 1442 bp for F, and 399 bp for X. **B**, ATP2B1 mRNA expression in aorta of 8-week–old mice (n=6 for each genotype) as quantified by quantitative RT-PCR using exon 10 and 11 amplification. Data are presented as mean and SE from 6 independent experiments ($P<0.0001$). **C** and **D**, ATP2B1 protein expression in aorta of VSMC ATP2B1 knockout (KO) mice and control mice estimated by immunoblot analysis (n=5 for each genotype). One representative of 5 independent experiments is shown. Data are presented as mean and SE. **E**, Birth rate of mating, which is expected to have the same ratio of births. The data were collected from 632 mice born by the mating of VSMC ATP2B1 KO mice and control mice. **F**, Growth curve of VSMC ATP2B1 KO mice and control mice. The data were all collected from male mice. Weights for each genotype represent mean and SE (n=12–24 for each genotype; C indicates control mice; K, VSMC ATP2B1 KO mice). The data collected show that there was no difference in body weight alterations.

alteration of body weight between VSMC ATP2B1 KO mice and control mice.

VSMC ATP2B1 KO Mice Showed Higher Blood Pressure Than Control Mice Under Resting Conditions

To ascertain whether deletion of ATP2B1 in VSMCs affects blood pressure, conscious VSMC ATP2B1 KO mice and control mice were subjected to blood pressure measurements by the tail-cuff method. All of the experiments were carried out in a blinded manner on male mice eating standard rodent chow (0.3% NaCl). Under resting conditions, VSMC ATP2B1 KO mice displayed higher systolic blood pressure than that of control mice at 8 and 22 weeks of age (Figure 2A). Heart rate did not differ significantly between the groups (data not shown).

VSMC ATP2B1 KO Mice Showed Higher Blood Pressure Assessed by 24-Hour Radiotelemetric System Than Control Mice

To confirm the effects of deletion of VSMC ATP2B1 on blood pressure and to analyze the circadian pattern of blood pressure, conscious VSMC ATP2B1 KO mice and control mice were subjected to blood pressure measurements by radiotelemetry. KO mice showed higher blood pressure than control mice at 14 weeks of age throughout the day (systolic blood pressure, Figure 2B; diastolic blood pressure, Figure S9A; mean blood pressure, Figure S9B), whereas circadian variations in heart rate did not differ significantly between the groups (Figure 2C).

Expressions of Calcium-Regulatory Genes in Cultured VSMCs of ATP2B1 Mice

The same as for the in vivo results, expression of both ATP2B1 (0.07-fold $P<0.0001$; Figure 3A) and Na^+-Ca^{2+} exchanger isoform 1 (NCX1) (0.3-fold $P<0.0001$; Figure 3B) mRNAs were decreased in cultured VSMCs of ATP2B1 KO mice aorta compared with those in the control mouse aorta. On the contrary, the expression of ATP2B4 mRNA was upregulated (1.9-fold $P<0.0001$; Figure 3C) in KO VSMCs.

Increased Intracellular Calcium Concentrations in VSMCs of ATP2B1 KO Mice

To investigate whether intracellular calcium concentration in VSMCs was altered through KO of the ATP2B1 gene, we used the Fura-2-acetoxymethyl ester fluorescence assay. As shown in Figure 3D, the intracellular calcium was higher in VSMCs of ATP2B1 KO mice (F340/F380 ratio of KO VSMCs: 0.631 ± 0.029; F340/F380 ratio of control VSMCs: 0.505 ± 0.022; $P<0.05$) at baseline condition. Furthermore, phenylephrine-induced peak increase in intracellular calcium concentration was also augmented in KO VSMCs than in

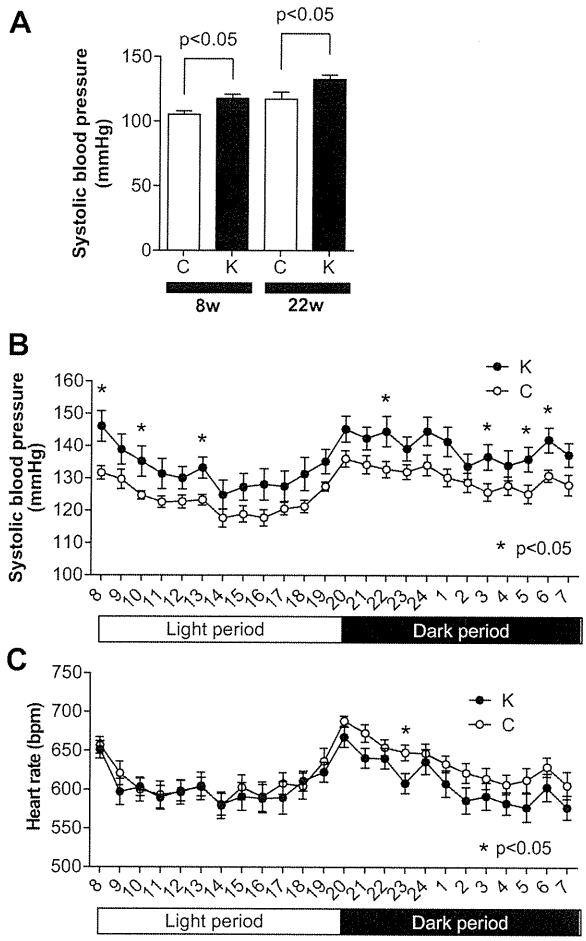


Figure 2. Blood pressure measured by tail-cuff method and radiotelemetric method. **A**, Systolic blood pressure was measured by tail-cuff method in 8-week–old knockout (KO) mice (117.7±3.4; n=15) and control mice (105.3±2.9; n=13), and 22-week–old vascular smooth muscle cell (VSMC) ATP2B1 KO mice (132.6±3.3; n=14) and control mice (117.3±5.4, n=11). Data are presented as mean and SE (C indicates control mice; K, VSMC ATP2B1 KO mice; 8w, 8-week–old; 22w, 22-week–old). **B**, Circadian patterns of systolic blood pressure in VSMC ATP2B1 KO mice (n=9) and control mice (n=9) on a 12-hour light (8:00 AM to 8:00 PM)/dark (8:00 PM to 8:00 AM) cycle are shown. Mice were studied on a normal-salt diet (0.3% NaCl). Values plotted are hourly means and SEs measured over 60 hours (C indicates control mice; K, VSMC ATP2B1 KO mice). **C**, Circadian patterns of heart rate in VSMC ATP2B1 KO mice (n=9) and control mice (n=9) on a 12-hour light (8:00 AM to 8:00 PM)/dark (8:00 PM to 8:00 AM) cycle are shown. Mice were studied on a normal-salt diet. Values plotted are hourly means and SEs measured over 60 hours.

control VSMCs (F340/F380 ratio of KO VSMCs: 1.187±0.068; F340/F380 ratio of control VSMCs: 0.805±0.034; $P<0.001$).

Vasoconstriction Was Accelerated by Phenylephrine Loading in Femoral Artery of KO Mice

We examined the vasoconstrictor response of femoral artery rings to phenylephrine. As summarized in Figure 4, femoral artery rings of KO mice were hyperreactive to the maximum concentration of phenylephrine (10^{-5} M) compared with those of control mice (KO: 84.1% KCl contraction; control: 54.4% KCl contraction; $P<0.05$; n=10).

Discussion

Implication of ATP2B1 in Blood Pressure Control and Function of ATP2B1 in VSMCs

This study showed that blood pressure was significantly higher in mice lacking ATP2B1 in VSMCs than that in wild mice. These results confirm the importance of the ATP2B1 gene in regulation of blood pressure. The ATP2B1 gene is one of the genes that we reported in 2008 as a gene for hypertension receptivity in a large-scale Japanese population, which has been confirmed recently in individuals of European descent, Koreans, and other Japanese. We first paid attention to the gene and made a strategy for creating a conditional KO model of the gene to confirm the relation between ATP2B1 and hypertension. VSMC ATP2B1 KO mice showed no significant change in birth rate and growth, although their expressions of ATP2B1 in the aorta and primary cultured VSMCs were markedly reduced, and they showed significantly higher blood pressure. We confirmed that the elevation of blood pressure in ATP2B1 KO mice was certain, with no relation to age and light-dark cycle. Furthermore, alteration in calcium homeostasis in VSMCs and increased vasoconstriction of femoral artery were observed in ATP2B1 KO mice. Recently, we showed that single nucleotide polymorphisms in the ATP2B1 gene cause phenotypic changes in human tissue.[10] ATP2B1 mRNA expression in human umbilical artery smooth muscle cells was significantly lower in those with a risk allele for hypertension than in those having no risk allele. The finding using human artery was consistent with those seen in mice lacking ATP2B1 in VSMCs. These findings support that KO of ATP2B1 in VSMCs caused blood pressure elevation.

The ATP2B1 gene encodes plasma membrane calcium ATPase isoform 1 (so-called PMCA1), which removes bivalent calcium ions from eukaryotic cells against very large concentration gradients and plays a critical role in intracellular calcium homeostasis. In mammals, calcium ATPase isoforms are encoded by ≥4 separate genes (ATP2B1 to B4),[17] and organ-specific mRNA expression of the isoforms has been reported. Using bladder smooth muscle cells, contractility measurements have documented the important role of ATP2B1 in the extrusion of Ca^{2+} after carbachol stimulation or depolarization with potassium chloride.[9] Although bladder smooth muscle expresses both ATP2B4 and ATP2B1, ATP2B1 inhibition caused 3-time increments in intracellular calcium concentration and contraction of bladder smooth muscle compared with ATP2B4 blockade. Thus, ATP2B1 rather than ATP2B4 may have an important role in calcium handling and regulation in contraction of smooth muscle cells. In vascular smooth muscle, ATP2B1 and ATP2B4 have been also shown to be expressed.[18] However, there were few reports evaluating the role of ATP2B1 in VSMCs. Thus, we decided to knock out the ATP2B1 gene of VSMCs to clarify the function of the ATP2B1 gene in hypertension theoretically in humans. In fact, VSMC-specific KO of ATP2B1