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Dietary Salt Intake Regulates WNK3–SPAK–NKCC1 Phosphorylation Cascade in Mouse Aorta Through Angiotensin II

Moko Zeniya, Eisei Sohara, Satomi Kita, Takahiro Iwamoto, Koichiro Susa, Takayasu Mori, Katsuyuki Oi, Motoko Chiga, Daiei Takahashi, Sung-Sen Yang, Shih-Hua Lin, Tatemitsu Rai, Sei Sasaki, Shinichi Uchida

Abstract—Na–K–Cl cotransporter isoform 1 (NKCC1) is involved in the regulation of vascular smooth muscle cell contraction. Recently, the with-no-lysine kinase (WNK)–STE20/SPS1-related proline/alanine-rich kinase (SPAK)–NKCC1 phosphorylation cascade in vascular smooth muscle cells was found to be important in the regulation of vascular tone. In this study, we investigated whether the WNK–SPAK–NKCC1 cascade in mouse aortic tissue is regulated by dietary salt intake and the mechanisms responsible. Phosphorylation of SPAK and NKCC1 was significantly reduced in the aorta in high-salt–fed mice and was increased in the aorta in low-salt–fed mice, indicating that the WNK–SPAK–NKCC1 phosphorylation cascade in the aorta was indeed regulated by dietary salt intake. Acute and chronic angiotensin II infusion increased phosphorylation of SPAK and NKCC1 in the mouse aorta. In addition, valsartan, an antagonist of angiotensin II type 1 receptor, inhibited low-salt diet–induced phosphorylation of SPAK and NKCC1, demonstrating that angiotensin II activates the WNK–SPAK–NKCC1 phosphorylation cascade through the angiotensin II type 1 receptor. However, a low-salt diet and angiotensin II together did not increase phosphorylation of SPAK and NKCC1 in the aorta in WNK3 knockout mice, indicating that activation of the WNK–SPAK–NKCC1 phosphorylation cascade induced by a low-salt diet and angiotensin II is dependent on WNK3. Indeed, angiotensin II–induced increases in blood pressure were diminished in WNK3 knockout mice. In addition, decreased response to angiotensin II in the mesenteric arteries was observed in WNK3 knockout mice. Our data also clarified a novel mechanism for regulation of vascular tonus by angiotensin II. Inhibition of this cascade could, therefore, be a novel therapeutic target in hypertension. **(*Hypertension*. 2013;62:872-878.)** • Online Data Supplement

Key Words: angiotensin II ■ aorta ■ NKCC1 ■ SPAK ■ WNK

Na–K–Cl cotransporter isoform 1 (NKCC1) is involved in the regulation of vascular smooth muscle cell contractions via intracellular Cl^- accumulation, membrane depolarization, and activation of voltage-gated Ca channels, leading to elevation of peripheral resistance.[1] Indeed, NKCC1 knockout mice showed lower blood pressure because of decreased vascular tonus only when mice were fed a low-salt diet, as compared with those fed a normal diet, although dietary salt intake does not affect blood pressure in wild-type mice.[2] This indicates that NKCC1 plays a key role in the regulation of myogenic tone in arteries by dietary salt intake.

Pseudohypoaldosteronism type II is an autosomal-dominant disease characterized by salt-sensitive hypertension, hyperkalemia, and metabolic acidosis.[3] Mutations in with-no-lysine kinase 1 (WNK1) and WNK4 have been reported to cause pseudohypoaldosteronism type II.[4] It has been demonstrated previously that the WNK kinase family phosphorylated and activated oxidative stress-responsive kinase 1 (OSR1) and STE20/SPS1-related proline/alanine-rich kinase (SPAK), and that these OSR1/SPAK kinases could phosphorylate and activate NKCC and Na–Cl cotransporter (NCC), which are solute carrier 12a (SLC12a) family cotransporters. This regulation of SLC12 family members by WNK–OSR1/SPAK signaling was confirmed in vivo in various genetically engineered mouse models.[5–10]

Recently, the WNK–OSR1/SPAK–NKCC1 phosphorylation cascade in vascular smooth muscle cells was also found to be important for maintenance of vascular tone. Bergaya et al,[8] as well as our group,[11] reported decreased NKCC1 phosphorylation in the aorta and decreased myogenic tone in the mesenteric arteries in $WNK1^{+/-}$ mice, indicating that WNK1 plays

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a substantial role in the maintenance of vascular tonus in the arteries. We have also shown in SPAK knockout mice that the phosphorylation of NKCC1 and the vascular tone responses to phenylephrine and bumetanide in the aorta were decreased.[12] These data demonstrate that WNK1 and SPAK constitute the WNK–SPAK–NKCC1 phosphorylation cascade within vascular smooth muscle cells. Interestingly, similar to NKCC1 knockout mice, WNK3 knockout mice showed lower blood pressure only when mice were fed a low-salt diet, although urinary excretion of Na was not significantly affected in WNK3 knockout mice, indicating that lower blood pressure in WNK3 knockout mice fed a low-salt diet is because of extrarenal mechanisms (ie, vascular tone).[13,14]

In this study, we investigated whether the WNK–SPAK–NKCC1 cascade in mouse aortic tissue is regulated by dietary salt intake, as well as the potential mechanisms. We further examined the involvement of WNK3 in these mechanisms. We demonstrated that a low-salt diet activates the WNK–SPAK–NKCC1 phosphorylation cascade in mouse aorta via angiotensin II (AngII). Furthermore, we found that WNK3 is involved in this mechanism, indicating that AngII physiologically regulates myogenic tone in the arteries through the WNK3–SPAK–NKCC1 phosphorylation cascade. Our data also clarified a novel mechanism for AngII-induced vascular contraction.

Materials and Methods

An expanded Methods section is available in the online-only Data Supplement.

Dietary Salt and Drug Infusion Study Protocols

For experiments examining the effects of dietary salt intake, C57BL/6J mice were placed on a high-salt diet (4% NaCl [wt/wt]), normal diet (0.9% NaCl [wt/wt]), or low-salt diet (0.01% [wt/wt]) for 1 week. All foods were obtained from Oriental Yeast Co, Ltd (Tokyo, Japan).

For the chronic AngII or aldosterone infusion model, male mice fed a normal diet were intraperitoneally administered AngII at a dose of 2 mg/kg per day or aldosterone at a dose of 0.07 mg/kg per day, as reported previously.[15,16] For the valsartan, an AngII receptor type 1 blocker, infusion model, male mice were fed a low-salt diet. For chronic intraperitoneal infusion, we used osmotic minipumps (Alzet Corporation, Cupertino, CA). For administration of the aldosterone receptor blocker eplerenone, eplerenone was administered for 7 days via chow (2.4 mg eplerenone per gram of chow) to achieve a dose of ≈400 mg/kg per day, as reported previously.[17] For the acute AngII infusion model, we implanted the infusion tube 5 days before administration of AngII, as reported previously,[15] and infused AngII at a dose of 25 μg/g. Mice were then euthanized at 30 minutes after the start of the infusion.

Immunoblotting

Protein lysates of thoracic aortas and kidneys were obtained as follows. The aorta was isolated carefully and frozen immediately with liquid nitrogen. After being crushed with a mortar, aortas were added to 150 μL of lysis buffer, as reported previously,[11] followed by centrifugation at 6000*g* at 4°C. Supernatant (120 μL) was then denaturated at 60°C for 20 minutes. Crude kidney membrane fraction (17 000*g*) was then prepared to measure the levels of phosphorylated NCC.

Primary antibodies used in this study were rabbit anti–phosphorylated SPAK[18]; rabbit anti-SPAK (Cell Signaling, Danvers, MA); rabbit anti–phosphorylated NKCC1 (T206)[12]; mouse anti-NKCC1 (T4)[19]; rabbit anti–phosphorylated NCC (T53, T58)[16]; rabbit anti-actin antibody (Cell Signaling); rabbit anti–phospho and total extracellular signal-regulated kinase 1/2 (ERK1/2) (Cell Signaling). Alkaline-phosphatase–conjugated anti-IgG antibodies (Promega, Madison, WI) were used as secondary antibodies for immunoblotting. WesternBlue (Promega) was used for the development of immunoblots. The relative intensities of immunoblot bands were determined by densitometry with ImageJ software.

Measurements of Arterial Diameter and Myogenic Tone

Wild-type mice and WNK3 knockout mice (8–10 weeks old) were euthanized by cervical dislocation. Small arteries from the superior mesenteric artery arcade were isolated and cannulated with small pipettes at both ends. Arterial external diameter and myogenic tone were measured in the isolated, pressurized arteries in physiological salt solution as described previously.[20–22] For measurement of diameter, the artery outer diameter was monitored continuously by a real-time edge-detection system (National Instruments). Myogenic tone was generated at an intraluminal pressure of 80 mm Hg unless otherwise noted. Pressurized arteries were infused with 1 μmol/L AngII for 5 minutes, and then 100 μmol/L bumetanide was added to arteries that had been preconstricted with AngII. At the end of each experiment, passive external diameter was measured in Ca-free physiological salt solution.

Results

Dietary Salt Intake Regulates Phosphorylation of NKCC1 and SPAK in Mouse Aorta

To investigate whether dietary salt intake regulates the WNK–SPAK–NKCC1 phosphorylation cascade in mouse aortic tissue, we examined the levels of phosphorylation of SPAK and NKCC1 in the aorta of mice fed high-, normal-, and low-salt diets. As shown in Figure 1, phosphorylation of SPAK at the WNK phosphorylation sites was reduced significantly in the aorta in high-salt–fed mice and was increased in the aorta in low-salt–fed mice. Similarly, NKCC1 phosphorylation at the SPAK phosphorylation site was also reduced in

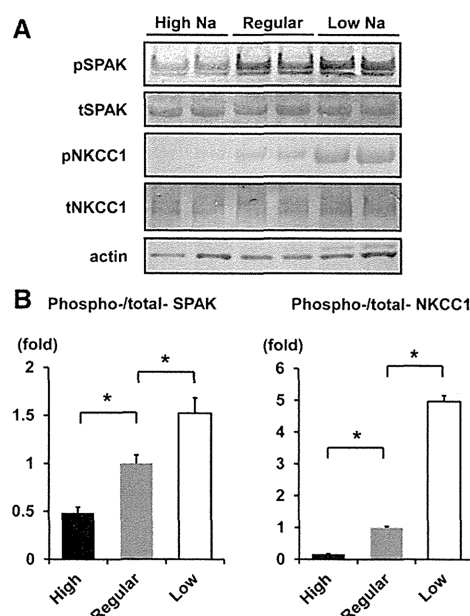

Figure 1. Dietary salt regulates phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and Na–K–Cl cotransporter isoform 1 (NKCC1) in mouse aorta. **A**, Representative immunoblots of total (t) and phosphorylated (p) SPAK and NKCC1 in aortas from mice fed high-, normal-, and low-salt diets. SPAK and NKCC1 phosphorylation in mouse aorta was increased by a low-salt diet and decreased by a high-salt diet, respectively. **B**, Densitometry analyses (n=8). *$P<0.05$.

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the high-salt diet group and was increased in the low-salt diet group. Phosphorylation of OSR1 was not regulated by dietary salt intake (Figure S1 in the online-only Data Supplement). These data indicate that the WNK–SPAK–NKCC1 phosphorylation cascade in the aorta was indeed regulated by dietary salt intake.

Low-Salt Diet Does Not Increase Phosphorylation of NKCC1 and SPAK in WNK3 Knockout Mouse Aorta

Recently, we reported that WNK3 knockout mice showed lower blood pressure only when fed a low-salt diet, although urinary excretion of Na was not affected significantly in WNK3 knockout mice,[13] indicating that WNK3 is able to regulate blood pressure based on dietary salt intake by vascular tonus. As shown in Figure 2A, we confirmed the expression of WNK3 in mouse aorta by reverse transcription polymerase chain reaction. Therefore, we examined phosphorylation of SPAK and NKCC1 in WNK3 knockout mice fed normal and low-salt diets. As shown in Figure 2B and 2C, WNK3 knockout mice fed a normal diet did not show decreased phosphorylation of SPAK and NKCC1 in their aortic tissue, as compared with wild-type mice. In contrast, as we suspected, WNK3 knockout mice fed a low-salt diet showed impaired low-salt diet–induced phosphorylation of SPAK and NKCC1, indicating that WNK3 plays a role in this mechanism.

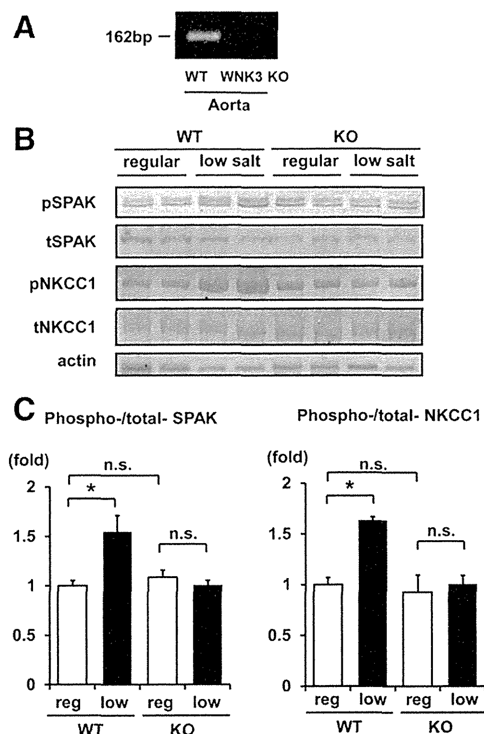

Figure 2. Phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and Na-K-Cl cotransporter isoform 1 (NKCC1) in aorta from wild-type (WT) and with-no-lysine kinase 3 (WNK3) knockout (KO) mice fed a normal diet or a low-salt diet. **A**, Expression of WNK3 in mouse aorta was confirmed by reverse transcription polymerase chain reaction. **B**, Representative immunoblots of total (t) and phosphorylated (p) SPAK and NKCC1 in aortas from WT mice and WNK3 KO mice fed a normal or a low-salt diet. Low-salt diet–induced phosphorylation of SPAK and NKCC1 was impaired in aortas from WNK3 KO mice. **C**, Densitometry analyses (n=8). $^*P<0.05$. low/low salt indicates low-salt diet; n.s., not significant; and reg, regular diet.

AngII Activates WNK3–SPAK–NKCC1 Phosphorylation Cascade in Mouse Aorta

We then investigated the mechanism(s) of this regulation. We focused on AngII and aldosterone because these components in the renin–angiotensin–aldosterone system are regulated by dietary salt intake. In addition, both AngII and aldosterone are well-known physiological regulators of the WNK–SPAK–NCC phosphorylation cascade in the kidney.[9,15,16,23] As angiotensin II type 1 receptor (AT1R) is abundantly expressed in vascular smooth muscle cells,[24] we first examined whether AngII regulates the WNK–SPAK–NKCC1 cascade in mouse aortic tissue. As shown in Figures 3 and 4, both acute and chronic AngII infusion significantly increased phosphorylation of SPAK and NKCC1 in wild-type mouse aorta. Valsartan, an antagonist of AT1R, inhibited low-salt diet–induced phosphorylation of SPAK and NKCC1 (Figure 5). These data demonstrate that AngII activates the WNK3–SPAK–NKCC1 phosphorylation cascade through the AT1R. In contrast, neither acute nor chronic AngII infusion increased phosphorylation of SPAK and NKCC1 in aortic tissue from WNK3 knockout mice (Figures 3 and 4). Increases in pERK1/2 confirmed that WNK3 knockout mice do not lack the AngII response in aorta (Figure S2).[25] These data indicate

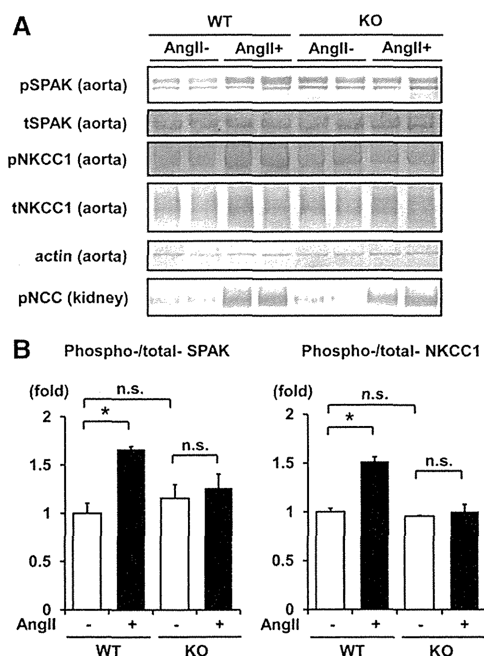

Figure 3. Chronic angiotensin II (AngII) infusion increased phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and Na-K-Cl cotransporter isoform 1 (NKCC1) in wild-type (WT) mouse aorta, but not in with-no-lysine kinase 3 (WNK3) knockout (KO) mouse. **A**, Representative immunoblots of total (t) and phosphorylated (p) SPAK and NKCC1 in aorta from WT mice and WNK3 KO mice treated with AngII for a week. Chronic AngII infusion increased phosphorylation of SPAK and NKCC1 in WT mice aorta, but did not increase them in WNK3 KO mice aorta. Increased Na–Cl cotransporter (NCC) phosphorylation in the kidney demonstrated that AngII infusion was effective. **B**, Densitometry analyses (n=8). $^*P<0.05$. n.s. indicates not significant.

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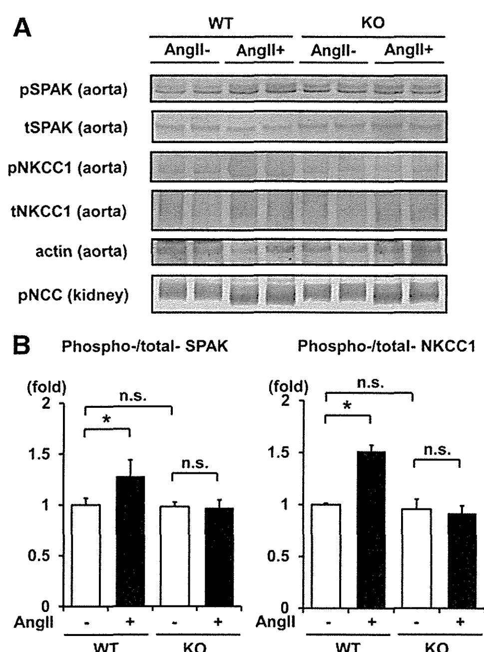

Figure 4. Acute angiotensin II (AngII) infusion increased phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and Na–K–Cl cotransporter isoform 1 (NKCC1) in wild-type (WT) mouse aorta, but not in with-no-lysine kinase 3 (WNK3) knockout (KO) mouse. **A**, Representative immunoblots of total (t) and phosphorylated (p) SPAK and NKCC1 in aortas from WT and WNK3 KO mice at 30 minutes after AngII infusion. Acute AngII infusion increased phosphorylation of SPAK and NKCC1 in WT mouse aorta, but not in WNK3 knockout mouse aorta. Increased Na–Cl cotransporter (NCC) phosphorylation in the kidney demonstrated that AngII infusion was effective. **B**, Densitometry analyses (n=3). **P*<0.05. n.s. indicates not significant.

that the activation of the WNK–SPAK–NKCC1 phosphorylation cascade by low salt and AngII is dependent on WNK3.

Next, to assess whether aldosterone regulates the WNK–SPAK–NKCC1 phosphorylation cascade in aortic tissue in vivo, we infused aldosterone into mice and examined the phosphorylation of SPAK and NKCC1. Although successful aldosterone infusion was confirmed by increased phosphorylation of NCC in the kidney, aldosterone did not increase phosphorylation of SPAK and NKCC1 in the mouse aorta (Figure S3). Similarly, eplerenone, a selective aldosterone receptor antagonist, did not decrease phosphorylation of SPAK and NKCC1 (Figure S4). These in vivo data suggest that the WNK3–SPAK–NKCC1 phosphorylation cascade in vascular smooth muscle cells is regulated by AngII through AT1R, but not by aldosterone.

Impaired AngII-Induced Hypertension and Vasoconstriction in WNK3 Knockout Mice

To investigate whether this AngII-induced activation of WNK3–SPAK–NKCC1 phosphorylation cascade is physiologically functional, we infused AngII to WNK3 knockout mice and checked blood pressure. As reported previously, systolic blood pressure in WNK3 knockout mice did not show any significant differences when compared with wild-type mice under a normal diet (103.2±1.1 versus 102.8±0.7 mm Hg; n=6; Figure 6A). However, when mice were treated with AngII, although systolic blood pressure in wild-type mice increased as expected, the increases in blood pressure in WNK3 knockout mice by AngII were lower than those in wild-type littermates (125.0±1.6 versus 106.4±1.2 mm Hg; n=9; *P*<0.01). Similarly, in telemetry tracing, an elevation of systolic blood pressure after AngII infusion was observed in wild-type mice, but not in WNK3 knockout mice (Figure S5).

Furthermore, we measured mesenteric arterial diameter to estimate peripheral arterial contraction. Myogenic tone did not exhibit significant differences between wild-type (20.7±3.4% of passive external diameter; n=5) and WNK3 knockout mice (22.4±3.2% of passive external diameter; n=5). However, as shown in Figure 6B, contraction of mesenteric arteries in WNK3 knockout mice by AngII was smaller than that of wild-type mice. This AngII-induced contraction was reduced by bumetanide, an NKCC inhibitor, in wild-type mice (Figure 6C), indicating that NKCC1 plays a role in this mechanism. As expected, the reduction in AngII-induced vasoconstriction by bumetanide was smaller in WNK3 knockout mice when compared with wild-type mice, confirming that the WNK3–SPAK–NKCC1 phosphorylation cascade is physiologically important for vasoconstriction by AngII.

Discussion

The renin–angiotensin system is a central component of the physiological regulation of blood pressure. AngII is a well-known primary effector hormone of this system and mediates the immediate physiological effects of vasoconstriction.[2] In this study, we found a novel mechanism for the regulation of vascular tonus by AngII through the WNK3–SPAK–NKCC1 phosphorylation cascade (Figure 7). Previously, it was shown that NKCC1 inhibition by high ceiling diuretics suppresses contractions triggered by modest depolarization and

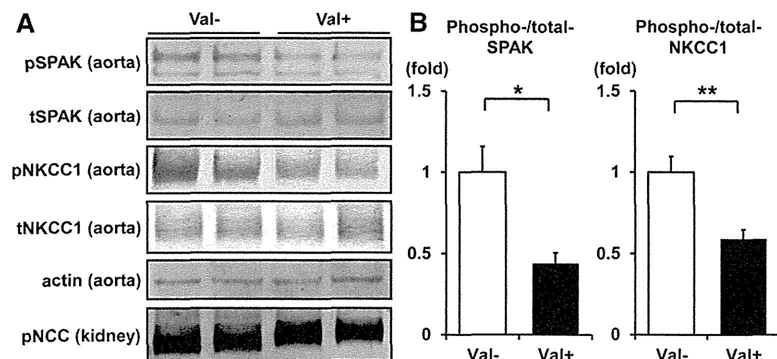

Figure 5. Valsartan (Val), angiotensin II type 1 receptor (AT1R) blocker, suppressed phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and Na–K–Cl cotransporter isoform 1 (NKCC1) in mouse aorta. **A**, Representative immunoblots of total (t) and phosphorylated (p) SPAK and NKCC1 in aortas from mice fed a low-salt diet with valsartan for a week. Phosphorylation of SPAK and NKCC1 in aorta of mice with valsartan was decreased. Decreased Na–Cl cotransporter (NCC) phosphorylation in the kidney demonstrated that AT1R blocker treatment was effective. **B**, Densitometry analyses (n=4). **P*<0.05; and ***P*<0.01.

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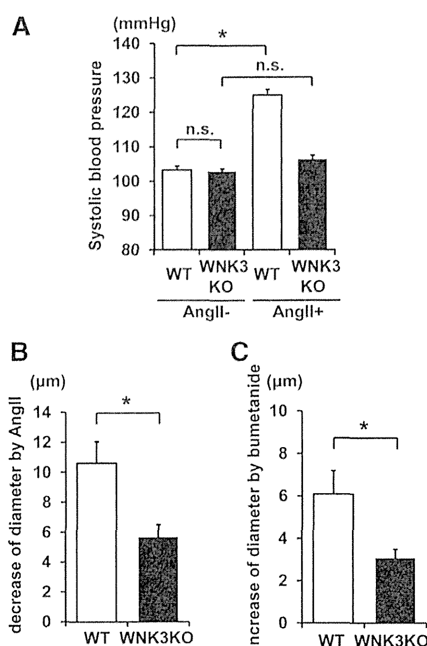

Figure 6. Impaired angiotensin II (AngII)–induced hypertension and vasoconstriction in with-no-lysine kinase 3 (WNK3) knockout (KO) mice. **A**, Blood pressure of wild-type (WT) and WNK3 KO mice with or without AngII infusion. AngII-induced increases of systolic blood pressure in WNK3 KO mice were lower than those in WT mice. **P*<0.01. **B** and **C**, Effects of WNK3 deletion on AngII-induced contraction of small mesenteric arteries. **B**, AngII-induced vasoconstriction of mesenteric arteries from WT mice and WNK3 KO mice. AngII-induced contraction of mesenteric arteries from WNK3 KO mice was smaller than those from WT mice (n=5). **P*<0.05. **C**, Increases in diameter of mesenteric arteries by additional infusion of 100 μmol/L bumetanide after AngII treatment. Reductions in AngII-induced vasoconstriction by bumetanide were smaller in WNK3 KO mice when compared with WT mice (n=5). **P*<0.05. n.s. indicates not significant.

myogenic tone of vascular smooth muscle cells,[22,26] including myogenic tone of renal afferent arterioles.[27] The WNK–SPAK–NKCC1 phosphorylation cascade in afferent arterioles could be a potential feedback mechanism for regulating renal blood flow by altered salt intake. Further investigations into

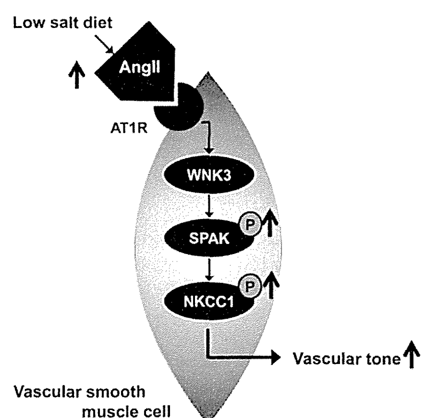

Figure 7. Proposed with-no-lysine kinase 3 (WNK3)–mediated mechanism of vasoconstriction by angiotensin II (AngII). AngII increases phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and Na–K–Cl cotransporter isoform 1 (NKCC1) through WNK3, leading to increased vascular tone. AT1R indicates angiotensin II type 1 receptor.

WNK signaling in renal afferent arteriole would provide new insights in this field.

In vascular smooth muscle cells, NKCC1 is reported to play an essential role in the regulation of vascular tonus.[2,28] External stimuli, such as vasoactive hormones, induce Ca release from intracellular Ca stores. This opens Ca-dependent Cl channels in plasma membranes, leading to depolarization. This depolarization opens voltage-dependent Ca channels in plasma membranes, resulting in induction of further Ca entry and contraction of smooth muscles. In this scheme, NKCC1 is important for accumulating intracellular Cl^-, enabling depolarization, leading to the opening of voltage-dependent Ca channels. Because phosphorylation of NKCC1 at SPAK phosphorylation sites causes NKCC1 activation, the WNK–SPAK–NKCC1 phosphorylation cascade is important for vasoconstriction.

In this study, we demonstrated that dietary salt intake regulates the WNK–SPAK–NKCC1 phosphorylation cascade in mouse aorta. Our data demonstrated that a low-salt diet activates the WNK–SPAK–NKCC1 phosphorylation cascade in mouse aortic tissue. This phenomenon is reasonable for the physiological maintenance of blood pressure in vivo because this phosphorylated NKCC1 in vascular smooth muscle cells leads to increases in vascular tone to avoid decreases in blood pressure by sodium depletion in the body.

Moreover, through investigations into physiological regulators of this low-salt diet–induced activation of WNK–SPAK–NKCC1, we found that AngII is the main physiological regulator of the WNK–SPAK–NKCC1 phosphorylation cascade in vascular smooth muscle cells. In the kidney, both aldosterone and AngII are strong physiological regulators of the WNK–OSR1/SPAK–NCC phosphorylation cascade. However, in mouse aortic tissue, aldosterone did not activate WNK–SPAK signaling. Instead, AngII activated the WNK–SPAK–NKCC1 phosphorylation cascade through the AT1R. This could be because of abundant expression of AT1R in vascular smooth muscle cells.

To the best of our knowledge, WNK1 and WNK3 are the WNK kinases expressed in vascular smooth muscle cells.[8,11] Both WNK1 and WNK3 have been reported to phosphorylate downstream SPAK and NKCC1,[29–33] WNK3 knockout mice were reported to show lower blood pressure only when fed a low-salt diet although urinary excretion of Na is not significantly affected in WNK3 knockout mice.[13] This indicates that WNK3 is involved in the regulation of the WNK–SPAK–NKCC1 phosphorylation cascade in vascular smooth muscle cells by dietary salt changes. In this study, we demonstrated that both a low-salt diet and AngII infusion did not activate the WNK–SPAK–NKCC1 phosphorylation cascade in aortic tissue of WNK3 knockout mouse. Moreover, AngII-induced increases in blood pressure were diminished in WNK3 knockout mice. In addition, decreased response to AngII in the mesenteric arteries was observed in WNK3 knockout mice. These results indicate clearly that WNK3 plays a substantial role in the mechanism of AngII-induced NKCC phosphorylation. In contrast, although WNK1 knockout mice showed decreased phosphorylation of SPAK and NKCC1 in mouse aorta under a normal-salt diet, Bergaya et al[8] reported that WNK1 plays a role in vasoconstriction by adrenergic stimulation through

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adrenergic receptor-1, but not in vasoconstriction by AngII stimulation in vivo. Taken together, these results suggest that WNK1 and WNK3 play different roles in vascular smooth muscle cells; WNK1 could be important for maintenance of basal activity of the WNK–SPAK–NKCC1 phosphorylation cascade and for the response to adrenergic stimulation. In contrast, WNK3 could be important for the response to stimulation by AngII. Further investigation will be necessary.

The detailed mechanisms underlying WNK3 activation by AngII in vascular smooth muscle cells remain to be determined. One possible mechanism is that AngII increases WNK3 expression. However, quantitative reverse transcription polymerase chain reaction revealed that WNK3 mRNA in mouse aorta was not affected by dietary salt intake (data not shown). Recently, Kelch-like3 (KLHL3) and Cullin3 were also identified to cause pseudohypoaldosteronism type II,[34,35] and we found that KLHL3 induces Cullin3-mediated WNK4 ubiquitination.[36,37] Defective interaction between KLHL3 and WNK4 because of pseudohypoaldosteronism type II–causing mutations resulted in increased levels of WNK4 protein, indicating that protein levels of WNK kinases are crucial for activity of the WNK–OSR1/SPAK–SLC12a cotransporter cascade. Therefore, although WNK3 mRNA was not increased in the aorta in mice fed a low-salt diet, WNK3 protein could be upregulated through a degradation pathway. To confirm this, detection of WNK3 protein expression in mouse aorta is necessary; however, we have not yet detected WNK3 protein in mouse aorta with available antibodies against WNK3. As even acute infusion of AngII is able to activate the WNK–SPAK–NKCC1 phosphorylation cascade in mouse aorta, another possibility is that the WNK kinases are activated by rapid modifications through AngII, such as WNK4 phosphorylation at 1169S.[38]

OSR1 was reported to be one of the components in the WNK signaling cascade in vascular smooth muscle cells.[39] However, phosphorylation of OSR1 was not regulated by dietary salt in mouse aorta. Although OSR1 and SPAK phosphorylation by WNK kinase was almost identical in the kidney, recent articles have demonstrated that they play different roles depending on the expression segment within the nephron.[12,39–41] SPAK, but not OSR1, may play a major role in the AngII-induced activation of the WNK–SPAK–NKCC1 phosphorylation cascade in vascular smooth muscle cells.

Perspectives

In this study, we demonstrated that dietary salt intake regulates the WNK–SPAK–NKCC1 phosphorylation cascade in mouse aortic tissue. AngII stimulation via AT1R was found to be the main regulator of this signal. Furthermore, we demonstrated that WNK3 plays a major role in this mechanism. Our data also clarified a novel mechanism for regulating vascular tonus by AngII. Inhibition of this cascade could be a novel therapeutic target in hypertension.

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We thank Chieko Iijima for assistance in the experiments and Toru Sugiyama for helpful discussion.

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Disclosures

None.

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Novelty and Significance

What Is New?

- It was determined that dietary salt intake regulates the with-no-lysine kinase (WNK)–STE20/SPS1-related proline/alanine-rich kinase (SPAK)–Na–K–Cl cotransporter isoform 1 (NKCC1) phosphorylation cascade in mouse aortic tissue through angiotensin II. Furthermore, we demonstrated that WNK3 plays a major role in this mechanism. This is the first report to demonstrate how the WNK3–SPAK–NKCC1 phosphorylation cascade is physiologically regulated in vivo. In addition, we clarified for the first time the physiological role of WNK3 in vivo.

What Is Relevant?

- Regulation of the WNK3–SPAK–NKCC1 phosphorylation cascade in arteries is physiologically important for the maintenance of blood pressure against sodium depletion in the body. Moreover, our data also clarified a novel mechanism for regulation of vascular tonus by angiotensin II.

Summary

In this study, we demonstrated that dietary salt intake regulates the WNK–SPAK–NKCC1 phosphorylation cascade in mouse aortic tissue. Angiotensin II stimulation via angiotensin II type 1 receptor was found to be the main regulator of this signal. Furthermore, we demonstrated that WNK3 plays a major role in this mechanism. Our data clarified a novel mechanism for regulation of vascular tonus by angiotensin II. Inhibition of this cascade could be a novel therapeutic target in hypertension.

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ONLINE SUPPLEMENT

Dietary salt intake regulates WNK3-SPAK-NKCC1 phosphorylation cascade in mouse aorta through angiotensin II

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Materials and Methods

Animals

Studies were performed on 12-week-old male C57BL/6J mice, or 12-week-old male WT and WNK3 KO mice[1]. Mice were raised under a 12-hour day and night cycle, and were fed a normal rodent diet and plain drinking water. The experiment was approved by the Animal Care and Use Committee of Tokyo Medical and Dental University.

mRNA extraction and RT-real time PCR analysis

Total RNA from aorta samples was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Total RNA was reverse-transcribed using Omniscript reverse transcriptase (Qiagen, Hilden, Germany). We investigated the presence of WNK3 with the following primers; WNK3 (sense; 5'-GCTGTTGCAACTTCCCCTAGT-3' (exon 1) and antisense; 5'-CCGTTGCTGCTCAGCTTTAG-3' (exon 2)).[1]

Blood pressure measurement

Blood pressure in restrained conscious mice at steady state was measured with a programmable tail-cuff sphygmomanometer (MK-2000A; Muromachi, Tokyo, Japan). The implantable radiotelemetry equipment for conscious, freely moving laboratory animals was purchased from Data Science International (St Paul, MN) and included an implantable transmitter (model TA11PA-C10), a receiver (model RPC-1), a data processing device (Data Exchange Matrix) and an ambient pressure reference monitor (APR-1). All data were computed using an analysis program (Dataquest ART4.31).

Statistical analysis

Statistical significance was evaluated using an un-paired t-test. All data are expressed as means ± SEM. When more than three groups were compared, one-way ANOVA with Fischer's post-hoc test was used. $P<0.05$ was considered to indicate statistical significance.

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Figure S1, Zeniya M, et al.

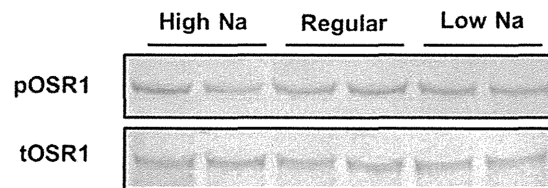

Figure S1. Phosphorylation of OSR1 was not regulated by dietary salt intake

(A) Immunoblots of total- and phosphorylated- OSR1 in aortas from mice fed high-, normal- and low-salt diets. Phosphorylation levels of OSR1 were not altered by dietary salt.

Figure S2, Zeniya M, et al.

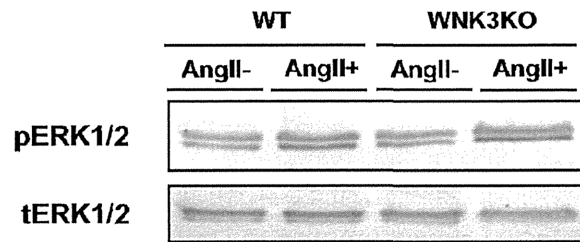

Figure S2. Phosphorylation of ERK1/2 in mouse aorta after AngII treatment. Phosphorylation of ERK1/2 increased in both of wild-type and WNK3 knockout mice aorta by AngII infusion, confirming that WNK3 knockout mice do not lack AngII response.

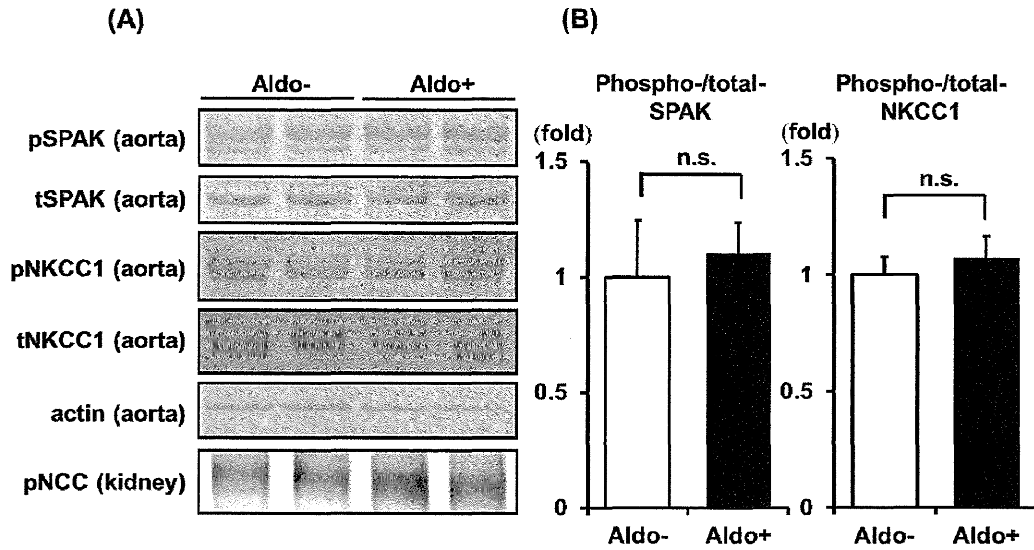

Figure S3. Aldosterone did not increase phosphorylation of SPAK and NKCC1 in mouse aorta

(A) Representative immunoblots of total- and phosphorylated- SPAK and NKCC1 in aortas from mice treated with aldosterone for a week. Aldosterone infusion for a week did not increase phosphorylation of SPAK and NKCC1 in mouse aorta. Increased NCC phosphorylation in the kidney demonstrated that aldosterone treatment was effective. (B) Densitometry analyses (n=12). Aldo; aldosterone.

Figure S4, Zeniya M, et al.

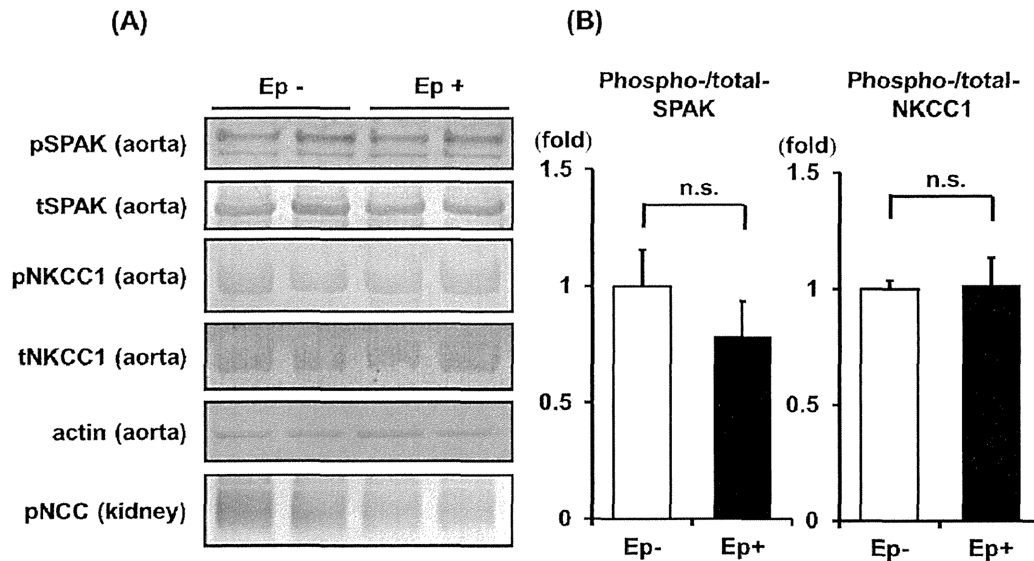

Figure S4. Eplerenone did not decrease phosphorylation of SPAK and NKCC1 in aorta

(A) Representative immunoblots of total- and phosphorylated- SPAK and NKCC1 in aortas from mice fed a low-salt diet with eplerenone. Eplerenone infusion for a week did not significantly suppress phosphorylation of SPAK and NKCC1 in mouse aorta. Decreased NCC phosphorylation demonstrated that eplerenone was effective. (B) Densitometry analyses (n=12). Ep; eplerenone.

Figure S5, Zeniya M, et al.

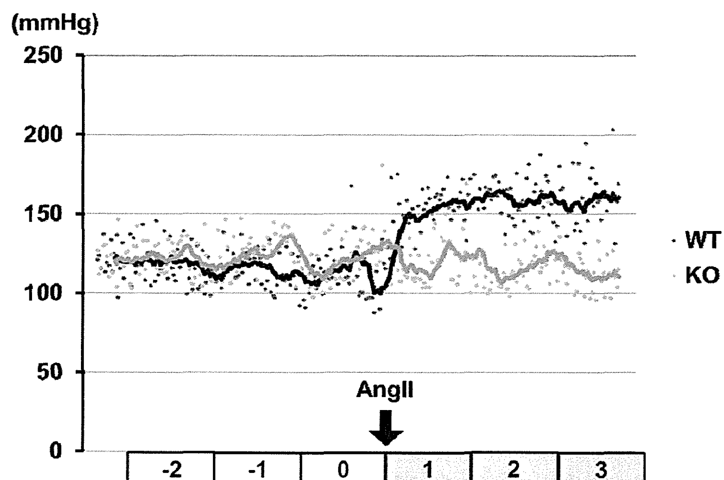

Figure S5. Radiotelemetry trace of systolic blood pressure in wild-type and WNK3 knockout mouse infused AngII

Elevation in systolic blood pressure after AngII infusion was observed in wild-type mouse, but not in WNK3 knockout mouse.

BASIC RESEARCH | www.jasn.org

Aberrant Glycosylation and Localization of Polycystin-1 Cause Polycystic Kidney in an AQP11 Knockout Model

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ABSTRACT

We previously reported that disruption of the aquaporin-11 (AQP11) gene in mice resulted in cystogenesis in the kidney. In this study, we aimed to clarify the mechanism of cystogenesis in AQP11(−/−) mice. To enable the analyses of AQP11 at the protein level *in vivo*, AQP11 BAC transgenic mice (Tg^{AQP11}) that express 3×HA-tagged AQP11 protein were generated. This AQP11 localized to the endoplasmic reticulum (ER) of proximal tubule cells in Tg^{AQP11} mice and rescued renal cystogenesis in AQP11(−/−) mice. Therefore, we hypothesized that the absence of AQP11 in the ER could result in impaired quality control and aberrant trafficking of polycystin-1 (PC-1) and polycystin-2 (PC-2). Compared with kidneys of wild-type mice, AQP11(−/−) kidneys exhibited increased protein expression levels of PC-1 and decreased protein expression levels of PC-2. Moreover, PC-1 isolated from AQP11(−/−) mice displayed an altered electrophoretic mobility caused by impaired *N*-glycosylation processing, and density gradient centrifugation of kidney homogenate and *in vivo* protein biotinylation revealed impaired membrane trafficking of PC-1 in these mice. Finally, we showed that the Pkd1(+/−) background increased the severity of cystogenesis in AQP11(−/−) mouse kidneys, indicating that PC-1 is involved in the mechanism of cystogenesis in AQP11(−/−) mice. Additionally, the primary cilia of proximal tubules were elongated in AQP11(−/−) mice. Taken together, these data show that impaired glycosylation processing and aberrant membrane trafficking of PC-1 in AQP11(−/−) mice could be a key mechanism of cystogenesis in AQP11(−/−) mice.

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Aquaporin-11 (AQP11) is a membrane-channel protein. Although AQP11 is reported to be permeable to the water molecule,[1–3] the permeability of AQP11 to other solutes remains unclear. AQP11(−/−) mice die in the neonatal period because of renal failure and retarded growth.[4,5] Moreover, AQP11(−/−) mice develop renal cysts, suggesting that AQP11 can play a role in cystogenesis.[4,5] However, the mechanisms of cystogenesis in AQP11(−/−) mice have yet to be clarified. One of the reasons for the difficulties in investigating AQP11 has been the lack of a good antibody for detecting endogenous AQP11 in mouse tissues.

Autosomal dominant polycystic kidney disease (PKD) is the most common inherited renal disorder, occurring in 1:400 to 1:1000 live births. It is characterized by gradual renal cyst development

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and expansion, ultimately resulting in massive kidney enlargement and ESRD. Among autosomal dominant PKD patients, 85%–90% of cases result from mutations in the PKD1 gene, whereas another 10%–15% of cases are accounted for by mutations in the PKD2 gene. PKD1 encodes polycystin-1 (PC-1), a 462-kD, 4303–amino acid integral membrane protein with 11 transmembrane domains, a long extracellular N terminus with multiple binding domains, and a short cytoplasmic C terminus that interacts with multiple proteins, including the protein product of PKD2, polycystin-2 (PC-2).[6] PC-2 is a significantly smaller 110-kD protein with six transmembrane domains. PC-1 and PC-2 are located in the plasma membrane and cilia of renal epithelia.[6–8]

To enable the analyses of AQP11 in mice at the protein level *in vivo*, we generated AQP11 BAC transgenic mice (Tg^{AQP11}) that express AQP11 tagged with 3×hemagglutinin (HA) sequence at its N terminus and showed that AQP11 localizes to the endoplasmic reticulum (ER) of proximal tubule cells *in vivo*. Moreover, to investigate the mechanisms of cystogenesis in AQP11(−/−) mouse kidneys, we focused on PC-1 and PC-2. Impaired glycosylation processing and membrane trafficking of PC-1 in AQP11(−/−) mouse kidneys were found, which could represent a key mechanism of cyst formation in AQP11(−/−) mice.

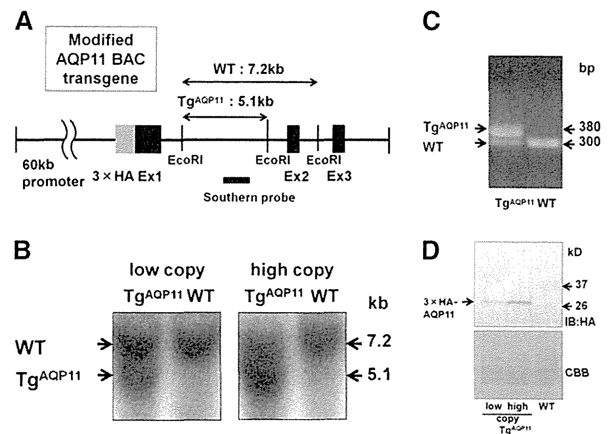

Figure 1. Generation of transgenic mice expressing 3×HA-tagged AQP11. (A) Structure of the modified AQP11 BAC transgene. 3×HA tag was fused to the N terminus of AQP11 in BAC. (B) Southern blot analysis of genomic DNA from wild-type (WT) and Tg^{AQP11} mice. (C) Genotyping analysis by PCR using genomic DNA derived from mouse tails. (D) Western blot of low- and high-copy Tg^{AQP11} mouse kidney homogenates probed with anti-HA antibody. 3×HA-AQP11 protein is indicated by arrows. Coomassie Brilliant Blue (CBB) staining was used as a loading control. IB, immunoblot.

RESULTS

Generation of 3×HA-Tagged AQP11 BAC Transgenic Mice

Transgenic mice that expressed N-terminal 3×HA-tagged AQP11 were generated. A BAC clone containing the whole exons of mouse AQP11 with its 60-kb promoter region was used. A 3×HA tag flanked by the N terminus of AQP11 was inserted in BAC (Figure 1A). As shown in Figure 1B, Southern blots were used to select founder transgenic mice, which were successfully bred to establish the low- and high-copy transgenic mouse lines. Genomic PCR was used for detection of transgene (Figure 1C). A Western blot of kidney homogenate probed with anti-HA antibody detected that the 3×HA-tagged AQP11 was expressed in the kidney, confirming that a 3×HA-tagged AQP11 transgenic mouse was generated (Figure 1D). Additionally, no significant differences in body weight were observed between wild-type mice and either low- or high-copy Tg^{AQP11} mice (Supplemental Figure 1).

3×HA-Tagged AQP11 Transgene Rescued Renal Cyst Formation and Retarded Growth in AQP11(−/−) Mice

To clarify that the 3×HA tag does not affect the function and subcellular localization of AQP11 *in vivo*, AQP11(−/−)Tg^{AQP11} mice, which express only 3×HA-tagged AQP11 protein, were generated. The 3×HA-tagged AQP11 transgene completely rescued renal cystogenesis in 3-week-old AQP11(−/−) mice (Figure 2, A and B, Supplemental Figure 2). Moreover, retarded growth in 3-week-old AQP11(−/−) mice was also rescued by the 3×HA-tagged AQP11 transgene (Figure 2C). These results showed that 3×HA-tagged AQP11 protein can function physiologically to replace native AQP11 protein *in vivo*.

Immunofluorescence of AQP11 in the Kidney

AQP11 localization in the Tg^{AQP11} mouse kidney was then examined. The immunofluorescence of Tg^{AQP11} mouse kidneys with anti-HA antibody revealed that AQP11 was present at the cortex, whereas no labeling was detected at the cortex of kidneys from wild-type littermates (Figure 3A). In the medulla, AQP11 labeling was absent in both wild-type and Tg^{AQP11} mice (Figure 3B). As shown in Figure 3C, double immunofluorescence with anti-HA and anti-AQP1 antibodies revealed that AQP11 was localized in the cytoplasm of proximal tubule cells. However, AQP11 staining was not detected in other segments of the kidney (Figure 3C), including the primary cilia. As expected from the observed proximal tubule localization of AQP11, the cysts in AQP11(−/−) mice originated mainly from the proximal tubules (Figure 3D).

AQP11 Localizes to ER *In Vivo*

To the best of our knowledge, the intracellular localization of AQP11 *in vivo* has yet to be reported. Double immunofluorescence with anti-HA antibody and organelle markers in the kidney revealed that 3×HA-tagged AQP11 was mainly colocalized with Lys-Asp-Glu-Leu (KDEL), an ER marker, and not with GM130, a Golgi marker, and Lamp2, a lysosome marker (Figure 4A). To confirm this ER localization of

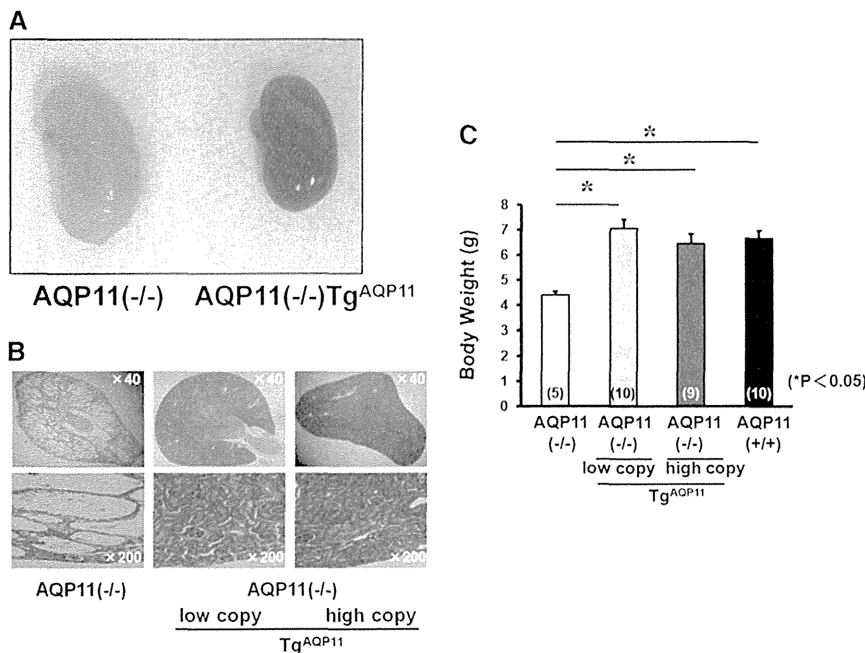

Figure 2. 3×HA-tagged AQP11 transgene rescued renal cyst formation and retarded growth in AQP11(−/−) mice. (A) Gross kidney and (B) histologic kidney sections of a 3-week-old AQP11(−/−) mouse and an AQP11(−/−)Tg^{AQP11} mouse. AQP11 transgene rescued renal cyst formation in AQP11(−/−) mice. (C) Body weight of 3-week-old AQP11(−/−), AQP11(−/−)Tg^{AQP11}, and AQP11(+/+) mice. AQP11 transgene rescued retarded growth in AQP11(−/−) mice. *$P<0.05$.

AQP11 *in vivo*, the ER fraction of the kidney was isolated from the Tg^{AQP11} mouse, and immunoblotting was performed. As shown in Figure 4B, robust expression of AQP11 was found in the ER fraction. These data clearly show ER localization of overexpressed transgenic AQP11 *in vivo*.

Loss of AQP11 Leads to Impaired Glycosylation Processing of PC-1

ER is the organelle that is involved in protein translation, translocation, protein folding, and *N*-glycosylation processing of many integral membrane proteins.[9] Because it is possible that AQP11 localizes to ER, we hypothesized that mechanisms of cystogenesis in the kidney of the AQP11(−/−) mouse were related to impaired quality control and aberrant trafficking of PC-1 and PC-2. PC-1 is a multidomain glycoprotein that is cleaved at a G protein–coupled receptor proteolytic site and divided into an N-terminal product (NTP) and a C-terminal product.[10] It has also been reported that mouse monoclonal antibody (7e12) detects endogenous PC-1 as two distinct glycoforms: endoglycosidase H-resistant (EndoH-resistant) and sensitive NTP forms.[11,12] As reported,[11,12] Western blot analyses of PC-1 with mouse monoclonal antibody (7e12) showed two NTP bands in mouse kidneys (Figure 5A). An increased protein expression level of PC-1 and a decreased protein expression level of PC-2 were also found in AQP11(−/−) mouse kidneys compared with wild-type mice (Figure 5). In addition, Western blot analyses of PC-1 detected an altered electrophoretic mobility of PC-1 in AQP11(−/−) mouse kidneys compared with wild-type mice, suggesting aberrant post-translational modification of PC-1, such as glycosylation (Figure 5A). In addition, the 3×HA-tagged AQP11 transgene rescued the altered electrophoretic mobility of PC-1 and the altered protein expression levels of PC-1 and PC-2 (Supplemental Figure 3). Therefore, to determine which type of protein modification is responsible for this altered electrophoretic mobility of PC-1, a deglycosylation assay was performed. Treatment of PC-1 with peptide-*N*-glycosidase F (PNGaseF), an enzyme that removes all N-linked sugars, reduced the size of the two products of abnormally modified PC-1 in AQP11 (−/−) mice to the same molecular mass as the deglycosylated PC-1 product of wild-type mice (Figure 6A), indicating that PC-1 was abnormally *N*-glycosylated in AQP11(−/−) mouse kidneys. This abnormal *N*-glycosylation of PC-1 in AQP11 (−/−) mouse kidneys was observed in the cortex but not the medulla (Supplemental Figure 4). In addition, we examined the effects of EndoH digestion on PC-1. EndoH resistance indicates the presence of mature complex glycans, which are typically required for protein trafficking to the cell surface. In wild-type mice, the upper PC-1 band was EndoH-resistant as previously reported (Figure 6A).[11] In contrast, in AQP11(−/−) mice, treatment with EndoH reduced the size of the abnormally glycosylated upper and lower bands, indicating that PC-1 from the AQP11(−/−) mouse kidney cortex is EndoH-sensitive (Figure 6A). These results suggested that the membrane trafficking of the abnormally glycosylated PC-1 in the AQP11(−/−) kidney was likely impaired. In contrast, the PNGaseF and EndoH assays on PC-2 and AQP1 showed no differences in molecular mass between wild-type and AQP11(−/−) mice before and after the treatment (Figure 6, B and C).

Impaired Membrane Trafficking of PC-1 in AQP11(−/−) Mouse Kidneys

Because PC-1 was found to be abnormally glycosylated in AQP11(−/−) mice, we hypothesized that membrane trafficking of PC-1 was impaired. To confirm this hypothesis, immunohistochemistry could not be used because of the lack of a good antibody. Therefore, ER and membrane compartments were prepared by density gradient centrifugation from wild-type and AQP11(−/−) kidney homogenates. Although PC-1 mainly localized at the ER, as previously

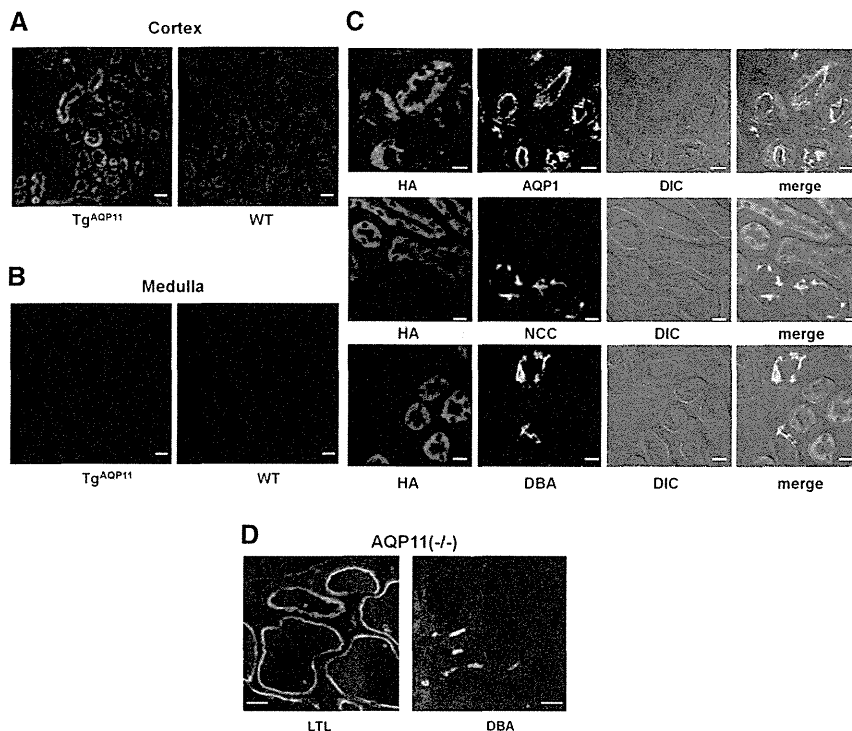

Figure 3. AQP11 protein expression in Tg^{AQP11} mouse kidney and the segmental origin of cysts in AQP11(−/−) mouse kidney. Immunofluorescence with HA antibody of 3-week-old Tg^{AQP11} and WT mouse kidneys: (A) cortex and (B) medulla. The immunostaining, image capture, and image processing were carried out under the same conditions. AQP11 was present only at the cortex. Scale bar, 20 μm. (C) Double immunofluorescence with HA antibody and AQP1 antibody (proximal tubule marker), NCC antibody (distal tubule marker), or dolichos biflorus agglutinin (DBA; collecting tubule and collecting duct marker) in the kidney of a 3-week-old Tg^{AQP11} mouse. AQP11 was localized in the cytoplasm of the proximal tubule cells. Scale bar, 10 μm. (D) Fluorescent staining with lotus tetragonolobus lectin (LTL; proximal tubule marker) and DBA in 3-week-old AQP11(−/−) mouse kidney. Segmental origin of cysts in AQP11(−/−) kidney was mainly proximal tubules. Scale bar, 50 μm.

reported,[13–15] protein expression of PC-1 in the plasma membrane fraction was clearly decreased in AQP11(−/−) mouse kidneys compared with wild-type mouse kidneys, suggesting that PC-1 trafficking to the plasma membrane was impaired (Figure 7, A and B). In addition, we used an *in vivo* protein biotinylation assay and showed that cell surface expression of PC-1 *in vivo* was clearly decreased in AQP11(−/−) kidneys compared with wild type (Figure 7C; Supplemental Figure 5). In contrast, membrane trafficking of PC-2 was still observed in AQP11(−/−) mice, although PC-2 levels in the membrane fraction were decreased (Supplemental Figure 6).

The Pkd1(+/−) Background Results in Increased Severity of PKD in the AQP11(−/−) Mouse

To confirm that loss of PC-1 function is a key mechanism involved in cystogenesis in AQP11(−/−) mice, the dosage of PC-1 was reduced using Pkd1(+/−) background in AQP11(−/−) mice. As expected, histologic examination of AQP11(−/−) mouse kidneys clearly revealed that the severity of cystic disease was markedly increased on the Pkd1(+/−) background in AQP11(−/−) littermates at postnatal day 12, indicating that loss of function of PC-1 is involved in the mechanism of cystogenesis in AQP11(−/−) mice (Figure 8A). We confirmed that the segmental origin of the cysts in Pkd1(+/−)AQP11(−/−) mice is mainly in the proximal tubules, consistent with AQP11(−/−) mice (Figure 8B). In addition, the kidney-to-body weight ratio (Figure 8C) and BUN (Figure 8D) showed significant increases in Pkd1(+/−) AQP11(−/−) mice compared with their counterparts.

Primary Cilia of Proximal Tubules Are Elongated in AQP11(−/−) Mice

Abnormal ciliary length has been reported in many renal cystic diseases.[11,16–21] Therefore, we examined the ciliary length of proximal tubules in AQP11(−/−) mice. Elongated primary cilia of proximal tubules were observed in AQP11(−/−) mice (Figure 9). In addition, 3×HA-tagged AQP11 transgene expression normalized the length of primary cilia in the AQP11(−/−) kidney, indicating that AQP11 may play a role in controlling ciliary length (Figure 9). However, the Pkd1(+/−) background did not alter the ciliary length of proximal tubules in AQP11(−/−) mice.

DISCUSSION

In this study, we generated Tg^{AQP11} mice to show that AQP11 localizes to the ER *in vivo*. Furthermore, we also confirmed that there were aberrant glycosylation processing and defective membrane trafficking of PC-1 in AQP11(−/−) mice, resulting in cyst formation in the kidney (Figure 10).

It is well known that PKD gene products localize to the primary cilia.[22] However, through the analysis of Tg^{AQP11} mice, AQP11 was found to be present in ER *in vivo* but not cilia. To date, only two nonciliary proteins other than AQP11 have been reported to be responsible for cystogenesis (glucosidase IIβ (GIIβ) and SEC63p) which are autosomal dominant polycystic liver disease–causing gene products. In ER, glycans are trimmed by glucosidases, and it has been reported that certain proteins fold improperly and fail to reach the Golgi in the absence of GII.[9] Loss of GIIβ and SEC63p led to cystogenesis in mouse liver and kidney.[22] Interestingly, both of them localize to ER as well as AQP11. GIIβ plays a role in