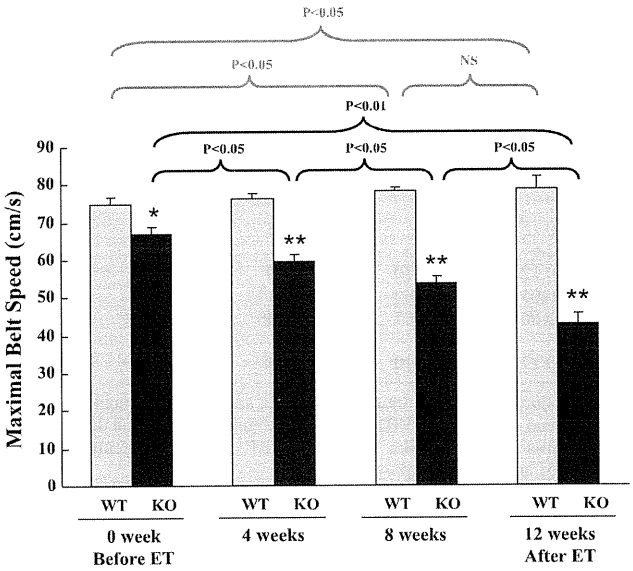

Fig. 1. The effect of endurance exercise training (ET) on maximal exercise ability in sarcalumenin (SAR) knockout (SARKO) mice. Maximal exercise ability, as evaluated by maximal belt speed, is already lower in SARKO mice than in wild-type (WT) mice before ET. During ET, maximal exercise ability gradually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

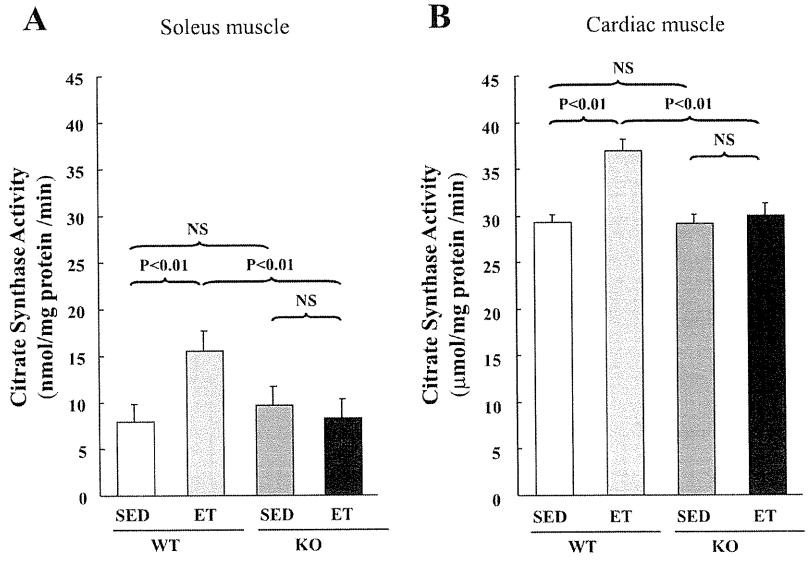

Fig. 2. Citrate synthase (CS) activity after ET. After ET, CS activity of soleus muscle (*A*) and cardiac muscle (*B*) was increased in ET-WT mice, but not in ET-SARKO mice. Values are means ± SE; n = 5 for each group. SED, sedentary; NS, not significant.

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.

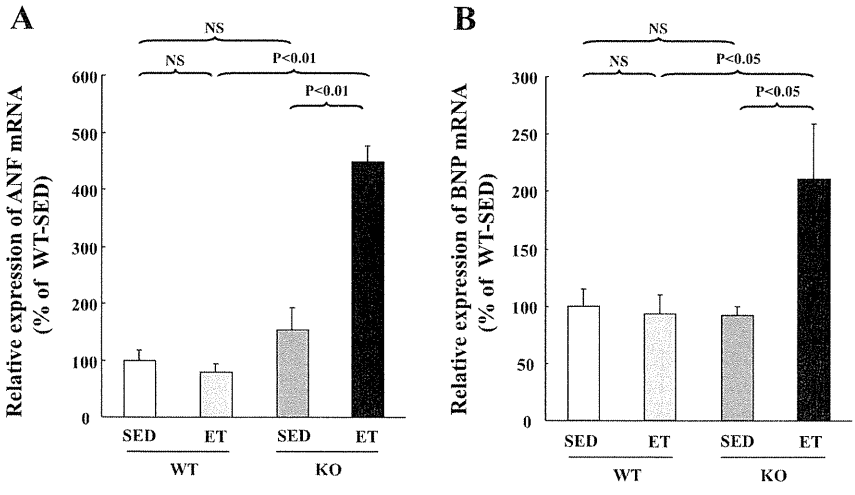

Fig. 3. Upregulation of atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) mRNAs in ET-SARKO mice. Quantitative RT-PCR analyses revealed that the expression levels of ANF (*A*) and BNP (*B*) mRNAs were significantly upregulated in the ventricles of ET-SARKO mice. The expression levels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

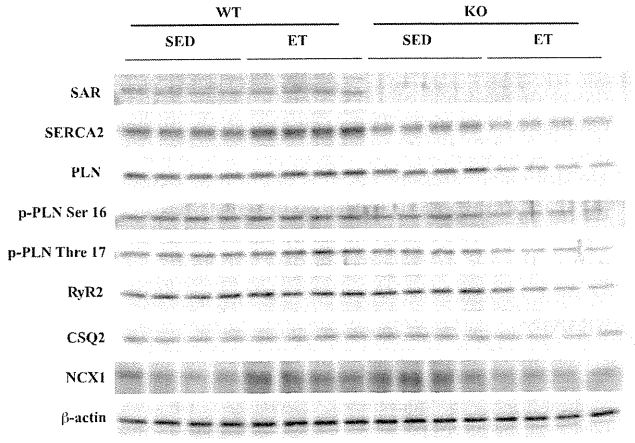

Fig. 4. The expression of Ca^{2+} handling proteins after ET. The expression levels of sarcalumenin (SAR), sarco(endo)plasmic reticulum Ca^{2+}-ATPase 2 (SERCA2), phospholamban (PLN), phosphorylated PLN (p-PLN), ryanodine receptor 2 (RyR2), calsequestrin 2 (CSQ2), and sodium/calcium exchanger 1 (NCX1)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

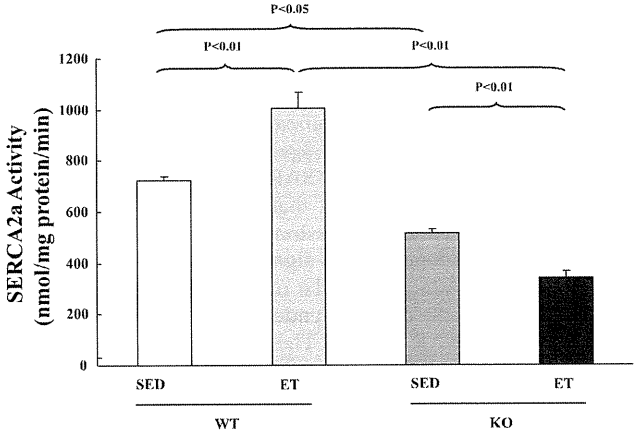

Fig. 5. SERCA2a activity after ET. SERCA2a activity was increased in ET-WT, whereas it was decreased in ET-SARKO after ET. Values are means ± SE; $n = 5$ for each group.

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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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.

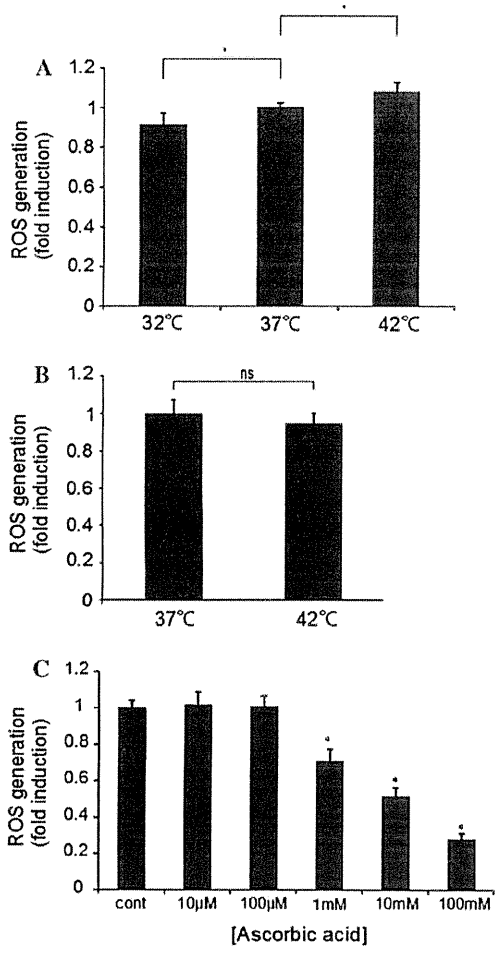


Fig. 1 ROS production in cancer cells and normal cells at different temperatures. **a** ROS production in cancer cells at 32, 37, and 42°C. Prostate cancer cells were incubated at different temperatures, followed by determination of ROS production (mean ± SEM; $n = 4$, $^*p < 0.05$). **b** ROS production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

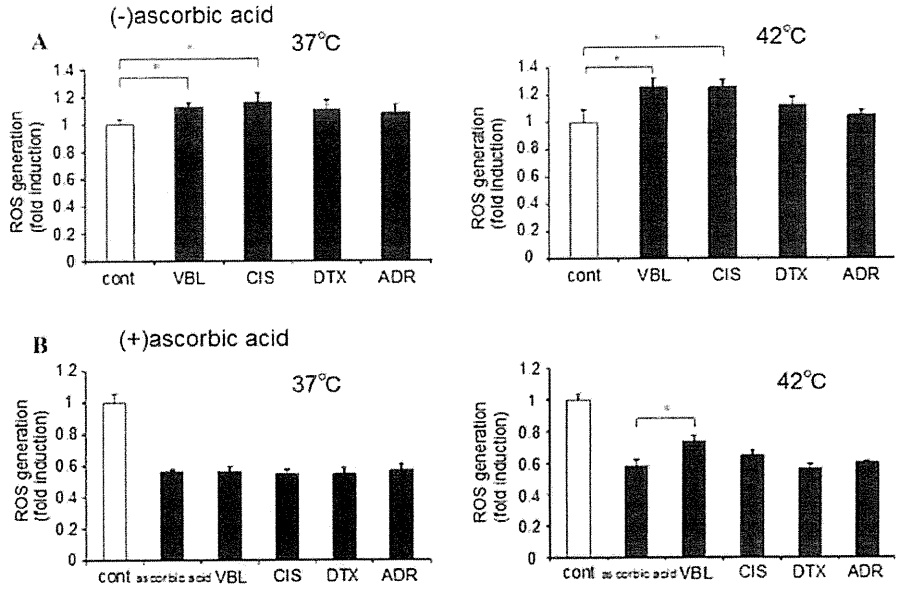


Fig. 2 Effect of anticancer drugs and ascorbic acid on ROS production. **a** ROS production was determined at 37 or 42°C in the presence of 200 nM VBL, 15 μM CIS, 7.5 μM DTX or 1 μM ADR (mean ± SEM; $n = 4$, $^*p < 0.05$). **b** ROS production was similarly determined in the presence of 1 mM ascorbic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

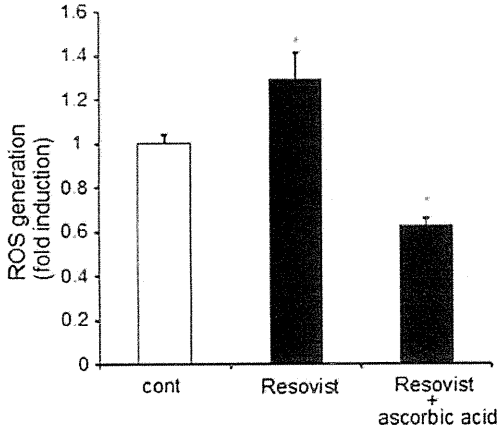


Fig. 3 Effect of Resovist on ROS production. ROS production was determined in the presence of 10 μM Resovist and/or 1 mM ascorbic acid at 37°C. Prostate cancer cells were incubated for 45 min, followed by ROS production assays (mean ± SEM; $n = 4$, $^*p < 0.05$)

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

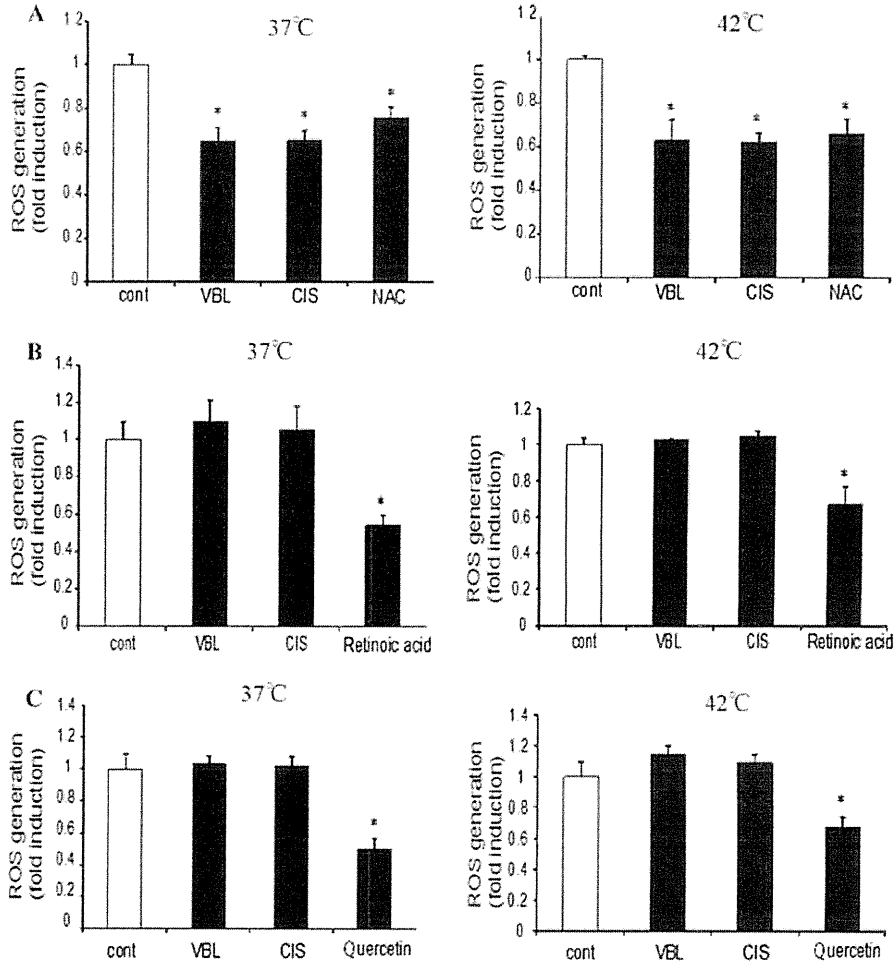


Fig. 4 Effect of various antioxidants on ROS production. ROS production was determined in the presence of 200 nM VBL or 15 μM CIS at 37 or 42°C. Various antioxidants, i.e., 10 mM NAC (*N*-acetyl-cysteine), 50 nM retinoic acid, 100 nM quercetin, 50 μM catechin, 100 nM lutein, and 20 μM, β-carotene, were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

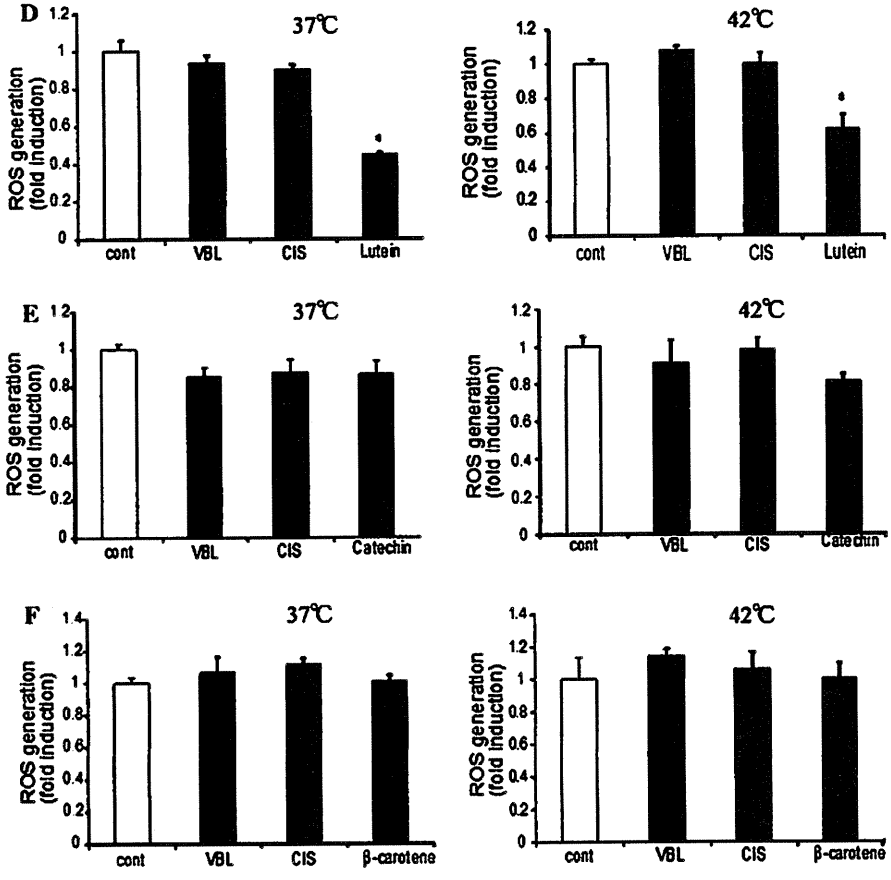
and VBL. Thus, both cancer chemotherapy and hyperthermic treatment enhanced ROS production, at least in prostate cancer cells.

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.

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Hypertension
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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

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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

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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

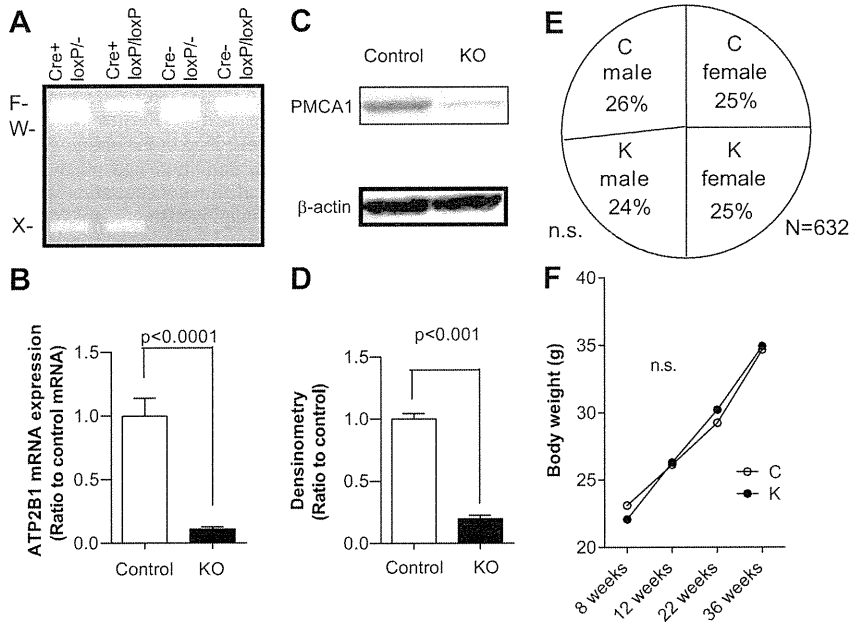

Figure 1. High efficiency, vascular smooth muscle cell (VSMC)-selective deletion of ATP2B1 gene. **A**, Tail DNA was prepared by standard methods, and the ATP2B1 gene was amplified by PCR using the forward primer 5′-CATCCTCTTTAGTTATTAAGGAAGCAGT-3′ (located in the intron before the first loxP site) and 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

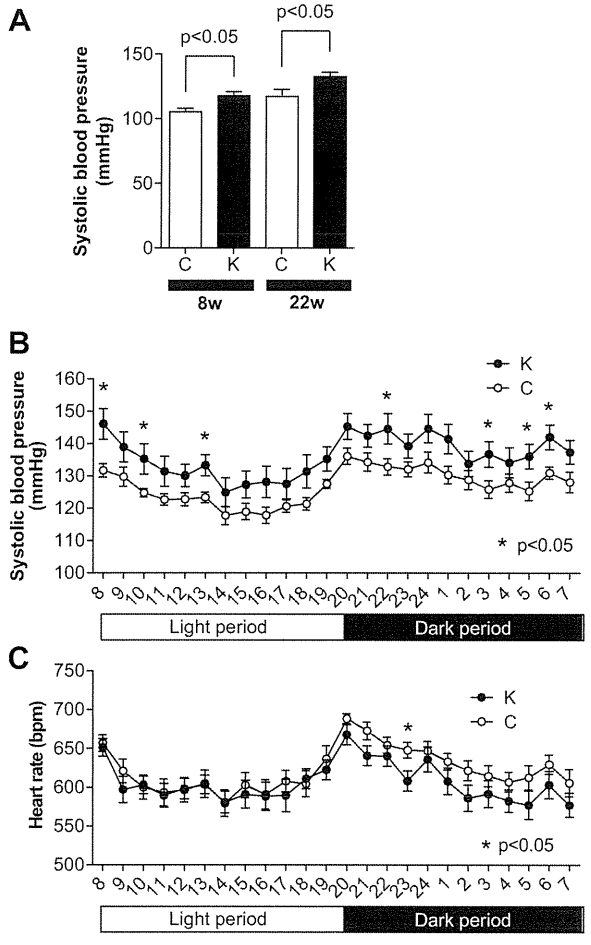

Figure 2. Blood pressure measured by tail-cuff method and radiotelemetric method. **A**, Systolic blood pressure was measured by tail-cuff method in 8-week–old knockout (KO) mice (117.7±3.4; n=15) and control mice (105.3±2.9; n=13), and 22-week–old vascular smooth muscle cell (VSMC) ATP2B1 KO mice (132.6±3.3; 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

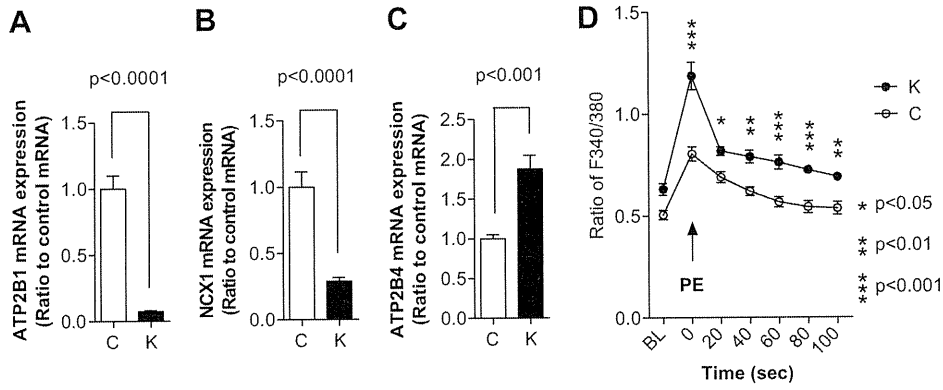

Figure 3. Altered gene expression and calcium transient in primary cultured vascular smooth muscle cells (VSMCs) of knockout (KO) mice. **A**, ATP2B1 mRNA expression in primary cultured VSMCs (n=8–10 for each genotype) quantified by quantitative RT-PCR (qRT-PCR) using exon 10 and 11 amplification. Data are presented as mean and SE ($P<0.0001$). **B**, NCX1 mRNA expression in primary cultured VSMCs (n=8–10 for each genotype) quantified by qRT-PCR. Data are presented as mean and SE ($P<0.0001$). **C**, ATP2B4 mRNA expression in primary cultured VSMCs (n=8–10 for each genotype) quantified by qRT-PCR. Data are presented as mean and SE ($P<0.0001$). **D**, Measurement of basal condition and phenylephrine-induced increase in intracellular calcium concentration of VSMCs were performed. Figure shows the time course of phenylephrine-stimulated change in intracellular calcium concentration in ATP2B1 KO VSMC and control VSMC mice (n=26–30 cells from 10 to 11 coverslips). ATP2B1 KO VSMC mice showed higher intracellular calcium concentration the entire time course before and after the phenylephrine stimulation. Data are displayed as ratio of F340/F380. Intracellular calcium concentration values over the entire cell were averaged to obtain the changes in the whole-cell calcium concentration. Data are presented as mean and SE (BL indicates baseline condition; PE, phenylephrine; K, ATP2B1 KO VSMC; C, control VSMC; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$).

showed an elevation in blood pressure associated with a rise in intracellular calcium and a decrease in NCX1 mRNA expression. Moreover, the vascular contractile response was increased in KO mice. These results suggest that ATP2B1 has important roles in calcium handling and contraction in VSMCs.

Important Relationship Between ATP2B1 and Other Calcium-Related Genes

NCX1, a calcium pump similar to ATP2B1, is known to play an important role in hypertension through its effect on VSMCs.[19,20] Interestingly, a recent report revealed that calcium clearance proteins, such as plasma membrane Ca^{2+}-ATPase (PMCA), sarcoplasmic reticulum Ca^{2+} ATPase, and NCX1, showed coordinated expression.[21] Furthermore, PMCA and NCX1 showed similar changes in expression in human arterial myocytes.[22] These recent findings suggest that ATP2B1 and NCX1 have a strong relationship and are modulated by the same system. Moreover, NCX1 and PMCA showed colocalization in the basolateral membrane of mouse distal convoluted cells in several studies.[23,24] Furthermore, several proteins associate with NCX1 and PMCA with an alteration in their activity.[25] On the contrary, ATP2B4 was upregulated in KO VSMCs. This upregulation of ATP2B4 seems to compensate for the decrease in expression of ATP2B1. These findings suggest that decreased expression of ATP2B1 and NCX1 is one of the possible mechanisms of increase in intracellular calcium concentration.

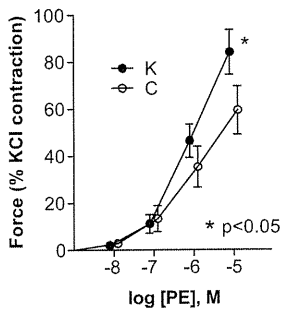

Figure 4. Phenylephrine-induced vasoconstriction of femoral artery rings. Isolated femoral artery rings obtained from knockout (KO) mice and control mice were stimulated with the α1-adrenoceptor agonist phenylephrine. Data are presented as mean and SE of 9 to 10 independent experiments. Force is expressed as the percentage of maximal contraction obtained by potassium-enriched solution.

Possibility of Alteration in Intracellular Calcium Homeostasis

In a recent study, a novel PMCA1 selective inhibitor, caloxin1b3, raised cytosolic calcium concentration in endothelial cells.[26] This finding supports the results that KO of ATP2B1 in VSMCs causes significant alterations in calcium-related gene expression and in intracellular calcium concentration. In the present study, KO VSMCs showed higher intracellular calcium concentration compared with the control, and a higher response was observed in response to the stimulation of phenylephrine compared with that of control VSMCs. Because increased intracellular calcium concentration may lead to blood pressure elevation via vasoconstriction,[27] the increased calcium concentrations seen in ATP2B1 KO VSMCs may be one of the possible mechanisms of high blood pressure seen in AP2B1 KO mice.

Increased Vasoconstriction Is One of the Possible Mechanisms for Blood Pressure Elevation

In the present study, we confirmed an increased contractile response to phenylephrine in femoral artery rings of KO

mice. Because phenylephrine activates inositol 1,4,5-triphosphate-induced intracellular calcium release and also stimulates voltage-independent calcium-permeable channels,[28] the alteration in contractile response may be attributed to alteration in intracellular calcium homeostasis. As shown in the present study, increased intracellular calcium concentration would augment the contractile capacity, which might increase the blood pressure in ATP2B1 KO mice. These findings strongly support the hypothesis that ATP2B1 gene is associated with blood pressure control in vivo.

Conclusion

We revealed that ATP2B1 KO in VSMCs increases the blood pressure in vivo study. Lack of ATP2B1 in VSMCs also increased intracellular calcium concentration and augmented the vascular contractility in ex vivo study. Our results clearly demonstrated that ATP2B1 gene expression in VSMCs is important in blood pressure regulation. Because the ATP2B1 gene has been reported to be a hypertension-susceptible gene by our systemic multiple candidate gene analyses, the present data suggest not only the importance of the ATP2B1 gene as a hypertension-related gene but also the value of the systemic multiple candidate gene approach and genome-wide association study in finding disease-related genes.

Perspectives

We made mice with conditional KO of ATP2B1 in VSMCs. However, the role of ATP2B1 in cells other than VSMCs in blood pressure control is not known. Thus, we need to further investigate the role of the ATP2B1 gene using other types of Cre mice and should make new organ-specific KO mice to analyze the role of the ATP2B1 gene in other conditions.

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Disclosures

N.H., Y.T., K.K., Y.K., H.U., T.M., and S.U. have been named as the inventors on a patent application by Ehime University, Shiga University of Medical Science, and Yokohama City University in work related to this study.

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