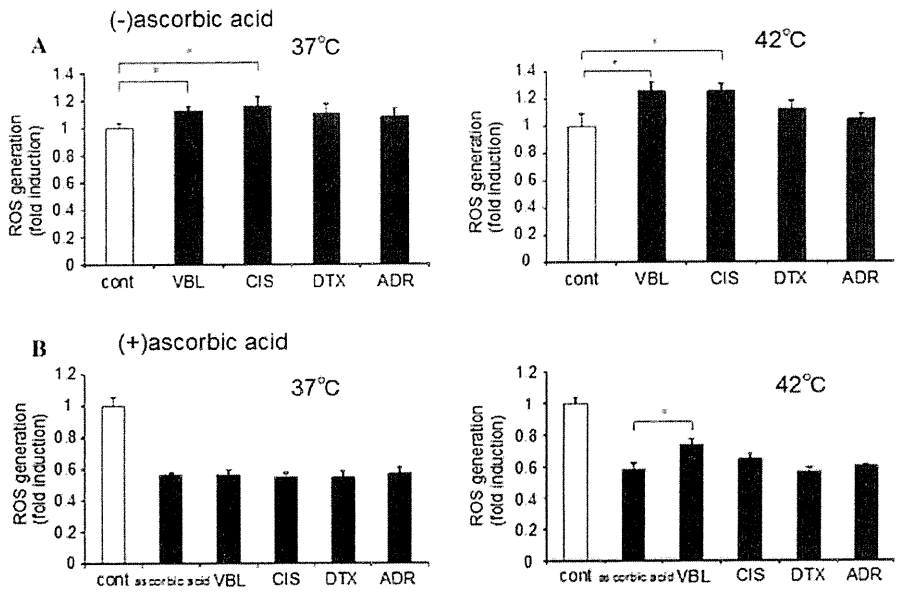


Fig. 2 Effect of anticancer drugs and ascorbic acid on ROS production. **a** ROS production was determined at 37 or 42°C in the presence of 200 nM VBL, 15 μM CIS, 7.5 μM DTX or 1 μM ADR (mean ± SEM; $n = 4$, $^*p < 0.05$). **b** ROS production was similarly determined in the presence of 1 mM ascorbic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

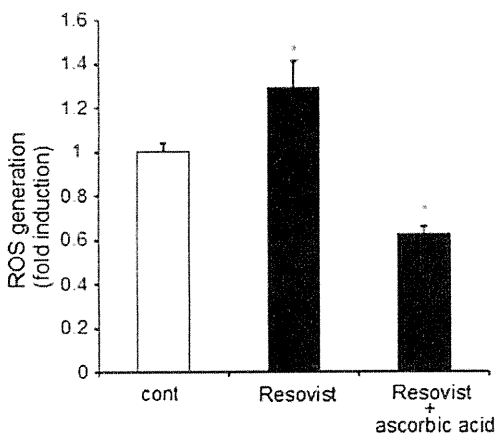


Fig. 3 Effect of Resovist on ROS production. ROS production was determined in the presence of 10 μM Resovist and/or 1 mM ascorbic acid at 37°C. Prostate cancer cells were incubated for 45 min, followed by ROS production assays (mean ± SEM; $n = 4$, $^*p < 0.05$)

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

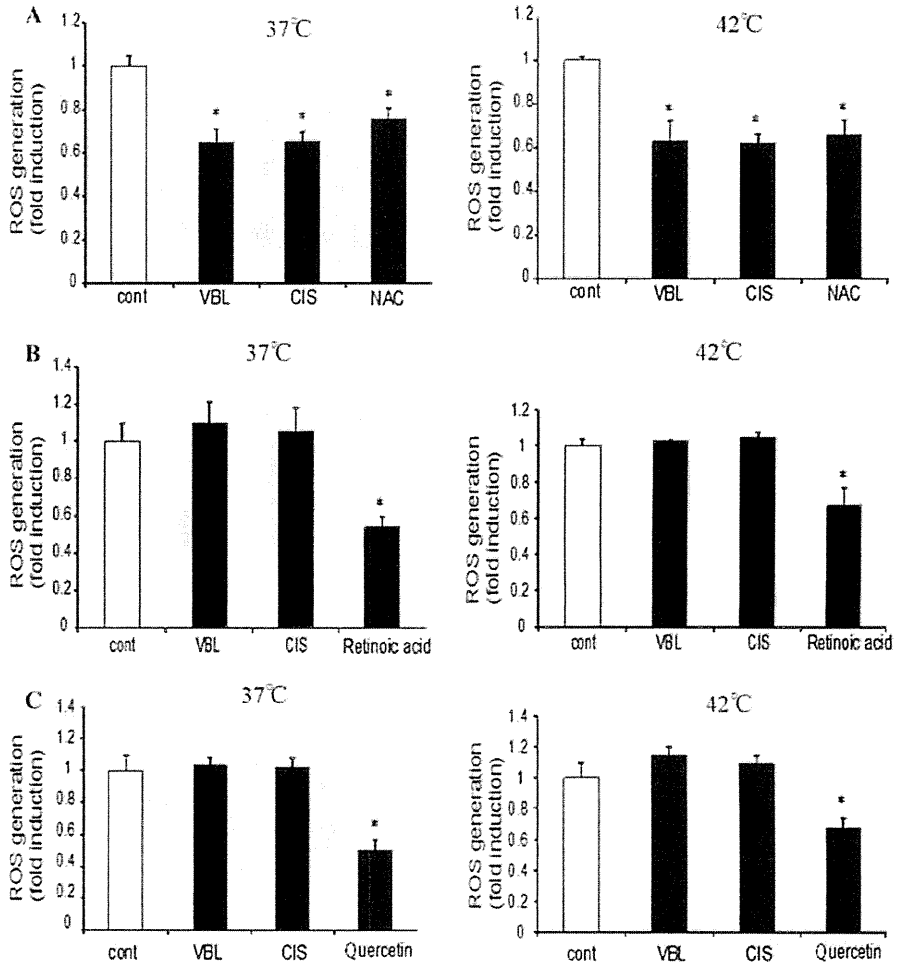


Fig. 4 Effect of various antioxidants on ROS production. ROS production was determined in the presence of 200 nM VBL or 15 μM CIS at 37 or 42°C. Various antioxidants, i.e., 10 mM NAC (*N*-acetyl-cysteine), 50 nM retinoic acid, 100 nM quercetin, 50 μM catechin, 100 nM lutein, and 20 μM, β-carotene, were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

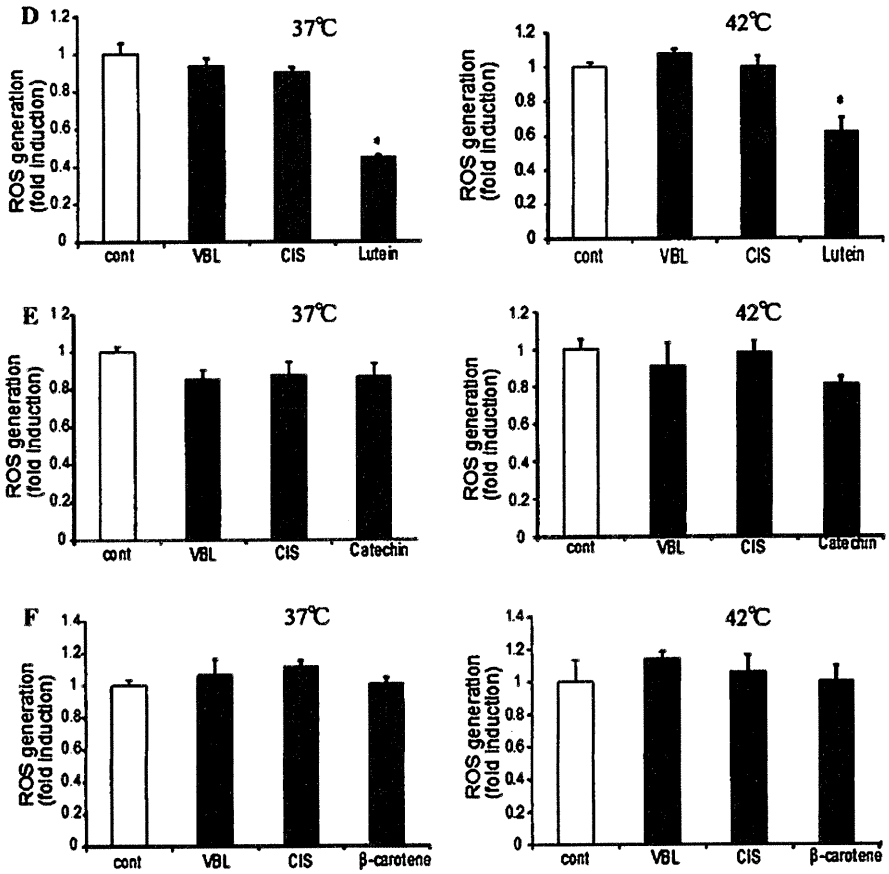
and VBL. Thus, both cancer chemotherapy and hyperthermic treatment enhanced ROS production, at least in prostate cancer cells.

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

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

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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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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.

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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

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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

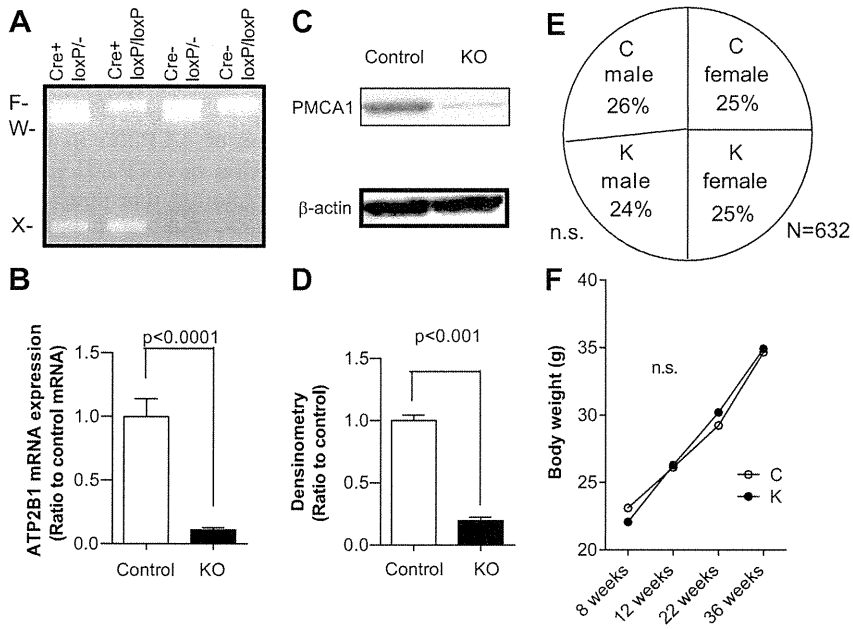

Figure 1. High efficiency, vascular smooth muscle cell (VSMC)-selective deletion of ATP2B1 gene. **A**, Tail DNA was prepared by standard methods, and the ATP2B1 gene was amplified by PCR using the forward primer 5′-CATCCTCTTTAGTTATTAAGGAAGCAGT-3′ (located in the intron before the first loxP site) and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

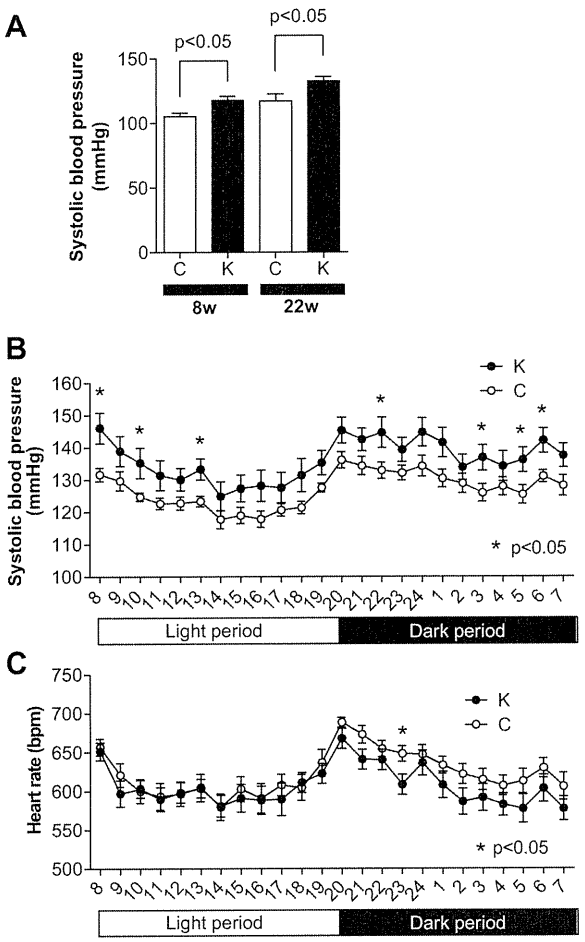

Figure 2. Blood pressure measured by tail-cuff method and radiotelemetric method. **A**, Systolic blood pressure was measured by tail-cuff method in 8-week–old knockout (KO) mice (117.7±3.4; n=15) and control mice (105.3±2.9; n=13), and 22-week–old vascular smooth muscle cell (VSMC) ATP2B1 KO mice (132.6±3.3;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

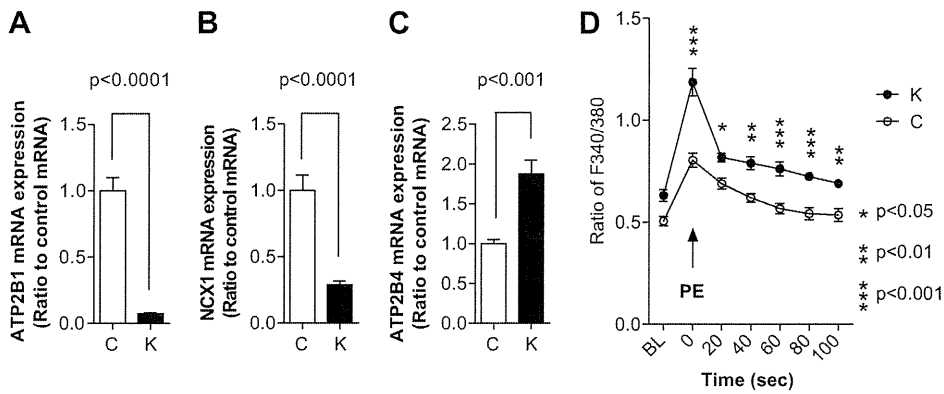

Figure 3. Altered gene expression and calcium transient in primary cultured vascular smooth muscle cells (VSMCs) of knockout (KO) mice. **A**, ATP2B1 mRNA expression in primary cultured VSMCs (n=8–10 for each genotype) quantified by quantitative RT-PCR (qRT-PCR) using exon 10 and 11 amplification. Data 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.

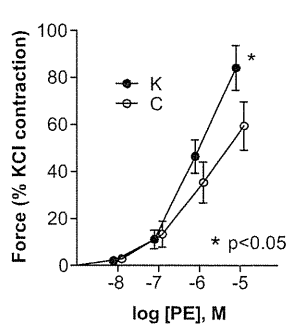

Figure 4. Phenylephrine-induced vasoconstriction of femoral artery rings. Isolated femoral artery rings obtained from knockout (KO) mice and control mice were stimulated with the α1-adrenoceptor agonist phenylephrine. Data are presented as mean and SE of 9 to 10 independent experiments. Force is expressed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

Title

Mice lacking hypertension candidate gene ATP2B1 in vascular smooth muscle cells show significant blood pressure elevation

Authors

Yusuke Kobayashi[1)], Nobuhito Hirawa[2)], Yasuharu Tabara[3)], Hidenori Muraoka[4)], Megumi Fujita[1)], Nobuko Miyazaki[1)], Akira Fujiwara[1)], Yasuhiro Ichikawa[5)], Yuichiro Yamamoto[2)], Naoaki Ichihara[1)], Sanae Saka[1)], Hiromichi Wakui[1)], Shin-ichiro Yoshida[1)], Keisuke Yatsu[2)], Yoshiyuki Toya[1)], Gen Yasuda[2)], Katsuhiko Kohara[3)], Yoshikuni Kita[6)], Kohtaro Takei[4)], Yoshio Goshima[4)], Yoshihiro Ishikawa[5)], Hirotsugu Ueshima[6)], Tetsuro Miki[3)], Satoshi Umemura[1)]

Affiliations

1. Department of Medical Science and Cardiorenal Medicine, Yokohama City University Graduate School of Medicine, Yokohama, Japan
2. Division of Nephrology and Hypertension, Yokohama City University Medical Center, Yokohama, Japan
3. Department of Geriatric Medicine, Ehime University Graduate School of Medicine, Toon, Japan
4. Department of Molecular Pharmacology and Neurobiology, Yokohama City University Graduate School of Medicine, Yokohama, Japan
5. Cardiovascular Research Institute, Yokohama City University Graduate School of Medicine, Yokohama, Japan
6. Department of Health Science, Shiga University of Medical Science, Otsu, Japan

METHODS

Animal Care

Animals were housed under a 12-hour day/night 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. In order to clarify the importance of the ATP2B1 gene in blood pressure regulation, we attempt to knock out the function of the ATP2B1 gene

of vascular smooth muscle cells. To generate conditional ATP2B1 KO mice, we utilized the Cre-loxP and FLP-FRT recombination system.

Cre-Mice

SM22-Cre mice [Tg(Tagln-cre)1Her/J, stock #004746] were obtained from The Jackson Laboratory (Bar Harbor, ME, USA). The SM22-Cre transgenic mice express Cre recombinase under control of the mouse transgelin (smooth muscle protein 22-alpha) promoter. Thus SM22-Cre mice knockout the gene that is sandwiched with loxP sites in vascular smooth muscle cell (VSMC) specifically. Mice engineered in this study were backcrossed onto the (C57BL/6J) genetic background for at least six generations.

ATP2B1 conditional knockout (KO) mouse

Conditional ATP2B1 KO mouse was generated by Cre/loxP and FLP-FRT recombination system. ATP2B1 is encoded by 21 exons on chromosome 10, and mice lacking exon 10 was reported to be an embryonic lethal. We therefore designed a new vector to knockout the exon 10 of ATP2B1 gene (Figure S1). This targeting vector contains a loxP-FRT-PGK-neo-FRT cassette with 5,572 bp of ATP2B1 homologous regions upstream of exon 10, and loxP site with 3,891 bp homologous regions downstream of exon 10. After linearization and electroporation into the C57BL/6 (RENKA) ES cell lines (Transgenic Inc., Kumamoto, Japan), 431 neo-resistant ES cell clones were obtained. Among the randomly selected 143 ES clones, homologous recombination was confirmed in six clones (#73583, #73584, #73588, #73612, #73693, #73697) by KpnI or PvuII digested Southern blot analysis using 5' probe, 3' probe or neo probe (Figure S2, S3, S4). To verify the recombination of loxP site downstream exon 10, PCR-amplified fragment length (using primer A and primer B) was analyzed by agarose-gel electrophoresis (Figure S5). Both wild-type allele (460 bp) and mutant allele (519 bp) was ascertained in all six clones. We next eliminated the PGK-neo region by transfection of Flp recombinase vector in to the #73588 clone (Figure S6). Flp/FRT recombination was verified by analyzing the length of PCR product amplified with primer C and primer D (Figure S7). Among 104 transfected clones, recombination was ascertained in the seven clones (#77743, #77775, #77936, #77937, #77956, #77961, #77964). KpnI or EcoRI digested Southern blot analysis using 5' probe, 3' probe, or DR probe further confirmed the Flp/FRT recombination in all seven clones (Figure S8). Three ATP2B1floxed ES cell clones (#77743, #77775, #77936) were aggregated with 8-cell stage embryos (ICR strain). The embryo was transferred into uterus of recipient females, and chimeras were delivered by Caesarean section at embryonic 17 day. The

chimeras were mated with wild-type mouse, and germline transmission was ascertained in 14 (9 male, and 5 female) of 31 F1 mouse.

Creation of the Vascular Smooth Muscle Cell-targeted ATP2B1 KO Mice

ATP2B1$^{loxP/loxP}$ mice were generated by the Cre-loxP and FLP-FRT recombination system. ATP2B1 is encoded by 21 exons on chromosome 10, and mice lacking exon 10 are reported to be embryo-lethal. We therefore designed a vector to knockout exon 10 of the ATP2B1 gene. To target inactivation of the ATP2B1 gene to VSMC, ATP2B1$^{loxP/loxP}$ mice were intercrossed with SM22-Cre transgenic mice expressing Cre recombinase under control of the mouse transgelin (smooth muscle protein 22-alpha) 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 at least 6 times.

Blood Pressure Measured by Radiotelemetric Method

Direct blood pressure measurement was performed by a radiotelemetric method in which a blood pressure transducer (PA-C10, Data Sciences International, USA) 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 [1].

Cell Culture of Mouse VSMC

The VSMC were aseptically isolated from thoracic aortic explants of an 8-week-old ATP2B1 KO mouse and its wild-type littermate as described previously [2]. VSMC were prepared by the explant method and cultured in Dulbecco's modified Eagle's Medium as described previously [3, 4]. VSMC multiplying in a medium containing FBS rapidly lose their contractile features and become "synthetic" cells [3]. Thus, the early passage cells (third to sixth passage) were used in the present study. Subconfluent VSMC were used in the following experiments. The serum of these cells were deprived for 24 hours and then stimulated as indicated. After the desired incubation period, cells were rinsed with ice-cold phosphate-buffered saline and then lysed and sonicated.

Western Blot Analysis of ATP2B1

Western blot analysis was performed as described previously [5, 6]. Briefly, tissue extracts

were used for electrophoresis, and membranes (Millipore, USA) were incubated with rabbit polyclonal anti-ATP2B1 antibody (MBL, Japan) and subjected to enhanced chemiluminescence (GE Healthcare, US). The images were analyzed quantitatively using a FUJI LAS3000 Image Analyzer (FUJI Film, Japan) for determination of the ATP2B1 protein level. To measure the aortic expression ratio of ATP2B1/β-actin, each ATP2B1 protein level was divided by the corresponding β-actin protein level obtained by re-probing, and thus derived from the same extract.

Isometric Tension of the Femoral Artery Vascular Rings

After the mice were anesthetized with pentobarbital, the femoral artery ring was isolated and placed in a tissue bath and kept at 37° C. Two tungsten wires (40 μm in diameter) were threaded into the lumen, and the preparation was mounted in a two-channel myograph (Dual Wire myograph system 410A; Danish Myo Technology, Aarhus, Denmark). One tungsten wire was connected to a micro-manipulator, and the other was connected to a force transducer. All of the vascular rings were initially stabilized for at least 60 min with a modified Krebs-Henseleit solution (Sigma-Aldrich) whose temperature was maintained at 37 °C by a heated water jacket. Isometric tension was continuously monitored using a PowerLab/8 SP system (ADInstruments, Inc., Colorado Springs, CO). After the vascular ring was relaxed, the resting tension was adjusted to 5 mN. After normalization, phenylephrine (Sigma-Aldrich) was added to stimulate vasoconstriction. The concentration of phenylephrine was increased from 10^{-8} M to 10^{-5} M. After a plateau vasoconstriction had been attained, phenylephrine was washed out. At the end of all experiments, vasoconstriction of the femoral artery ring was induced by potassium-enriched solutions containing (in mM): KCl 120, $CaCl_2$ 2.5, $MgSO_4$ 1.17, $NaHCO_3$ 25, KH_2PO_4 1.18, EDTA 0.027, Glucose 5.5.

RESULTS

VSMC ATP2B1 KO Mice showed Higher Blood Pressure Assessed by 24-hour Radiotelemetric System than Control Mice

To confirm the effects of deletion of vascular smooth muscle cell 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 (diastolic blood pressure; Figure S9A, mean blood pressure;

Figure S9B).

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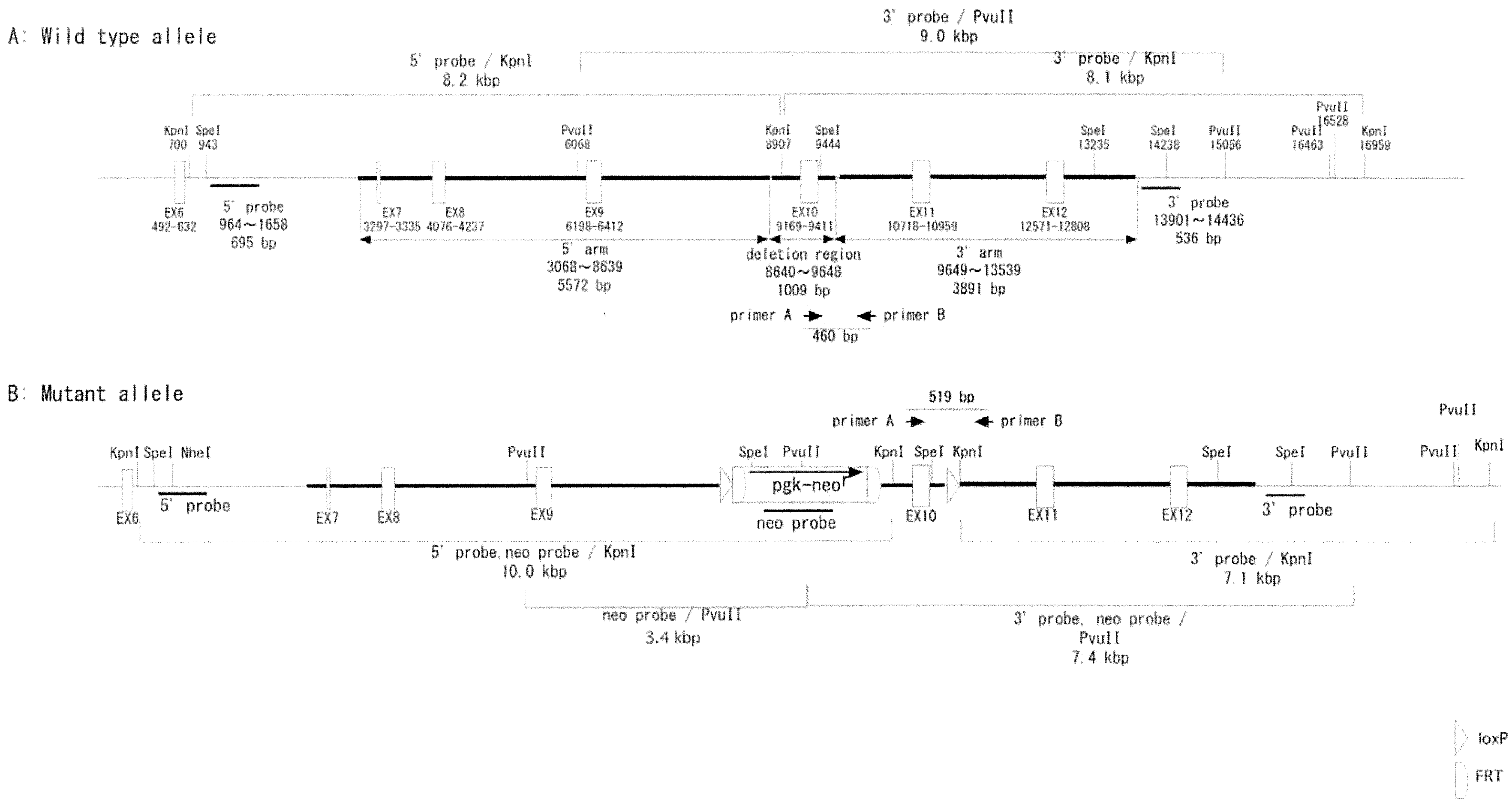
A: Wild type allele
3' probe / PvuII
9.0 kbp
5' probe / KpnI
8.2 kbp
3' probe / KpnI
8.1 kbp
KpnI 700
SpeI 943
PvuII 6068
KpnI 8907
SpeI 9444
SpeI 13235
SpeI 14238
PvuII 15056
PvuII 16463
PvuII 16528
KpnI 16959
EX6 492-632
5' probe 964～1658 695 bp
EX7 3297-3335
EX8 4076-4237
EX9 6198-6412
EX10 9169-9411
EX11 10718-10959
EX12 12571-12808
3' probe 13901～14436 536 bp
5' arm 3068～8639 5572 bp
deletion region 8640～9648 1009 bp
3' arm 9649～13539 3891 bp
primer A
primer B
460 bp
B: Mutant allele
519 bp
primer A
primer B
PvuII
KpnI SpeI NheI
PvuII
SpeI PvuII
KpnI SpeI KpnI
SpeI
SpeI
PvuII
PvuII
KpnI
pgk-neo[r]
EX6
5' probe
EX7
EX8
EX9
neo probe
EX10
EX11
EX12
3' probe
5' probe, neo probe / KpnI
10.0 kbp
3' probe / KpnI
7.1 kbp
neo probe / PvuII
3.4 kbp
3' probe, neo probe / PvuII
7.4 kbp
loxP
FRT

Figure S1 Vector design of ATP2B1 exon 10 conditional KO mouse

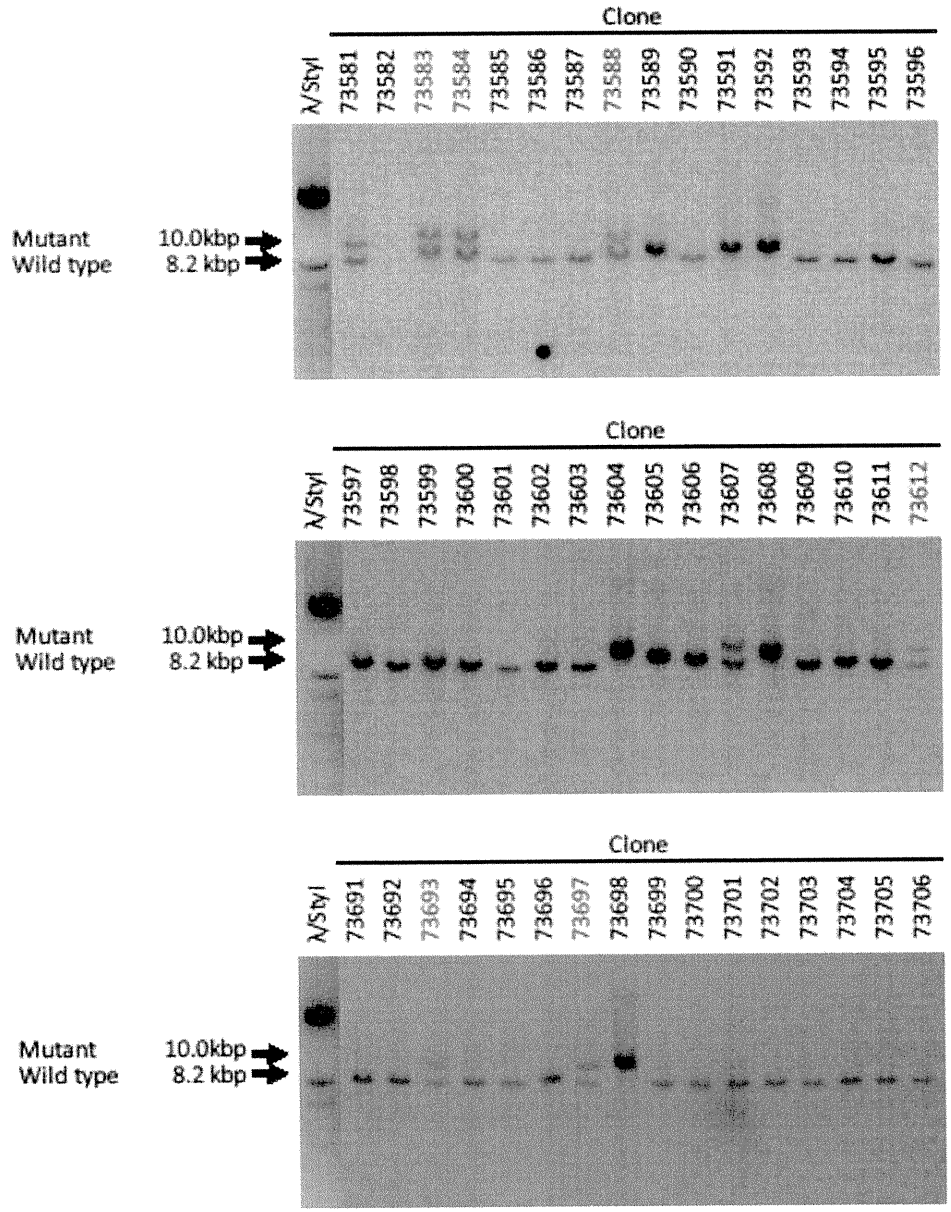

Figure S2 Kpn I-digested Southern blot analysis using 5' probe

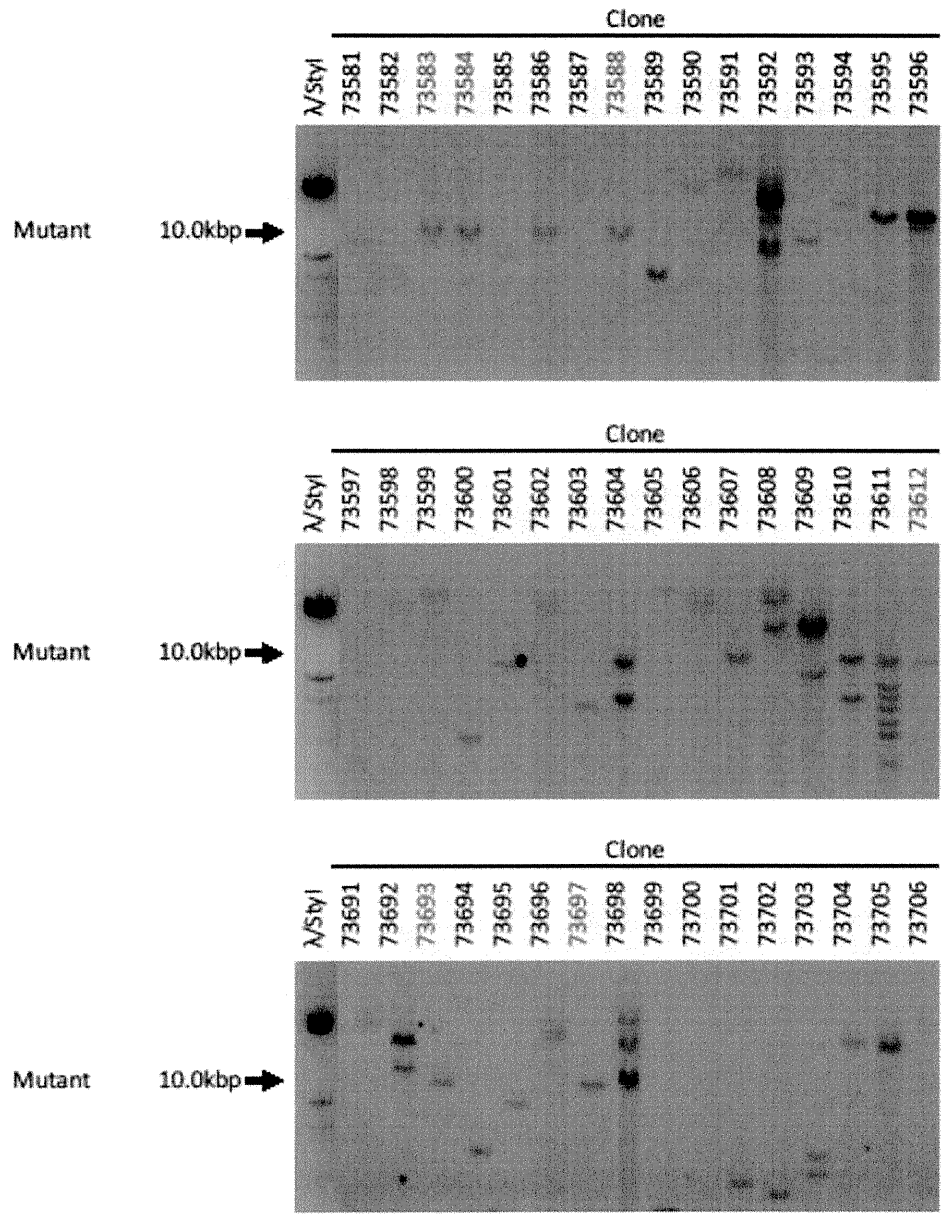

Figure S3 Kpn I-digested Southern blot analysis using neo probe