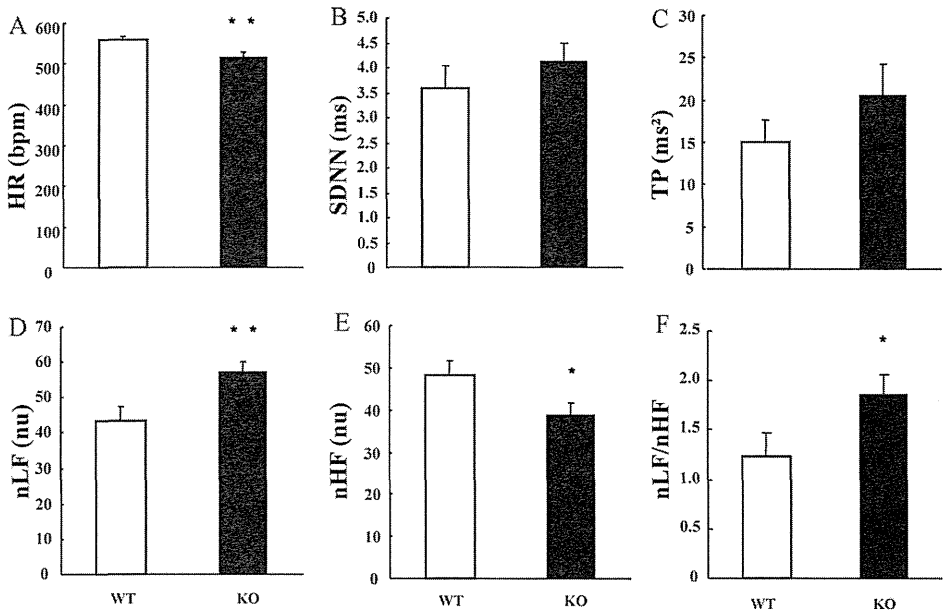


**Fig. 3.** Heart rate variability in senescent SARKO mice. The heart rate variability (HRV) was compared between senescent WT ($n = 6$) and senescent SARKO mice ($n = 8$) using telemetric ECG. (A) Mean heart rate (HR), (B) standard deviation of normal R-R intervals (SDNN), (C) total power (TP), (D)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

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

### 3.5. Senescent SARKO mice exhibited impaired exercise capacity

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

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

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

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

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

### 3.8. Senescent SARKO mice exhibited ER stress

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

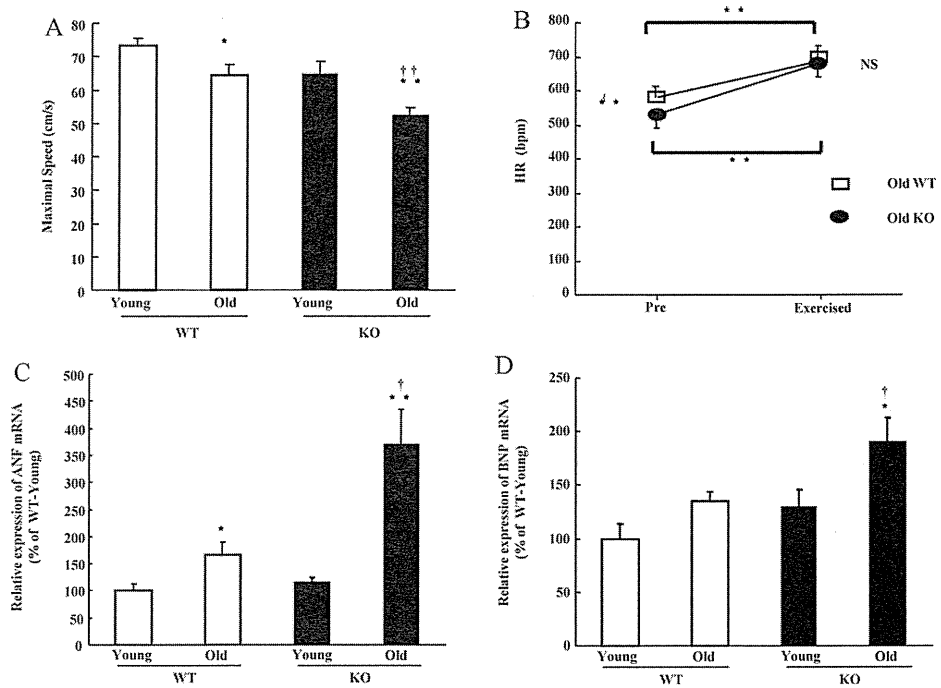


**Fig. 4.** Senescent SARKO mice exhibited impaired exercise capacity, and upregulation of ANF and BNP mRNAs. (A) Exercise capacity was examined by a treadmill-based exercise stress test in young WT ($n = 16$), senescent WT ($n = 12$), young SARKO ($n = 16$), and senescent SARKO mice ($n = 17$). * vs.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

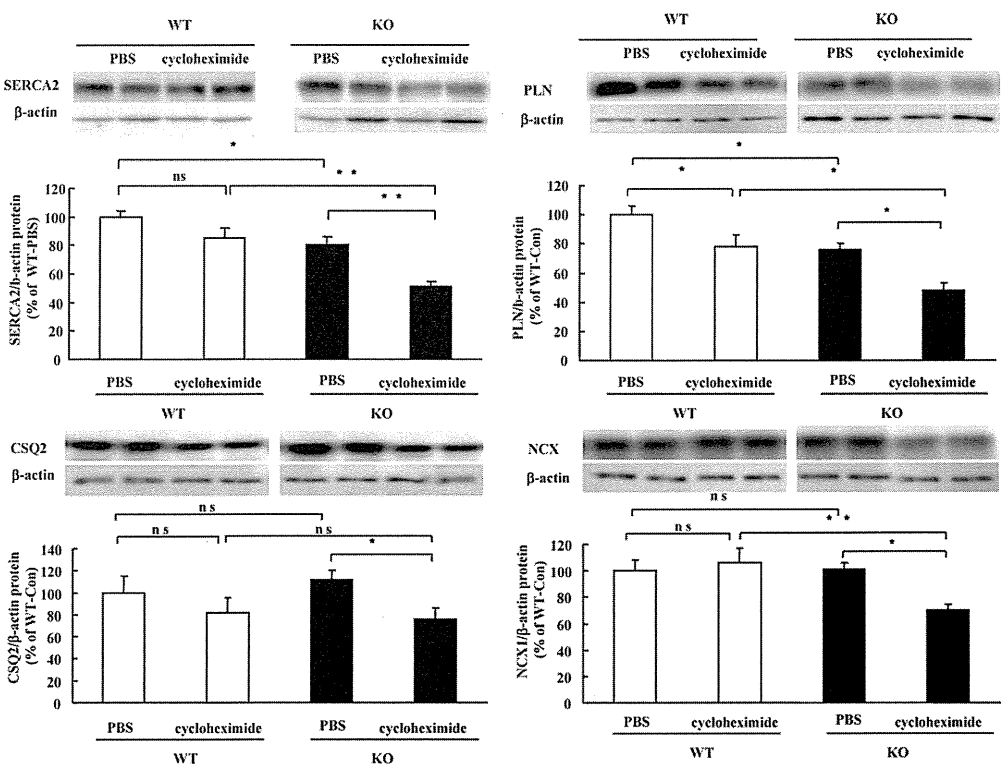


**Fig. 5.** Progressive decreases in SERCA2 and other $Ca^{2+}$ handling proteins after cycloheximide treatment in senescent SARKO mice. Quantification of the expression levels of SERCA2 (A), PLN (B), CSQ2 (C), and NCX1 (D) proteins. $n = 4$–6 for each group. Protein expression was normalized by β-actin.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

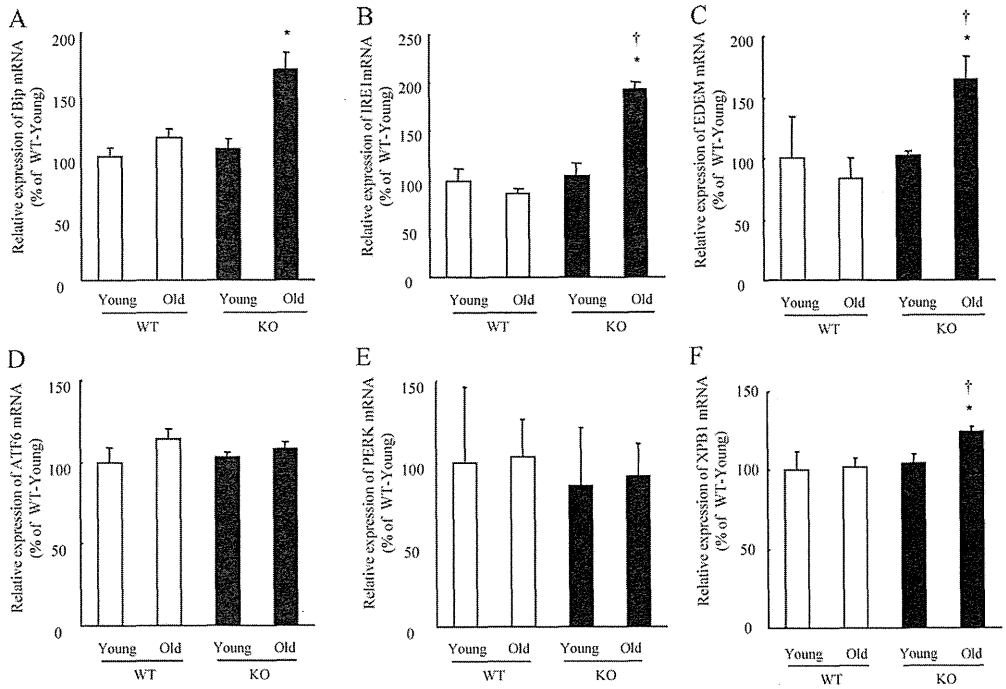


**Fig. 6.** A significant increase in activation of the ER stress pathway in senescent SARKO mice. Quantitative RT-PCR analyses revealed that the expression levels of Bip (A), IRE1 (B), EDEM (C), and XBP1 (F) mRNAs were significantly increased in the ventricles of senescent SARKO mice. Young WT mice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

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

## 4. Discussion

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

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

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

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

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

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

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

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

## Acknowledgments

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## Appendix A. Supplementary data

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

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Case Report

# Acute pulmonary embolism induced by renal obstruction with benign prostatic hyperplasia: Case report

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**KEY WORDS**
Acute pulmonary embolism;
Urinary distension;
Prostatic hyperplasia;
Deep vein thrombosis

**Summary**
*Background:* We report a rare case of acute pulmonary embolism (PE) induced by urinary retention and bladder distention with benign prostatic hyperplasia (BPH).
*Case report:* A 76-year-old male with BPH presented to the hospital with anuria of 24 h duration and abdominal distention. Physical examination revealed tenderness and distention of the lower abdomen and a swollen right leg. Echocardiography after urethral catheterization showed a large free-floating thrombus traversing back and forth through the tricuspid orifice. Computed tomographic angiography demonstrated filling defects at the level of the right inter lobar pulmonary artery and the segmental branches of both pulmonary arteries, indicating acute PE. The patient was treated with heparin and warfarin for three weeks to ensure the resolution of the pulmonary embolus. After the resolution of all symptoms, the patient was discharged without further complication.

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*Conclusion:* This case suggested that a distended bladder is a potential risk factor for the development of deep vein thrombosis and PE.


## Introduction

Pulmonary embolism (PE) is a blockage of one or more arteries in lungs by air, fat, tumor tissue, or thrombus. Approximately 90% of PE arises from deep vein thrombosis (DVT) [1]. DVT risk factors include surgery, hospitalization, immobilization, smoking, obesity, age, medication, thrombophilia, or pregnancy [2]. Although bladder distention could also cause obstruction of veins in the pelvis, and thus subsequent DVT [3–5], it is rare for bladder distention to induce PE. In this regard, it remains unknown whether benign prostatic hyperplasia (BPH), which can also induce bladder distention [6], can lead to PE. Here, we report a PE case with a history of BPH accompanied by severe urinary retention and bladder distention.

## Case report

A 76-year-old man was brought to our hospital with lower abdominal distention. He had noticed the symptoms 4 days prior to the visit. His past medical history was significant for chronic atrial fibrillation without chronic anticoagulation therapy. He had no prior history of heart failure or thrombosis. He never smoked and his family history was negative for clotting disorder or thrombosis.

On admission, his body mass index was 24 kg/m$^2$. Vital signs included a mildly elevated blood pressure at 134/79 mmHg, with irregular pulses at a rate of 70 beats/min, and peripheral oxygen saturation of 98% on room air. His body temperature at the time of presentation was 37 °C. Physical examination was unremarkable except for a tender and rigid abdomen without guarding or rebound as well as a swollen right lower leg. Laboratory tests were consistent with dehydration, renal dysfunction, and inflammation as follows. Significant findings included a white blood cell count of 16,300 cells/μl. C-reactive protein was 9.50 mg/dl. Blood urea nitrogen was 88 mg/dl and serum creatinine was 2.34 mg/dl. Qualitative analysis of urine showed the presence of white blood cells. Prothrombin and partial thromboplastin times were normal, but D-dimer was increased (86 μg/ml: normal range < 1 μg/ml). The levels of plasma protein C and protein S were within normal limits, and anti-cardiolipin antibody was negative. The serum level of prostatic specific antigen was increased (14.1 μg/ml: normal range < 4 μg/ml). Chest X-ray showed no abnormality. Electrocardiogram was consistent with atrial fibrillation, and unchanged from prior recording taken a month earlier. Echocardiography was significant for mild concentric left ventricular hypertrophy with normal left ventricular function, and no thrombosis in his heart and no signs of right ventricular pressure overload were found. Abdominal ultrasound examination revealed an extended urinary bladder. Ultrasound examination for vein of lower extremity showed a thrombus in the right femoral vein and no thrombus in the left. Taken together, urinary distention secondary to BPH, obstruction of pelvic vein within the pelvis by the distended urinary bladder, urinary tract infection, acute post-renal failure, and right DVT were suspected. Urinary catheterization was then performed to relieve the urinary obstruction.

However, when echocardiography was repeated 1 h after the urinary catheterization, we found a large floating thrombus within the right atrium, with frequent back and forth movement through the tricuspid orifice, which was not observed previously (Fig. 1A and B). Mild pressure overload of the right ventricle was also observed at this time. The patient was immediately brought to our intensive care unit. Follow-up echocardiography, however, showed that the observed thrombus in the right atrium had disappeared. Emergent computed tomographic (CT) angiography revealed clots at the level of right distal pulmonary artery, and the segmental branches of both pulmonary arteries (Fig. 2A–C). Pelvis CT showed that there was BPH. Chest and abdominal CT showed that there were no thrombi in either of the femoral veins or the inferior vena cava. Screening for malignancy with CT was negative.

Pulmonary perfusion scintigraphy showed diffuse filling defects in both lungs, which were consistent with findings of CT angiography (Fig. 3A and B). Lower extremity perfusion scintigraphy showed a delay of disappearance of $^{99m}$Tc-MAA in his right leg compared to the left leg (Fig. 3C and D). Accordingly, we diagnosed that the patient had developed PE.

Treatment for PE with continuous intravenous infusion of heparin (14,000 units/day) and oral warfarin (3 mg/day) was started. We did not perform intravenous infusion of urokinase or tissue plasminogen activator because the patient showed no sign of hemodynamic instability or severe right ventricular pressure overload. On day 5 during the hospitalization, heparin was discontinued because the prothrombin time/international normalized ratio had reached 2.0. On day 14, CT showed reduced size of the thrombus (20% of the initial size). Regarding BPH, both suppressive therapy for BPH and intermittent self-catheterization were initiated. On day 19, the patient became symptom free and was discharged.

## Discussion

There are few reports of urinary distention-induced DVT [3–5,7], although urinary retention itself can lead to increased venous pressure in the pelvis [7]. Accordingly, it is not well accepted that BPH can serve as a risk factor for PE through urinary retention and bladder distention. A review reported that 9 of 15 urinary distention cases, which led to DVT, had prostatic enlargement [8,9]. Meinardi et al. previously reported a case of BPH-induced DVT with protein S deficiency [5]. These authors concluded that BPH alone may be insufficient to induce PH unless other risk factors, such as thrombophilic defects, are accompanied. However,

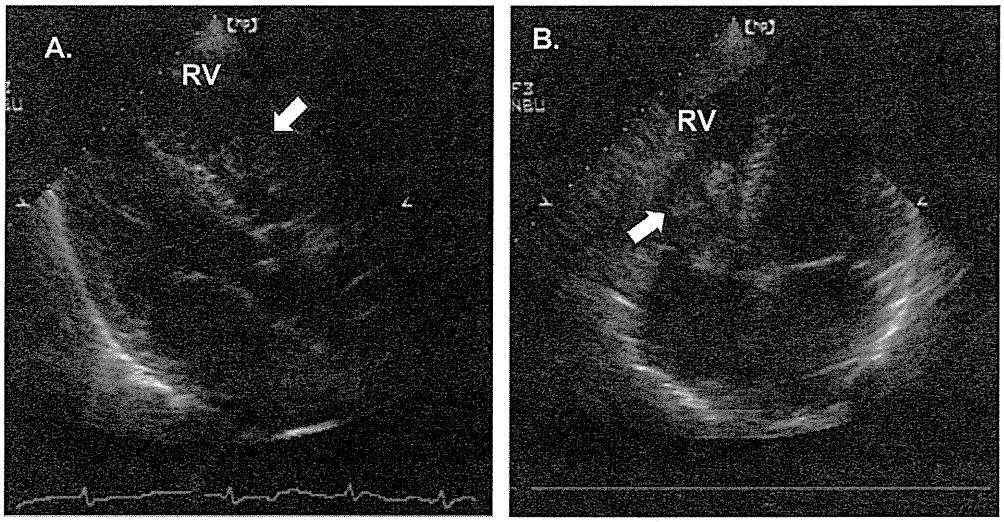


**Figure 1** Echocardiogram of axis view (A) and four chamber view (B). A giant floating thrombus (3 cm × 3 cm, white arrows) in the right atrium and ventricle frequently moved back and forth through the tricuspid orifice. RV, right ventricle.

as shown in the current and another case [10], BPH alone can induce DVT without any other risk factors. It is thus tentative to speculate that BPH should be recognized as a potential risk factor for DVT via urinary bladder distention. In this case, it should be noted that hypercoagulability, due to increased inflammation along with urinary tract infection, might contribute to thrombus formation. More interestingly, bilateral lower extremity swelling is usual with such bladder distention; a previous review summarized that 14 of 15 patients had bilateral swelling [8] and that the occurrence of PE following DVT was uncertain. This is in contrast to the current case, which showed unilateral leg swelling

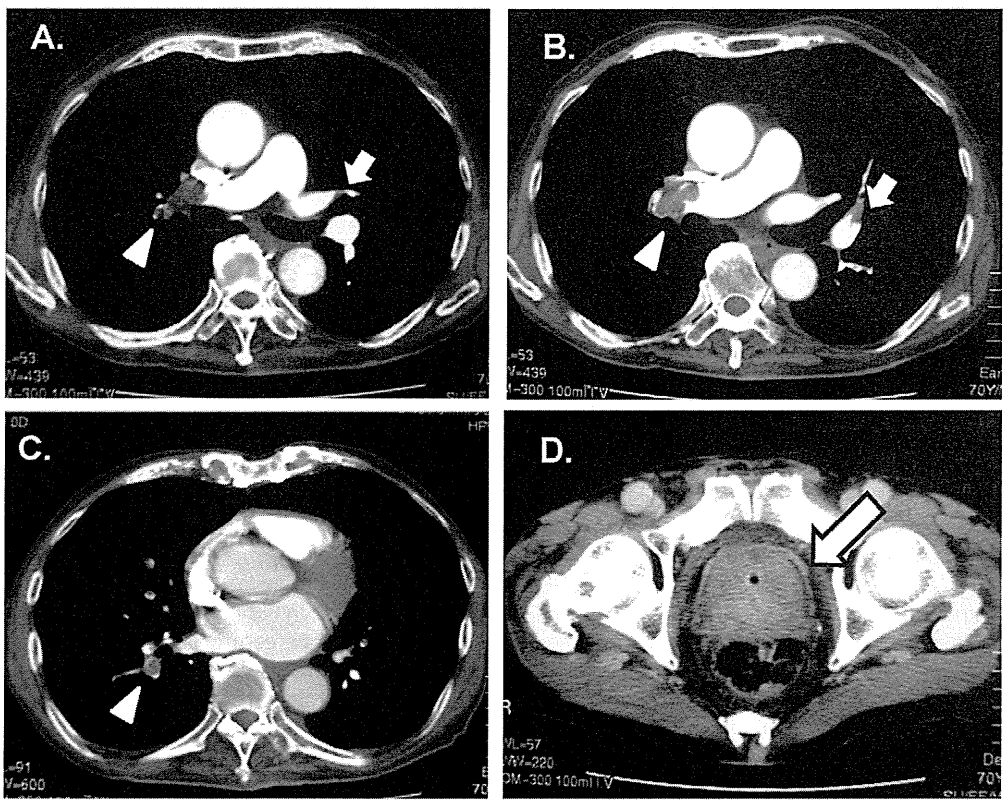


**Figure 2** Chest computed tomography (CT) shows clots and disturbed blood flow in right distal pulmonary artery and left segmental branch (A—C). Clots are shown by arrowheads. Pelvic CT shows enlarged prostate (D, arrow).

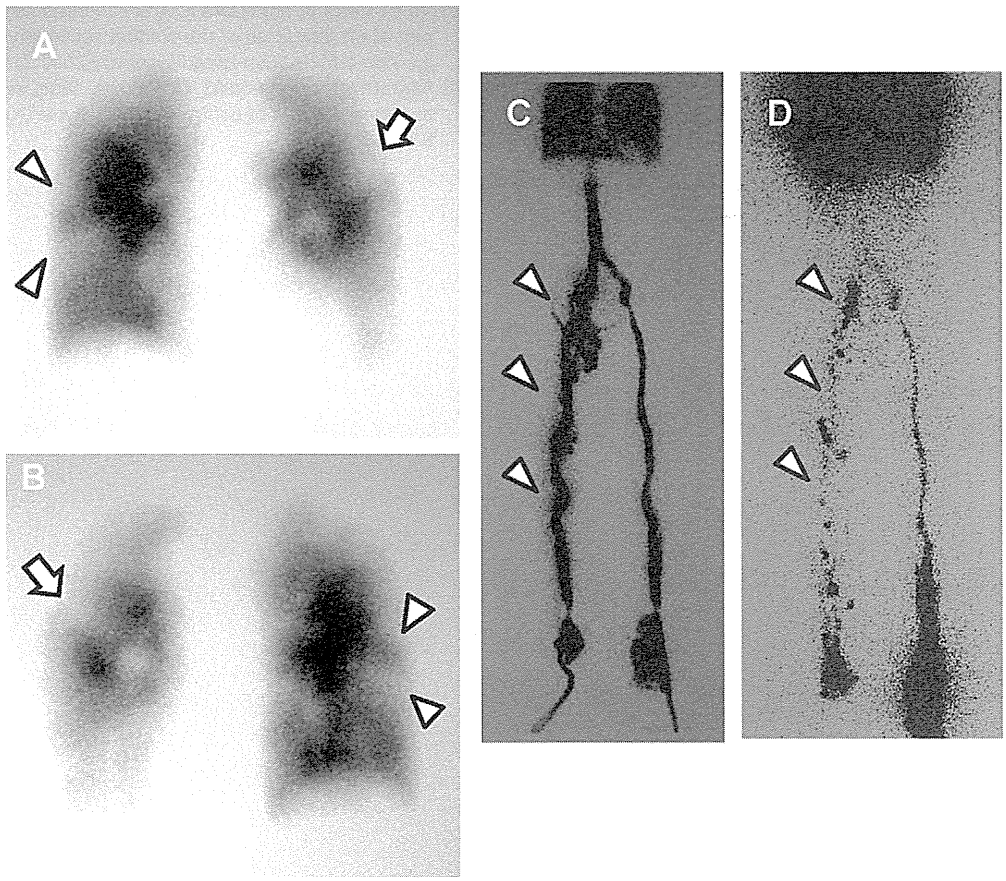


**Figure 3** Pulmonary perfusion scintigraphy showed filling defects in both lung [A (anterior view) and B (posterior view)]. Perfusion scintigraphy of lower extremities showed a delay of disappearance of $^{99m}$Tc-MAA in his right leg compared to his left leg [C (early phase) and D (delay phase)].

followed by PE. When unilateral leg swelling is observed with urinary distention, DVT and potentially PE might be considered as well. Before urinary catheterization was performed, formation of thrombus due to distended bladder was not listed as a differential diagnosis because it is clinically rare. Placing temporary inferior vena cava might be effective for inhibition of PE, thus should be considered in the case of leg swelling concurrent with distended bladder.

Prognosis of PE is typically poor. The conventional treatments for PE include urokinase, heparin, tissue plasminogen activator, and warfarin [2]. We used warfarin in this case but not urokinase or tissue plasminogen activator because hemodynamic instability or severe right ventricular pressure overload were not found. Consequently, the patient was discharged without further complications. Factors that brought this fortunate outcome may include the PE onset within the hospital and thus the rapid initiation of heparin and warfarin therapy.

## Conclusion

BPH could potentially cause DVT and thus PE via bladder distention.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jccase.2011.10.004.

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# Acute myocardial infarction with isolated conus branch occlusion

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**Abstract** There are few reports of acute myocardial infarction (AMI) relating to the occlusion of the conus branch, most of which are iatrogenic in nature. So far as we are concerned, this is the first case of spontaneous AMI with isolated conus branch occlusion. Electrocardiogram (ECG) showed mild elevation of ST segment in leads $V_1$ through $V_3$. Cardiac makers of myocardial infarction were positive. Right coronary angiography revealed an isolated occlusion of the conus branch. Penetration of the guidewire in the occluded lesion was attempted, and recanalization was successfully achieved. The patient was discharged without any adverse events.


*Keywords:* Acute myocardial infarction; Conus branch; Occlusion; ST elevation; Spontaneous; Isolated

## Introduction

The conus branch of the right coronary artery (RCA) frequently provides blood supply to the outflow tract of right ventricle. Previous papers reported that characteristic electrocardiographic (ECG) changes (elevation of ST segment in the right precordial leads) appeared when the conus branch was occluded or narrowed. All these reported events occurred during coronary angiography/angioplasty or cardiac surgery in the RCA.[1-5] Here, unlike these iatrogenic occlusions, we show the first case of spontaneous occlusion of the conus branch. The isolated conus branch occlusion led to acute myocardial infarction (AMI), and we treated the case with successful recanalization using penetration with a guidewire, instead of balloon/stent catheter.

## Case report

A 78-year-old Japanese man awoke with retrosternal chest pain. The patient had been on home oxygen therapy for severe pulmonary emphysema and been on maintenance hemodialysis for the past 2 years. He had no history of coronary artery disease, diabetes, thrombosis, or atrial fibrillation and had never received anticoagulation therapy. Other risk factors included a history of tobacco use and smoking 20 cigarettes a day for 60 years. Family history was negative for clotting disorder, stroke, or coronary artery disease.

One hour after the onset of the chest pain, the patient visited an outside clinic. The electrocardiogram showed elevation of ST segment in the precordial leads ($V_1$ through $V_3$) (Fig. 1A). Sublingual nitroglycerin was given, and the patient was transferred to the emergency room of our hospital. At presentation, the patient had sustained chest pain that was not reduced by the nitroglycerin given at the outside clinic.

On admission, the blood pressure was 152/66 mm Hg and the heart rate was 82 beats per minute. The result of the physical examination was unremarkable. The electrocardiogram showed mild ST-segment elevation in the leads $V_1$ through $V_3$ (Fig. 1B). The maximum elevation of ST segment

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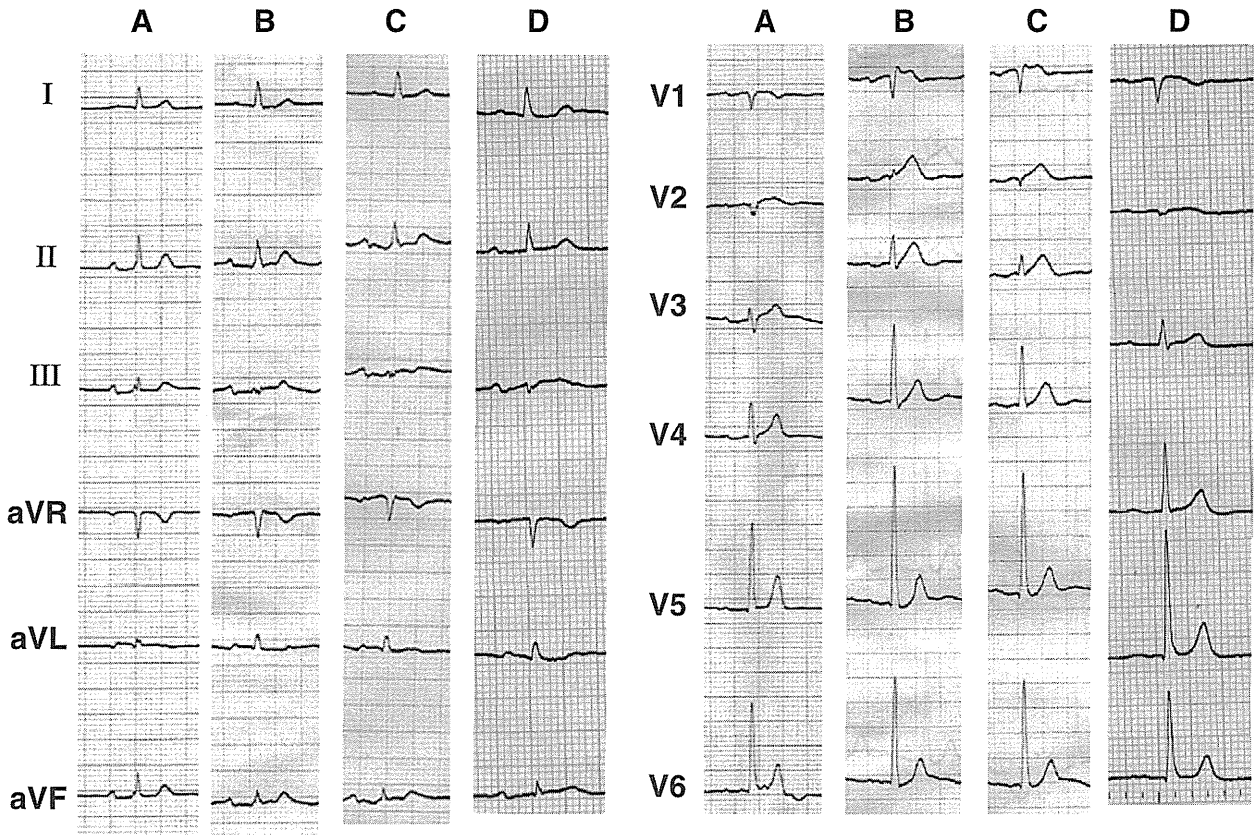


Fig. 1. (A) Electrocardiogram of the conus branch occlusion. (B) Electrocardiogram of the patient showed atrioventricular block 1 month before the onset of AMI. (C) Elevation of ST segment in leads $V_1$ through $V_3$ was seen in the outside clinic after sublingual nitroglycerin was given, and (D) resolution of ST-segment elevation after the recanalization using the guidewire.

was 2 mm in the lead $V_2$ (Fig. 1C) recorded 3 hours after the onset of the chest pain as compared with the ECG 1 month before the admission (Fig. 1A). White blood cell count was 3600 cells/$\mu$l. C-active protein level was 2.69 mg/dL. Serum creatinine level was 6.48 mg/dL. Cardiac markers were as follows: creatinine kinase level of 138 IU/L, creatinine kinase-MB fraction of 15 IU/L, and troponin T level of 0.37 ng/mL. Echocardiography showed relatively normal segmental wall motions of both right and left ventricles. At that point, acute coronary syndrome was strongly suspected, and coronary angiography was immediately performed. Complete occlusion of the conus branch of the RCA was found (Fig. 2A and B). Because coronary angioplasty was considered to be difficult in this lesion, penetration of guidewire was attempted. The guidewire was easily crossed through the occluded lesion (Fig. 2C), and Thrombolysis in Myocardial Infarction Trial (TIMI) grade III flow was restored as demonstrated in the following coronary angiography (Fig. 2D), suggesting successful recanalization. The resolution of chest pain was immediate. Intravenous infusion of heparin with oral warfarin (3 mg/day) and aspirin (100 mg/day) was initiated. The electrocardiogram taken at 24 hours after recanalization showed the resolution of ST elevations in leads $V_1$ through $V_3$ without the development of Q-wave or T-wave inversion (Fig. 1D). Creatinine kinase level peaked at 1066 IU/L with an MB fraction of 137 IU/L 1 day after the recanalization. On day 13, the patient was discharged with aspirin and warfarin. Although CHADS2 score is 1, we added warfarin to the patient because we considered the possibility of his hypercoagulability due to sever emphysema and his maintenance of hemodialysis. It was also likely that thromboembolism caused myocardial infarction, not arteriosclerosis.

The characteristic ECG changes (elevation of ST segment in leads $V_1$ through $V_3$) observed in this case are consistent with those in the previous reports of conus branch occlusion.[3,5] Matthews[5] reported that conus branch was occluded by injection to RCA during angiography. Eichhofer et al[4] reported that stent implantation in the right proximal coronary artery resulted in conus branch occlusion. The electrocardiogram showed similar pattern in the precordial leads ($V_1$ through $V_3$). These reports did not mention treatments, presumably because it is considered difficult to perform angioplasty or stent implantation for conus branch occlusion due to the small diameter of the artery. Successful balloon angioplasty in the conus branch was reported in a case of occlusion by suture material after mitral valve reconstruction.[2] On the contrary, occlusion of the conus branch is known to show similar ECG pattern as those seen with Brugada syndrome, that is, elevations of the ST segment in the right precordial leads ($V_1$ through $V_3$). There are 2 reports of Brugada-like ECG changes due to conus branch vasospasms with acetylcholine, which resulted in ventricular fibrillation or ventricular tachycardia.[3,6] Because Brugada syndrome can lead to ventricular fibrillation,

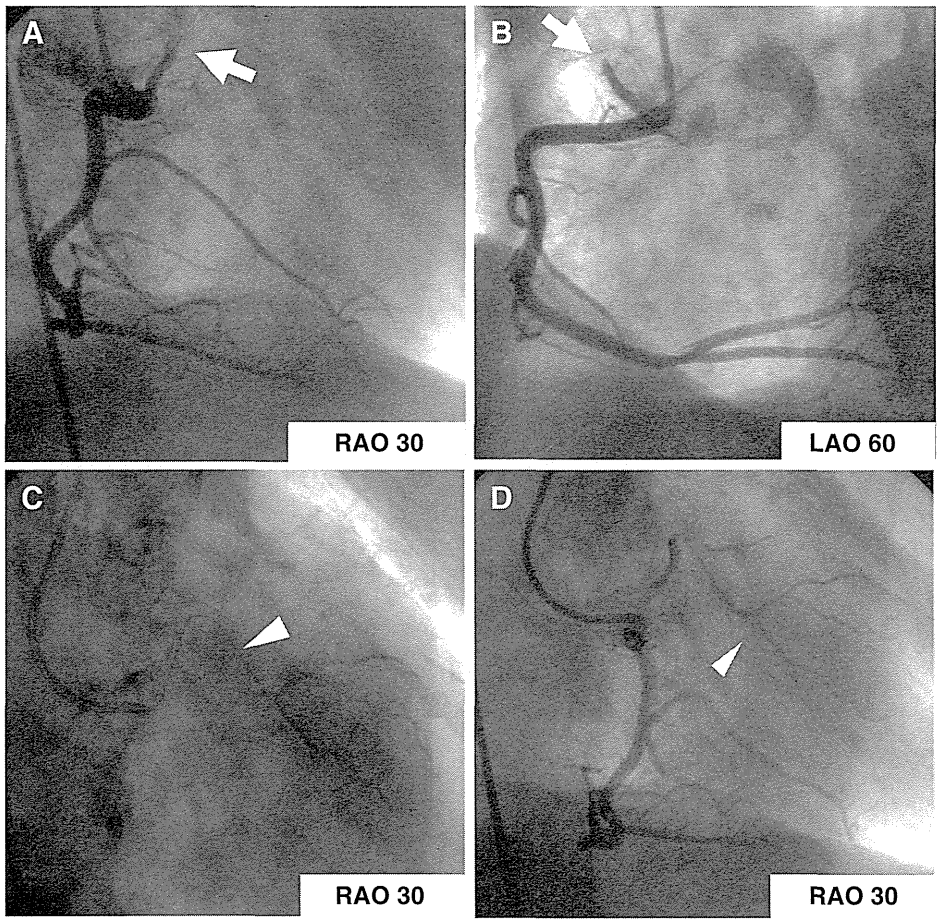


Fig. 2. Coronary angiograms before and after the penetration of guidewire into the conus branch occlusion. The conus branch of the RCA showed total occlusion (arrows in A and B). The TIMI flow grade was 0 at this point. A guidewire was penetrated into the conus branch lesion (arrowhead in C), and grade III TIMI flow was observed (D). RAO indicates right anterior oblique; LAO, left anterior oblique.

and thus sudden death, treatment of conus branch occlusion is of clinical importance. In our case, penetration of a guidewire was attempted, and blood flow was restored without development of ventricular fibrillation or tachycardia. To our knowledge, this is the first report of successful recanalization with a guidewire in a conus branch occlusion. Because insertion of a guidewire into the conus branch may be easier than that of balloon/stent catheter, guidewire penetration should be considered as the first option for treatment of isolated conus branch occlusion. Furthermore, conus branch occlusion described in the previous reports were unexceptionally iatrogenic in nature; spontaneous occlusion like our case has not been reported. Nonetheless, all cases, including the current case, showed similar ECG changes, that is, ST elevation in the right precordial leads ($V_1$ through $V_3$) and moderate elevation in creatinine kinase (300-1000 IU/L). Meanwhile, the conus artery in about 50% of human hearts is not a branch of the RCA but arises from the right sinus ostium of Valsalva directly. Therefore, when ST elevations in the right precordial leads are observed, not only the conus branch of RCA but also the conus artery occlusion should be considered. Nonetheless, when angioplasty is considered to be difficult in either cases, penetration with a guidewire could be an alternative optional for treatment. Because this artery is important in terms of critical ventricular arrhythmia development, such treatment should be performed immediately even though the putative irrigation area may not be very large.

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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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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-$\alpha$) 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 $\geq$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 $\mu$mol/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

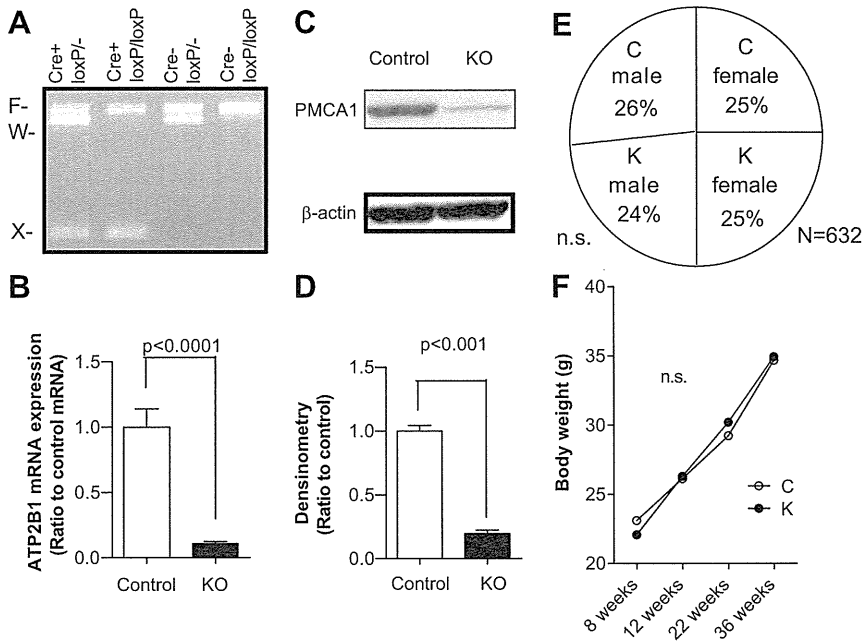


**Figure 1.** High efficiency, vascular smooth muscle cell (VSMC)-selective deletion of ATP2B1 gene. **A**, Tail DNA was prepared by standard methods, and the ATP2B1 gene was amplified by PCR using the forward primer 5′-CATCCTCTTTAGTTATTAAGGAAGCAGT-3′ (located in the intron before the first loxP site)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

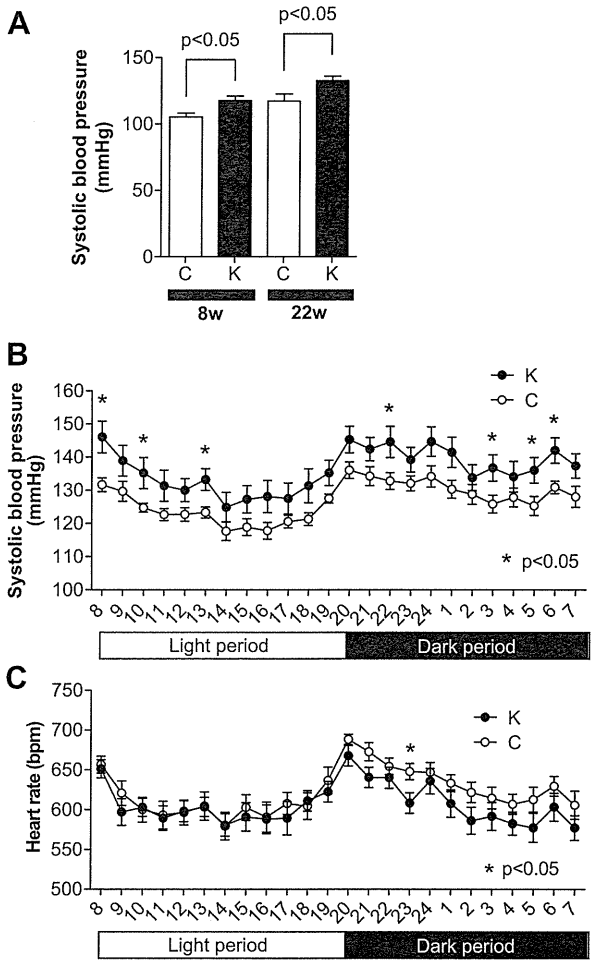


**Figure 2.** Blood pressure measured by tail-cuff method and radiotelemetric method. **A**, Systolic blood pressure was measured by tail-cuff method in 8-week–old knockout (KO) mice (117.7±3.4; n=15) and control mice (105.3±2.9; n=13), and 22-week–old vascular smooth muscle cell (VSMC) ATP2B1 KO mice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

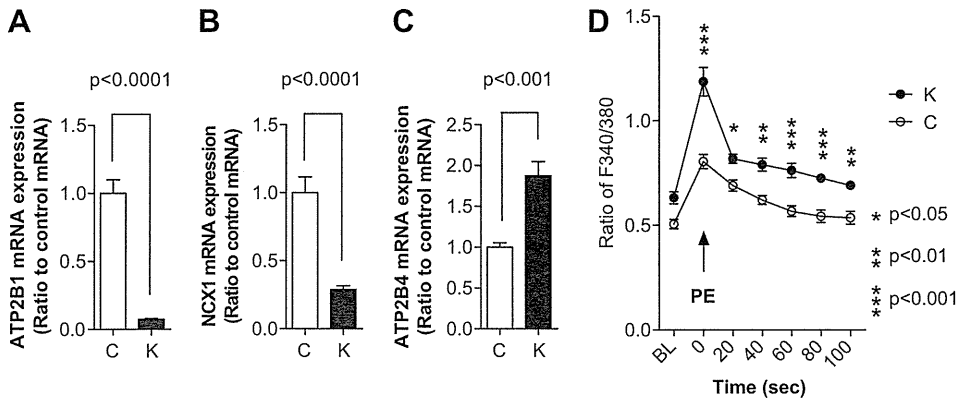


**Figure 3.** Altered gene expression and calcium transient in primary cultured vascular smooth muscle cells (VSMCs) of knockout (KO) mice. **A**, ATP2B1 mRNA expression in primary cultured VSMCs (n=8–10 for each genotype) quantified by quantitative RT-PCR (qRT-PCR) using exon 10 and 11 amplification.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

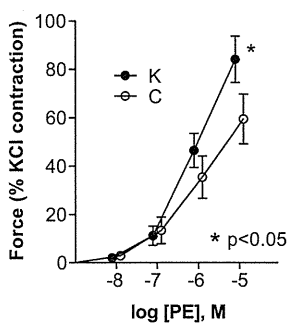


**Figure 4.** Phenylephrine-induced vasoconstriction of femoral artery rings. Isolated femoral artery rings obtained from knockout (KO) mice and control mice were stimulated with the $\alpha$1-adrenoceptor agonist phenylephrine. Data are presented as mean and SE of 9 to 10 independent experiments. Force 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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