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ARTICLE

Reduced secretion of parathyroid hormone and hypocalcemia in systemic heterozygous *ATP2B1*-null hypertensive mice

Yosuke Ehara[1] · Nobuhito Hirawa[2] · Kouichiro Sumida[1] · Akira Fujiwara[2] · Minako Kagimoto[1] · Yuki Ooki-Okuyama[1] · Megumi Fujita[1] · Mari Katsumata[1] · Yusuke Kobayashi[1] · Sanae Saka[2] · Ikuma Katou[3] · Keisuke Yatsu[1] · Satoshi Umemura[1] · Kouichi Tamura[1]

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Abstract

The ATP2B1 gene is associated with hypertension. We previously reported that systemic heterozygous ATP2B1-null (ATP2B1$^{+/-}$) mice exhibited hypertension due to impaired endothelial nitric oxide synthase (eNOS) activity and decreased nitric oxide (NO) production. The ATP2B1 gene encodes plasma membrane calcium ATPase 1 (PMCA1), which has been thought to regulate only intracellular Ca^{2+} concentration. However, recently, it has been suggested that *ATP2B1* works not only at cellular levels, but also throughout the entire body, including in the calcium metabolism, using small intestine-specific *ATP2B1* knockout mice. To clarify the roles of *ATP2B1* in the entire body and the effects of *ATP2B1* on blood pressure, we examined the alterations of calcium related factors in ATP2B1$^{+/-}$ mice. ATP2B1$^{+/-}$ mice exhibited hypocalcemia. The expression of *ATP2B1* in the kidney and small intestine decreased, and hypercalciuria was confirmed in ATP2B1$^{+/-}$ mice. The intact-PTH levels were lower, and bone mineral density was increased in these mice. These results suggest that hypocalcemia is mainly a result of inhibited bone resorption without compensation by PTH secretion in the case of *ATP2B1* knockout. Moreover, NO production may be affected by reduced PTH secretion, which may cause the increase in vascular contractility in these mice. The *ATP2B1* gene is important for not only intra-cellular calcium regulation but also for calcium homeostasis and blood pressure control.

Introduction

Many studies have reported that the *ATP2B1* gene is one of the candidate genes for hypertension. We reported the association between high blood pressure and the *ATP2B1* gene in the Japanese population through the Millennium Genome Project [1, 2]. Furthermore, the *ATP2B1* gene was found to be associated with hypertension in people of European [3] or Korean decent [4], and other populations worldwide [5].

ATP2B1 encodes plasma membrane calcium ATPase 1 (PMCA1). PMCA comprises four different isoforms (PMCA1-4) that are encoded by four independent genes. PMCA1 and PMCA4 are expressed ubiquitously, whereas PMCA2 and PMCA3 are mainly present in neuronal tissue [6, 7]. PMCA1 plays a role in discharging Ca^{2+} from the inside to the outside of the cell, and strictly regulates the intracellular Ca^{2+} concentration [1, 8, 9].

We previously reported that ATP2B1$^{+/-}$ mice exhibited enhanced vasoconstriction and elevated blood pressure, and the phenotype was associated with impaired endothelial nitric oxide synthase (eNOS) activity and decreased nitric oxide (NO) production [10]. In addition, vascular smooth muscle cell-specific *ATP2B1* knockout mice exhibited increased blood pressure, intracellular calcium concentration and vascular contractility [11], and these mice were highly susceptible to calcium channel blockers [12].

Recently, it was reported that small intestine-specific *ATP2B1* knockout mice had decreased bone mineral density without changes in serum calcium concentration [13]. This suggested that *ATP2B1* works not only at cellular levels, but

✉ Nobuhito Hirawa
hirawa@yokohama-cu.ac.jp

[1] Department of Medical Science and Cardiorenal Medicine, Yokohama City University Graduate School of Medicine, Yokohama, Kanagawa, Japan

[2] Department of Nephrology and Hypertension, Yokohama City University Medical Center, Yokohama, Kanagawa, Japan

[3] Department of Molecular Pathology, Yokohama City University Graduate School of Medicine, Yokohama, Kanagawa, Japan

also throughout the entire body, including in bone metabolism.

Ca^{2+} is one of the most important ions in animals. Calcium plays a crucial role in physiological functions, including cardiac muscle contraction, vascular muscle contraction, bone mineralization, fertilization, synaptic transmission, blood clotting, and neuronal function. Therefore, the calcium balance is tightly regulated by bone metabolism, and reabsorption in the kidney and small intestine, which are controlled by hormones such as parathyroid hormone (PTH) and 1α,25-hydydroxyvitaminD_3 (1α,25-(OH_2D_3) [14]. The effects of *ATP2B1* on bone metabolism, serum calcium concentration and PTH have not been clarified. In order to confirm the roles of *ATP2B1* in calcium homeostasis and blood pressure control, we examined alterations in phenotypes of bone, small intestine, kidney and parathyroid gland using $ATP2B1^{+/-}$ hypertensive mice, which demonstrated impaired eNOS activity and decreased NO production in vascular smooth muscle cells.

Methods

Animal care

Animals were housed under a 12-h light-dark cycle at 25 °C. Mice were examined at 12–15-weeks of age and fed a normal-salt diet (0.3% NaCl) with free access to drinking water. Biochemical analyses were performed on blood samples collected after cardiac puncture under deep anesthesia with intraperitoneal administration of pentobarbital sodium (32.4 mg/kg). Vital organs were removed after euthanasia. Experiments were conducted under the guidelines for animal experiments set by the Animal Experiment Committee of Yokohama City University School of Medicine.

Generation of systemic heterozygous *ATP2B1*-null ($ATP2B1^{+/-}$) mice

$ATP2B1^{+/-}$ mice were generated as previously described [10] using the Cre-loxP system because the *ATP2B1*-null mutant displays embryonic lethality [15]. Tie2 promotor can cause non-cell-specific deletion of floxed alleles through the germ line activation. Normally, two successive rounds of breeding are performed to generate mice carrying two floxed target-gene alleles and a transgene expressing Cre-recombinase tissue-specifically. However, this causes the conversion of the floxed allele to a null allele in the germ line, which is transmitted to offspring, which can result in global deletion irrespective of their Cre-recombinase genotype. Therefore, germ-line activation of the Tie2 promotors can cause non-cell-specific deletion of floxed alleles with a fixed probability [16]. $ATP2B1^{+/loxP}$:Tie2-Cre mice were created by mating $ATP2B1^{loxP/loxP}$ female mice with transgenic mice expressing Cre recombinase under control of the murine Tie2 promotor (Tie2-Cre mice) [17] (B6.Cg-Tg(Tie2-Cre)1Ywa/J, stock No.008863, Jackson Laboratory). $ATP2B1^{+/loxP}$:Tie2-Cre male offspring were then crossed with $ATP2B1^{loxP/loxP}$ female mice, and we created systemic heterozygous ATP2B1 null ($ATP2B1^{+/-}$:Tie2-Cre(−)) mice and genetic control ($ATP2B1^{+/+}$:Tie2-Cre(−)) mice. Animals included in this study were 12–15-week-old $ATP2B1^{+/-}$ mice, with $ATP2B1^{+/+}$ mice as controls.

Measurement of serum calcium, phosphorous and several calcium regulatory markers

Serum calcium and phosphorous were analyzed with the 7170 autoanalyzer (SRL, Inc., Tokyo, Japan). Serum intact-PTH, fibroblast growth factor 23 (FGF23), and osteocalcin were measured with the ELISA method by MTP-300 MICROPLATE READER and 1α,25-(OH_2D_3) was measured with the LC–MS/MS method by Nexera UHPLC (Oriental Yeast CO., LTD., Shiga, Japan).

Measurement of urine creatinine, calcium and 8-hydroxy-2′-deoxyguanosine (8-OHdG)

The concentrations of urine creatinine and calcium in the spot urine collected by bladder massage were assessed. Urine creatinine and calcium were measured with the enzyme method using the 7170 autoanalyzer (Oriental Yeast CO., LTD., Shiga, Japan), and the calcium creatinine ratio was evaluated. The concentration of urine 8-OHdG was measured with New 8-OHdG Check ELISA by MTP-300 Micro Plate Reader (Oriental Yeast CO., LTD., Shiga, Japan).

Real-time quantitative reverse transcription-PCR analysis

Total RNA was extracted from the kidney and intestine (duodenum) with ISOGEN (Nippon Gene). cDNA was synthesized using the SuperScript III First Strand System (Invitrogen). Real-time quantitative reverse transcription-PCR (RT-PCR) was performed by incubating the reverse transcribed product with Taqman PCR Master Mix and a designed Taqman probe (ATP2B1: Mm01232254_m1, ATP2B4: Mm01285597_m1, Na^+/Ca^{2+} exchanger 1 (NCX1): Mm01232254_m1, Transient Receptor Potential Vanilloid (TRPV) channel type6: Mm00499069_m1, renin: Mm02342887_mH, Applied Biosystems). RNA amounts are shown relative to the mRNA control (18s: 4319413E; Applied Biosystems).

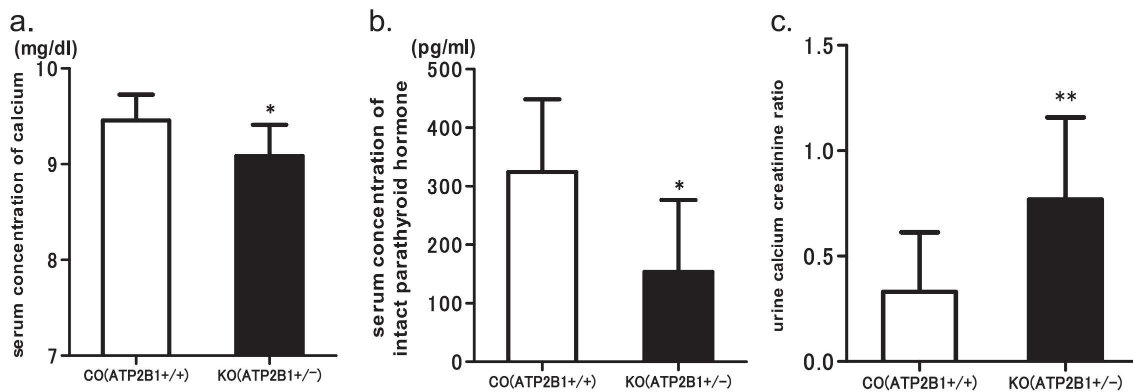


Fig. 1 Measurement of serum calcium, intact parathyroid hormone, and. urine calcium creatinine ratio. **a** Serum concentration of calcium in ATP2B1$^{+/-}$ mice ($n = 7$) and ATP2B1$^{+/+}$ mice ($n = 8$). **b** Serum concentration of intact parathyroid hormone in ATP2B1$^{+/-}$ mice and ATP2B1$^{+/+}$ mice ($n = 9$ for each group). **c** Urine calcium and creatinine were measured in ATP2B1$^{+/-}$ ($n = 12$) and ATP2B1$^{+/+}$ mice ($n = 11$). Data are mean±SEM. *t*-test *$p < 0.05$ versus the control group, **$p < 0.01$ versus the control group. ATP2B1±mice exhibited hypocalcemia and decreased intact-PTH. Urine calcium creatinine ratio was increased in ATP2B1±mice

Assessment of bone mineral density and bone pathology

Isolated femoral bone was assessed by peripheral quantitative computed tomography (pQCT) with XCT Research SA+ (Stratec Medizintechnik GmbH, Pforzheim, Germany) (Kureha-analyze center, Tokyo, Japan). Hematoxylin and eosin (HE) staining, tartrate-resistant acid (TRAP) staining, and von Kossa staining were performed. The number and properties of osteoblasts were confirmed by HE staining. The number and properties of osteoclasts were confirmed by TRAP staining.

Statistical analysis

For statistical analysis of differences between groups, the unpaired Student's *t*-test was used. All quantitative data are expressed as mean±SEM. *P*-values < 0.05 were considered significant.

Results

Generation of ATP2B1$^{+/-}$ mice

As we reported previously, ATP2B1$^{+/-}$ mice were generated by mating ATP2B1$^{+/+}$ mice: Male Tie2-Cre mice with ATP2B1$^{loxp/loxp}$ female mice. These mice were born and grew normally with no detectable abnormalities. Real-time quantitative-PCR analysis revealed approximately 70% lower ATP2B1 mRNA levels in tissues (brain, heart, lung, aorta, kidney, liver, spleen skeletal muscle and intestine) from ATP2B1$^{+/-}$ mice compared with in control mice. RT-PCR analysis demonstrated a deletion of exon 10 in ATP2B1$^{+/-}$ vascular smooth muscle cells. Expression of PMCA1 protein in kidneys from ATP2B1$^{+/-}$ mice was significantly lower than that in control mice on Western blotting [10].

Table 1 Measurement of serum phosphorous and several calcium regulatory markers

	CO(ATP2B1$^{+/+}$)	*n*	KO(ATP2B1$^{+/-}$)	*n*	*P*
Phosphorus (mg/dL)	7.7 ± 0.4	8	7.7 ± 0.2	9	NS
FGF23 (pg/mL)	114 ± 6	9	99 ± 5	9	NS
1α.25-(OH)$_2$D$_3$ (pg/mL)	23.6 ± 3.1	8	20.2 ± 3.2	8	NS
Osteocalcin (ng/mL)	94 ± 7	8	103 ± 6	8	NS

Measurement of serum phosphorous and several calcium regulatory markers. Serum phosphorous, 1α.25-(OH)$_2$D$_3$,FGF23 and osteocalcin concentrations were not significantly different between the two groups. Data are mean±SEM. *t*-test *$p < 0.05$ versus the control group

Serum calcium, phosphorous and calcium regulatory markers

The serum calcium concentration was significantly decreased in ATP2B1$^{+/-}$ mice compared with in ATP2B1$^{+/+}$ mice (Fig. 1a, 9.5 ± 0.1 versus 9.1 ± 0.1, $P < 0.05$). On the other hand, the serum phosphorous concentration was not different between the two groups (Table 1). Based on the above results, we examined alteration of calcium regulatory markers. The serum intact-PTH concentration was significantly decreased in ATP2B1$^{+/-}$ mice compared with in ATP2B1$^{+/+}$ mice (Fig. 1b, 324.4 ± 41.4 versus 153.6 ± 41.0, $P < 0.05$). However, 1α,25-(OH)$_2$D$_3$ and FGF23 concentrations were not significantly different between the two groups (Table 1).

Urinary excretion of calcium

The urine calcium creatinine ratio was significantly increased in ATP2B1$^{+/-}$ mice compared with in ATP2B1$^{+/+}$ mice (Fig. 1c, 0.33 ± 0.08 versus 0.77 ± 0.12, $P < 0.05$).

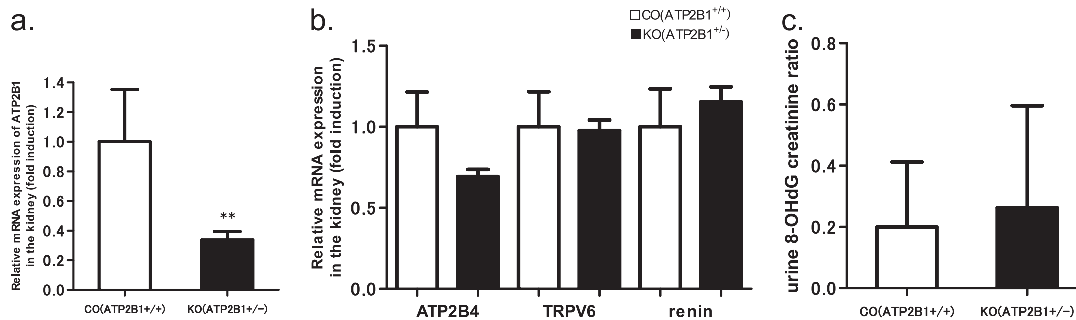


Fig. 2 Relative mRNA expression of ATP2B1, ATP2B4, TRPV6 and renin in the kidney, and urine 8-OHdG creatinine ratio. Relative mRNA expression levels of (**a**) ATP2B1, **b** ATP2B4, TRPV6 and renin were determined in the kidney of ATP2B1$^{+/+}$ and ATP2B1$^{+/-}$ mice ($n = 6$ for each group). ATP2B1 mRNA expression was decreased in the kidney of ATP2B1$^{+/-}$ mice. The mRNA expression of ATP2B4, TRPV6 and renin was not different between the two groups. Urine 8-OHdG and creatinine were measured in ATP2B1$^{+/-}$ ($n = 11$) and ATP2B1$^{+/+}$ mice ($n = 10$). Urine 8-OHdG creatinine ratio was not different between the two groups

Urinary oxidative-stress marker

The urine 8-OHdG creatinine ratio was not significantly different between the two groups (Fig. 2c).

The mRNA expression of ATP2B1, other calcium transporters and renin in the kidney

We performed qRT-PCR using kidney tissue from ATP2B1$^{+/-}$ and ATP2B1$^{+/+}$ mice to examine alterations in calcium regulator transporters and renin expression. The mRNA expression of *ATP2B1* was significantly decreased (Fig. 2a, 1.00 ± 0.14 versus 0.34 ± 0.02, $P < 0.05$) in ATP2B1$^{+/-}$ mice compared with in ATP2B1$^{+/+}$ mice. On the other hand, the mRNA expression of ATP2B4, TRPV6 and renin was not different between the two groups (Fig. 2b). Similarly, the urinary excretion of 8-OHdG was not different between the groups (Fig. 2c).

The mRNA expression of ATP2B1 and other calcium transporters in the small intestine

We performed qRT-PCR using small intestine tissue from ATP2B1$^{+/-}$ and ATP2B1$^{+/+}$ mice to examine alterations in calcium regulator transporter expression. The mRNA expression of *ATP2B1* was significantly decreased (Fig. 3a, 1.00 ± 0.12 versus 0.56 ± 0.0920, $P < 0.05$) in ATP2B1$^{+/-}$ mice compared with in ATP2B1$^{+/+}$ mice. On the other hand, the mRNA expression of TRPV6 and NCX1 was not different between the two groups (Fig. 3b).

Bone mineral density and bone mass

We performed pQCT using femoral bone of ATP2B1$^{+/-}$ and ATP2B1$^{+/+}$ mice in order to clarify the effects of *ATP2B1* reduction on bone metabolism in the entire body. The bone mineral density was significantly increased in ATP2B1$^{+/-}$ mice compared with in ATP2B1$^{+/+}$ mice (Fig. 4, 645.2 ± 9.2 versus 689.0 ± 12.9, $P < 0.05$). Consistent with the increase in bone mineral density, von Kossa staining of femoral bone revealed increased bone mass in ATP2B1$^{+/-}$ mice (Fig. 4).

Osteoblasts with eosinophilic cytoplasm and osteoclasts

We performed HE staining using femoral bone of ATP2B1$^{+/-}$ and ATP2B1$^{+/+}$ mice to confirm the cell properties and number of osteoblasts. The number of osteoblasts with eosinophilic cytoplasm in the growth plate was significantly increased in ATP2B1$^{+/-}$ mice compared with in ATP2B1$^{+/+}$ mice (Fig. 5a, b, 9 ± 3 versus 33 ± 8, $P < 0.05$). This indicates that osteogenesis is promoted in ATP2B1$^{+/-}$ mice. We performed TRAP staining using femoral bone of ATP2B1$^{+/-}$ and ATP2B1$^{+/+}$ mice to clarify the cell properties and number of osteoclasts. The number of osteoclasts was not different between the two groups (Fig. 5c, d).

Discussion

In the present study, we found that ATP2B1$^{+/-}$ mice had decreased intact-PTH levels despite hypocalcemia. The expression of *ATP2B1* in the kidney and small intestine decreased, furthermore femoral bone mineral density and urinary calcium excretion increased. Thus, we confirmed that the altered expression of the *ATP2B1* gene, which is thought to be responsible for regulating the intracellular calcium concentration, also affected the calcium metabolism throughout the entire body.

Ryan et al. [13] reported that the serum calcium concentration in small intestine-specific ATP2B1-deficient mice was not different from that in control mice. We previously confirmed that the vascular smooth muscle-specific

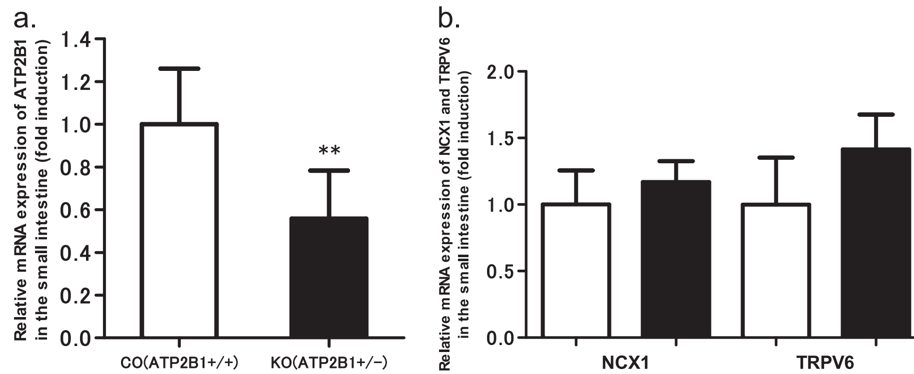


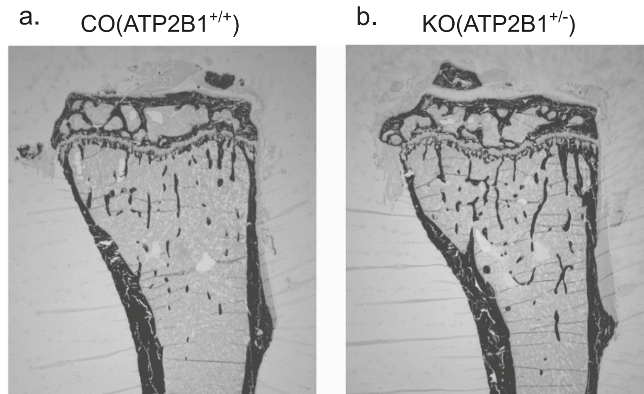
Fig. 3 Relative mRNA expression of calcium transporters in the small intestine. Relative mRNA expression levels of (**a**) ATP2B1 were determined in the small intestine of ATP2B1$^{+/+}$ and ATP2B1$^{+/-}$ mice ($n = 6$ for each group). ATP2B1 mRNA expression was decreased in ATP2B1$^{+/-}$ mice. Relative mRNA expression levels of (**b**) NCX1 and TRPV6 were examined in the small intestine of ATP2B1$^{+/+}$ ($n = 6$) and ATP2B1$^{+/-}$ mice ($n = 6$). The mRNA expression of TRPV6 and NCX1 was not different between the two groups. Data are mean ±SEM. *t*-test $*p < 0.05$ versus the control group

ATP2B1-deficient mice had no alterations in serum calcium levels (unpublished data). However, ATP2B1$^{+/-}$ mice exhibited hypocalcemia in the present study. As the parathyroid gland may be stimulated by the reduced calcium concentration [18], the hypocalcemia observed in ATP2B1$^{+/-}$ mice should be corrected by the increased PTH secretion as a compensatory mechanism. However, the PTH values in ATP2B1$^{+/-}$ mice were lower than those in control mice. This suggests that serum PTH production was suppressed by knockout of *ATP2B1* in the parathyroid gland. We confirmed that ATP2B1 expression in all organs in ATP2B1$^{+/-}$ mice was decreased, and that vascular smooth muscle intracellular Ca^{2+} concentrations were increased when ATP2B1 expression was decreased in VSMC. Similarly, ATP2B1 expression in the parathyroid gland may also decrease, increasing the intracellular Ca^{2+} concentration. Parathyroid cells have a pathway to reduce PTH secretion by elevating the intracellular Ca^{2+} concentration, which induces arachidonic acid (AA) through activation of cytosolic phospholipase A2 (cPLA2) [19–22]. Through the Ca^{2+}-cPLA2-AA mechanisms, PTH secretion may be suppressed in ATP2B1$^{+/-}$ mice. We attempted to verify the expression level of *ATP2B1* in the parathyroid gland in ATP2B1$^{+/-}$ mice, but isolation of the gland and evaluation of expression were difficult because of its small size [23].

There are some reports that suggest the relationship between PTH and blood pressure. Kalinowski et al. [24] reported that hPTH [1–34] and hPTHrP [1–34] activate NO production in endothelial cells, and experimental data suggested that PTH and PTHrp exert their vasorelaxant action via cAMP-dependent inhibition of L-type Ca^{2+} channel currents in VSMC [25, 26]. Thus, the decreased PTH secretion may cause decreased NO production, as observed in ATP2B1$^{+/-}$ mice [10]. On the other hand, there are numerous reports demonstrating the vasodilatory effects of exogenously administered PTH. Saglikes et al. [27] found that PTH blunts the vasoconstrictor effects of naturally occurring agonists, such as norepinephrine and angiotensin II, and this action is mediated via the production of vasodilating prostaglandins. Thus, the decreased PTH secretion may cause hypertension via decreased vasodilators in ATP2B1$^{+/-}$ mice.

The relationship between hypercalciuria and blood pressure was also reported. Kamijo et al. [28] noted the importance of hypercalciuria in spontaneously hypertensive rats. In this study, we observed a significant increase in urinary calcium excretion, and a significant decrease in ATP2B1 expression in the kidney in ATP2B1$^{+/-}$ hypertensive mice [10, 15]. On the other hand, the expression of ATP2B4, NCX1 was not altered. As PMCA1 exists on the basolateral side of the organ, it may directly or indirectly influence the serum calcium concentration. Through PMCA or NCX1, Ca^{2+} is thought to be extruded at the basolateral side by binding to $CaBP_{28k}$ and/or $CaBP_{9k}$ [29, 30] in the late distal convoluted tubule (DCT) and the connecting tubule (CNT), which is regulated by hormones, including PTH and active $1\alpha,25\text{-}(OH)_2D_3$ [31–33]. The result that ATP2B4 has not altered suggests that ATP2B1 is more important than ATP2B4 in calcium regulation. Then, it is suggested that decreased expression of ATP2B1 in the kidney is mainly associated with increased urinary calcium excretion.

Pointer et al. [34] also reported that calcium excretion is associated with lower renal cortical interstitial fluid calcium, which is associated with high blood pressure, as observed in Dahl salt-sensitive (DS) rats. Furthermore, Tojo et al. [35, 36] found that eNOS in the renal vasculature was significantly decreased in the DS rats. Thus, a similar mechanism may be involved in the hypertension exhibited by ATP2B1$^{+/-}$ mice because eNOS expression was decreased, as described in our previous study [10]. One the other hand, the expression of renin in the kidney and urine 8-OHdG creatinine ratio were not different between two



Bone mineral density (mg/cm^3)			P
Diaphyseal region	645.2 ± 9.2	689.0 ± 12.9	0.0154
Metaphyseal region	449.7 ± 14.5	490.0 ± 18.2	0.0437

Fig. 4 Von Kossa staining and bone mineral density in femoral bone by peripheral quantitative computed tomography (pQCT) (mg/cm3). Von Kossa staining and bone mineral density in **a** ATP2B1$^{+/+}$ and **b** ATP2B1$^{+/-}$ mice ($n = 8$ for each group). Data are mean±SEM. *t*-test $*p < 0.05$ versus the control group. Bone mineral density and bone mass were significantly increased in ATP2B1$^{+/-}$ mice compared with in ATP2B1$^{+/+}$ mice

groups, which suggest that renal RAS systems is not important to blood pressure contorl in ATP2B1$^{+/-}$ mice.

As ATP2B1$^{+/-}$ mice exhibited hypocalcemia, we pursued other mechanisms related with calcium metabolism. The absorption of calcium in the small intestine may play an important role in calcium homeostasis. Intracellular Ca^{2+} binding proteins, such as $CaBP_{9k}$, facilitate the movement of Ca^{2+} across enterocytic cells [37], and basal-lateral PMCA [38] and NCX [39] assist in the removal of Ca^{2+} from the cell to the extracellular fluid. In this study, we confirmed that *ATP2B1* was significantly decreased and $CaBP_{9k}$ was significantly increased in the small intestine of ATP2B1$^{+/-}$ mice. However, the expression levels of TRPV6 and NCX1 calcium regulatory proteins were not altered. Therefore, PMCA1 plays an important role in calcium metabolism in the small intestine, and its uptake into the blood is likely decreased due to the reduced *ATP2B1* expression level. However, Ryan et al. [13] reported that small intestine-specific *ATP2B1*-deficient mice exhibited decreased bone mineral density without altered PTH level or serum calcium concentration. *ATP2B1* in the small intestine may have little effect on serum calcium concentration, but why bone mineral density decreased in intestine-specific ATP2B1 KO mice remains unknown. Thus, we also examined alterations of bone phenotypes in ATP2B1$^{+/-}$ mice.

In ATP2B1$^{+/-}$ mice, the bone mineral density was markedly increased. Moreover, we confirmed that bone mass increased in ATP2B1$^{+/-}$ mice by von Kossa staining. In addition, an increased number of osteoblasts with eosinophilic cytoplasm was found in ATP2B1$^{+/-}$ mice, suggesting that bone production was increased [40]. However, there was no significant difference in the shape or number of osteoclasts. In the ATP2B1$^{+/-}$ mice, the bone mass was increased and PTH secretion was decreased. The decreased PTH levels were reasonable for the increased bone mass observed in the present study because it is known that PTH activates osteoclasts and suppresses the proliferation of osteoblasts [41–43]. However, despite the decreased PTH secretion, there was an increase in osteoblasts and no change in osteoclasts. This suggests that *ATP2B1* is directly involved in bone formation and bone resorption independently of PTH. As there were alterations in PTH values and bone density in ATP2B1$^{+/-}$ mice, we predicted that 1α,25-$(OH)_2D_3$ was also changed. However there were no significant changes in 1α,25-$(OH)_2D_3$ concentrations.

ATP2B1 gene polymorphisms are known to affect the expression level of *ATP2B1* in human umbilical cord blood vessels. Indeed, patients with a polymorphism causing decreased *ATP2B1* expression exhibited high blood pressure [2]. Similarly, in whole-body heterozygous *ATP2B1*-deficient mice in the previous study, expression of *ATP2B1* was decreased and blood pressure was elevated due to decreased eNOS activity and NO production [10]. This study also confirmed that ATP2B1$^{+/-}$ mice had hypocalcemia. The absorption of calcium in the kidney was decreased and suppression of bone resorption was suggested in these hypertensive mice. These effects were considered to be partly due to the reduction in PTH secretion by the *ATP2B1* knockout, and these results suggested that reduced PTH secretion and hypercalciuria are associated with elevated blood pressure via NO reduction in ATP2B1$^{+/-}$ mice.

There are several limitations in this study. As the parathyroid gland was too small, we were unable to clarify the mechanisms of *ATP2B1* on PTH secretion using the parathyroid gland in detail. This needs to be evaluated in a future study. Although this study helped to elucidate the reason for NO reduction in ATP2B1$^{+/-}$ mice, further study is needed in the future.

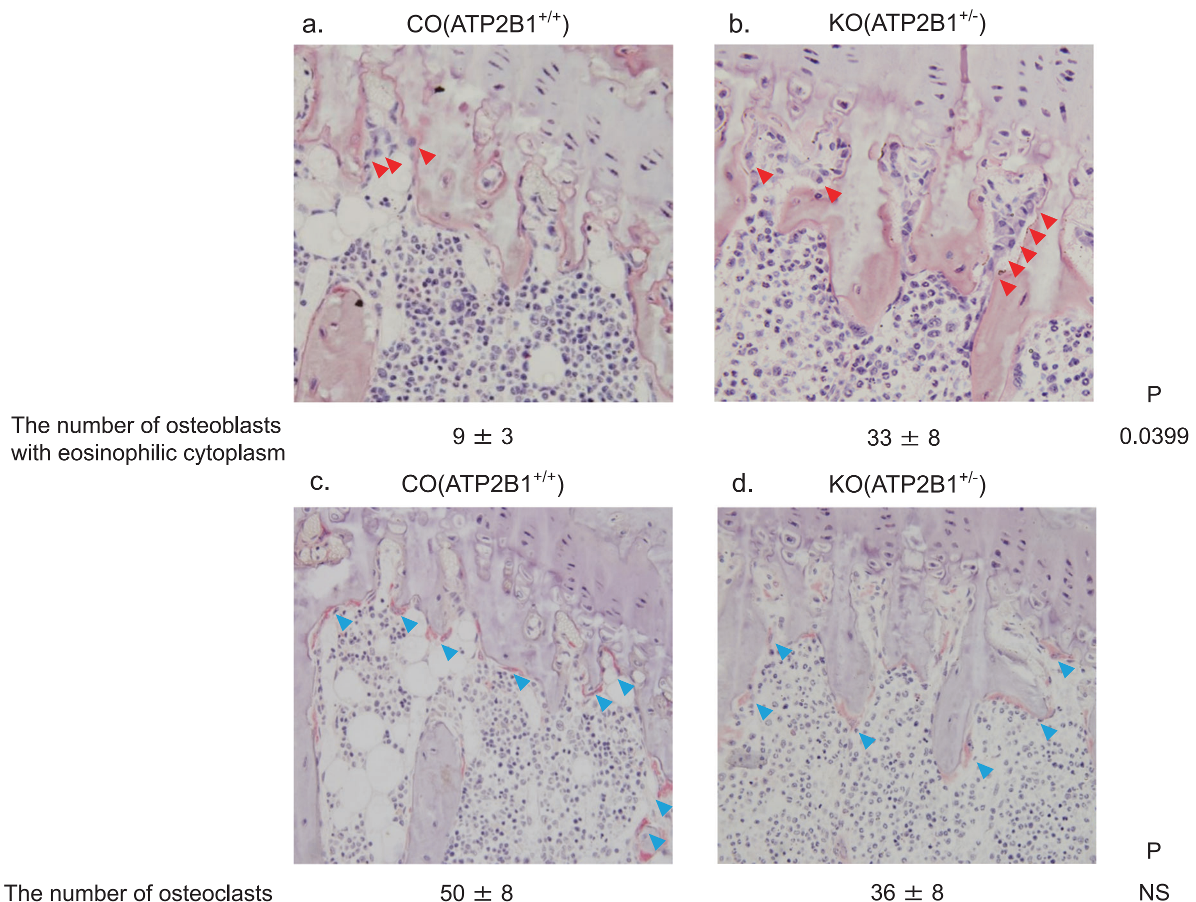


Fig. 5 The number of osteoblasts with eosinophilic cytoplasm on HE staining, and the number of osteoclasts on TRAP staining. HE staining and the number of osteoblasts with eosinophilic cytoplasm in (**a**) ATP2B1$^{+/+}$ mice and (**b**) ATP2B1$^{+/-}$ mice ($n = 3$ for each group). TRAP staining and the number of osteoclasts in (**c**) ATP2B1$^{+/+}$ mice and (**d**) ATP2B1$^{+/-}$ mice ($n = 3$ for each group). Data are mean±SEM. *t*-test $*p < 0.05$ versus the control group. The number of osteoblasts with eosinophilic cytoplasm in the growth plate was significantly increased in ATP2B1$^{+/-}$ mice. The number of osteoclasts was not different between the two groups

In conclusion, systemic heterozygous *ATP2B1*-null mice are thought to be a useful model for examining the pathology of calcium metabolism and hypertension. The *ATP2B1* gene is important for not only intra-cellular calcium regulation but also for calcium homeostasis and blood pressure.

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Compliance with ethical standards

Conflict of interest NH received a research grant from Pfizer, and SU received lecture fees from Takeda, Behringer, Daiichi-Sankyo, MSD, received manuscript fees from Sunmark publishing, and received scholarship donations from Takeda, Pfizer, Asteras, Daiichi-Sankyo, Behringer and AstraZeneka. KT received research grants from AstraZeneca, Ono Pharmaceutical and Tsumura. NH and SU have been name as the inventors on a patent based on work on ATP2B1 SNPs and hypertension. The remaining authors declare no conflicts of interest.

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