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 (17000*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 mmHg 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

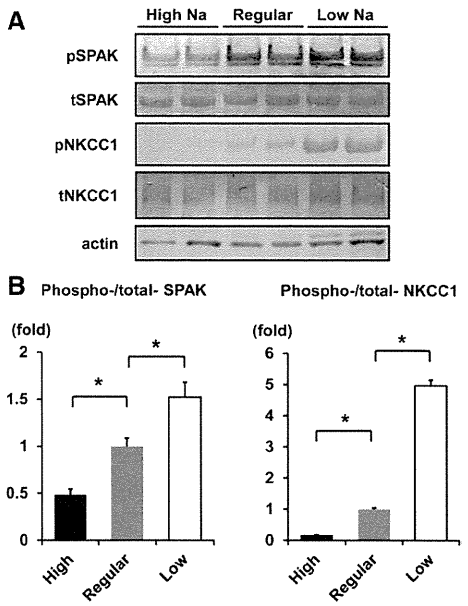

Figure 1. Dietary salt regulates phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and Na–K–Cl cotransporter isoform 1 (NKCC1) in mouse aorta. **A**, Representative immunoblots of total (t) and phosphorylated (p) SPAK and NKCC1 in aortas from mice fed high-, normal-, and low-salt 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). *$P<0.05$.

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.

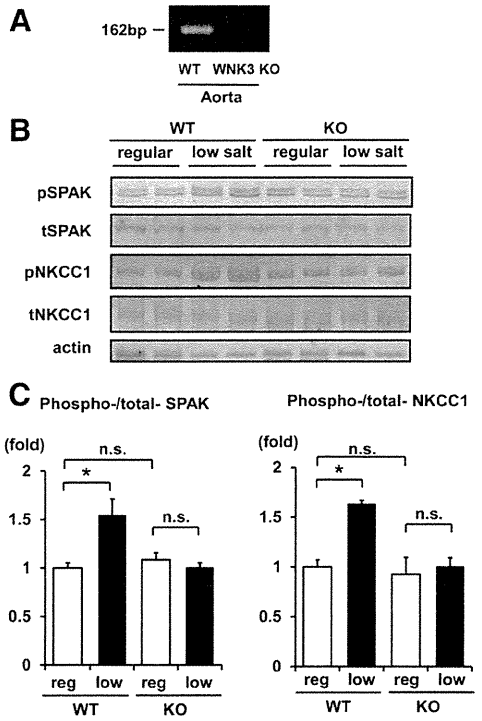

Figure 2. Phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and Na–K–Cl cotransporter isoform 1 (NKCC1) in aorta from wild-type (WT) and with-no-lysine kinase 3 (WNK3) knockout (KO) mice fed a normal diet or a low-salt diet. **A**, Expression of WNK3 in mouse aorta was confirmed 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

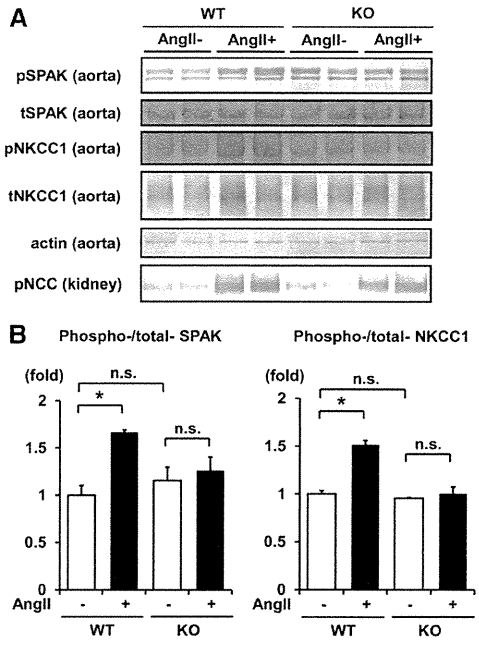

Figure 3. Chronic angiotensin II (AngII) infusion increased phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and Na–K–Cl cotransporter isoform 1 (NKCC1) in wild-type (WT) mouse aorta, but not in with-no-lysine kinase 3 (WNK3) knockout (KO) mouse. **A**, Representative immunoblots 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.

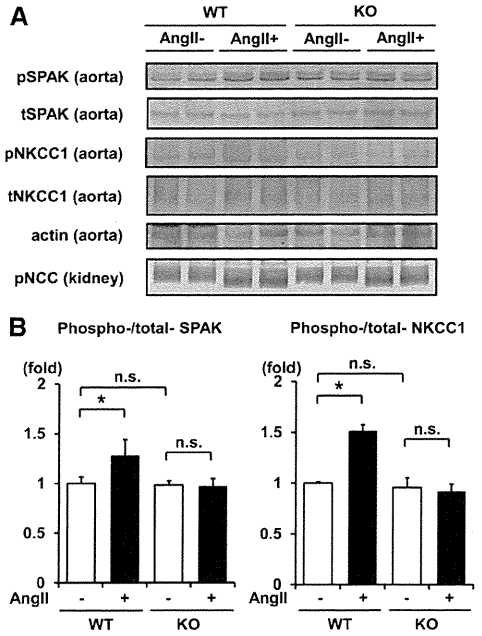

Figure 4. Acute angiotensin II (AngII) infusion increased phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and Na–K–Cl cotransporter isoform 1 (NKCC1) in wild-type (WT) mouse aorta, but not in with-no-lysine kinase 3 (WNK3) knockout (KO) mouse. **A**, Representative immunoblots 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

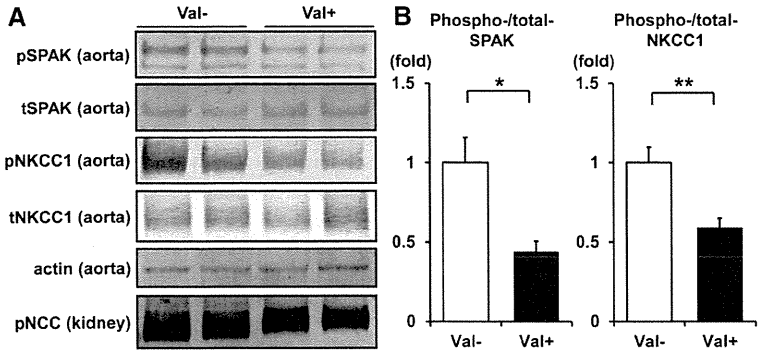

Figure 5. Valsartan (Val), angiotensin II type 1 receptor (AT1R) blocker, suppressed phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and Na–K–Cl cotransporter isoform 1 (NKCC1) in mouse aorta. **A**, Representative immunoblots of total (t) and phosphorylated (p) SPAK and NKCC1 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.

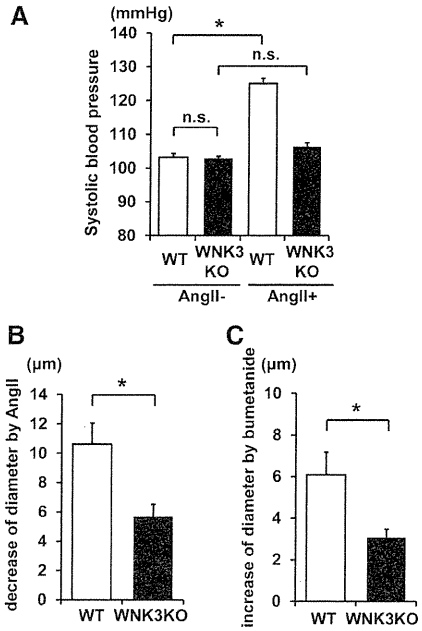

Figure 6. Impaired angiotensin II (AngII)–induced hypertension and vasoconstriction in with-no-lysine kinase 3 (WNK3) knockout (KO) mice. **A**, Blood pressure of wild-type (WT) and WNK3 KO mice with or without AngII infusion. AngII-induced increases of systolic blood pressure in WNK3 KO mice were lower 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

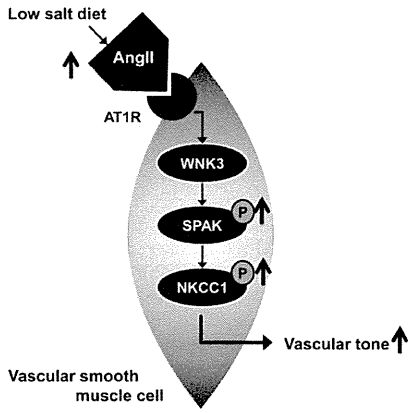

Figure 7. Proposed with-no-lysine kinase 3 (WNK3)–mediated mechanism of vasoconstriction by angiotensin II (AngII). AngII increases phosphorylation of STE20/SPS1-related proline/alanine-rich kinase (SPAK) and Na–K–Cl cotransporter isoform 1 (NKCC1) through WNK3, leading to increased vascular tone. AT1R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

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.

Acknowledgments

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

Sources of Funding

This study was supported, in part, by Grants-in-Aid for Scientific Research (S, A) from the Japan Society for the Promotion of Science; Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan; a Health and Labor Sciences Research Grant from the Ministry of Health, Labor, and Welfare; the Salt Science Research Foundation (grant no. 1228); the Takeda Science Foundation; Banyu Foundation Research Grant; and the Vehicle Racing Commemorative Foundation.

Disclosures

None.

References

1. Orlov SN, Koltsova SV, Tremblay J, Baskakov MB, Hamet P. NKCC1 and hypertension: role in the regulation of vascular smooth muscle contractions and myogenic tone. *Ann Med*. 2012;44(Suppl 1):S111–S118.
2. Meyer JW, Flagella M, Sutliff RL, Lorenz JN, Nieman ML, Weber CS, Paul RJ, Shull GE. Decreased blood pressure and vascular smooth muscle tone in mice lacking basolateral Na(+)-K(+)-2Cl(-) cotransporter. *Am J Physiol Heart Circ Physiol*. 2002;283:H1846–H1855.
3. Gordon RD. Syndrome of hypertension and hyperkalemia with normal glomerular filtration rate. *Hypertension*. 1986;8:93–102.
4. Wilson FH, Disse-Nicodème S, Choate KA, et al. Human hypertension caused by mutations in WNK kinases. *Science*. 2001;293:1107–1112.
5. Yang SS, Morimoto T, Rai T, Chiga M, Sohara E, Ohno M, Uchida K, Lin SH, Moriguchi T, Shibuya H, Kondo Y, Sasaki S, Uchida S. Molecular pathogenesis of pseudohypoaldosteronism type II: generation and analysis of a Wnk4(D561A/+) knockin mouse model. *Cell Metab*. 2007;5:331–344.
6. Ohta A, Rai T, Yui N, Chiga M, Yang SS, Lin SH, Sohara E, Sasaki S, Uchida S. Targeted disruption of the Wnk4 gene decreases phosphorylation of Na-Cl cotransporter, increases Na excretion and lowers blood pressure. *Hum Mol Genet*. 2009;18:3978–3986.
7. Chiga M, Rafiqi FH, Alessi DR, Sohara E, Ohta A, Rai T, Sasaki S, Uchida S. Phenotypes of pseudohypoaldosteronism type II caused by the WNK4 D561A missense mutation are dependent on the WNK-OSR1/SPAK kinase cascade. *J Cell Sci*. 2011;124(Pt 9):1391–1395.
8. Bergaya S, Faure S, Baudrie V, Rio M, Escoubet B, Bonnin P, Henrion D, Loirand G, Achard JM, Jeunemaitre X, Hadchouel J. WNK1 regulates vasoconstriction and blood pressure response to a 1-adrenergic stimulation in mice. *Hypertension*. 2011;58:439–445.
9. Castañeda-Bueno M, Cervantes-Pérez LG, Vázquez N, Uribe N, Kantesaria S, Morla L, Bobadilla NA, Doucet A, Alessi DR, Gamba G. Activation of the renal Na+:Cl- cotransporter by angiotensin II is a WNK4-dependent process. *Proc Natl Acad Sci USA*. 2012;109:7929–7934.
10. Rafiqi FH, Zuber AM, Glover M, Richardson C, Fleming S, Jovanovic S, Jovanovic A, O'Shaughnessy KM, Alessi DR. Role of the WNK-activated SPAK kinase in regulating blood pressure. *EMBO Mol Med*. 2010;2:63–75.
11. Susa K, Kita S, Iwamoto T, Yang SS, Lin SH, Ohta A, Sohara E, Rai T, Sasaki S, Alessi DR, Uchida S. Effect of heterozygous deletion of WNK1 on the WNK-OSR1/ SPAK-NCC/NKCC1/NKCC2 signal cascade in the kidney and blood vessels. *Clin Exp Nephrol*. 2012;16:530–538.
12. Yang SS, Lo YF, Wu CC, Lin SW, Yeh CJ, Chu P, Sytwu HK, Uchida S, Sasaki S, Lin SH. SPAK-knockout mice manifest Gitelman syndrome and impaired vasoconstriction. *J Am Soc Nephrol*. 2010;21:1868–1877.
13. Oi K, Sohara E, Rai T, Misawa M, Chiga M, Alessi DR, Sasaki S, Uchida S. A minor role of WNK3 in regulating phosphorylation of renal NKCC2 and NCC co-transporters in vivo. *Biol Open*. 2012;1:120–127.
14. Mederle K, Mutig K, Paliege A, Carota I, Bachmann S, Castrop H, Oppermann M. Loss of WNK3 is compensated for by the WNK1/SPAK axis in the kidney of the mouse. *Am J Physiol Renal Physiol*. 2013;304:F1198–F1209.
15. Talati G, Ohta A, Rai T, Sohara E, Naito S, Vandewalle A, Sasaki S, Uchida S. Effect of angiotensin II on the WNK-OSR1/SPAK-NCC phosphorylation cascade in cultured mpkDCT cells and in vivo mouse kidney. *Biochem Biophys Res Commun*. 2010;393:844–848.
16. Chiga M, Rai T, Yang SS, Ohta A, Takizawa T, Sasaki S, Uchida S. Dietary salt regulates the phosphorylation of OSR1/SPAK kinases and the sodium chloride cotransporter through aldosterone. *Kidney Int*. 2008;74:1403–1409.
17. Nishida H, Sohara E, Nomura N, Chiga M, Alessi DR, Rai T, Sasaki S, Uchida S. Phosphatidylinositol 3-kinase/Akt signaling pathway activates the WNK-OSR1/SPAK-NCC phosphorylation cascade in hyperinsulinemic db/db mice. *Hypertension*. 2012;60:981–990.

18. Sohara E, Rai T, Yang SS, Ohta A, Naito S, Chiga M, Nomura N, Lin SH, Vandewalle A, Ohta E, Sasaki S, Uchida S. Acute insulin stimulation induces phosphorylation of the Na-Cl cotransporter in cultured distal mpkDCT cells and mouse kidney. *PLoS ONE*. 2011;6:e24277.
19. Lytle C, Xu JC, Biemesderfer D, Forbush B 3rd. Distribution and diversity of Na-K-Cl cotransport proteins: a study with monoclonal antibodies. *Am J Physiol*. 1995;269(6 Pt 1):C1496–C1505.
20. Iwamoto T, Kita S, Zhang J, Blaustein MP, Arai Y, Yoshida S, Wakimoto K, Komuro I, Katsuragi T. Salt-sensitive hypertension is triggered by Ca2+ entry via Na+/Ca2+ exchanger type-1 in vascular smooth muscle. *Nat Med*. 2004;10:1193–1199.
21. Zhang J, Lee MY, Cavalli M, Chen L, Berra-Romani R, Balke CW, Bianchi G, Ferrari P, Hamlyn JM, Iwamoto T, Lingrel JB, Matteson DR, Wier WG, Blaustein MP. Sodium pump alpha2 subunits control myogenic tone and blood pressure in mice. *J Physiol (Lond)*. 2005;569(Pt 1):243–256.
22. Koltsova SV, Kotelevtsev SV, Tremblay J, Hamet P, Orlov SN. Excitation-contraction coupling in resistance mesenteric arteries: evidence for NKCC1-mediated pathway. *Biochem Biophys Res Commun*. 2009;379:1080–1083.
23. Subramanya AR, Yang CL, McCormick JA, Ellison DH. WNK kinases regulate sodium chloride and potassium transport by the aldosterone-sensitive distal nephron. *Kidney Int*. 2006;70:630–634.
24. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. *Am J Physiol, Cell Physiol*. 2007;292:C82–C97.
25. Inagami T, Eguchi S. Angiotensin II-mediated vascular smooth muscle cell growth signaling. *Braz J Med Biol Res*. 2000;33:619–624.
26. Anfinogenova YJ, Baskakov MB, Kovalev IV, Kilin AA, Dulin NO, Orlov SN. Cell-volume-dependent vascular smooth muscle contraction: role of Na+, K+, 2Cl- cotransport, intracellular Cl- and L-type Ca2+ channels. *Pflugers Arch*. 2004;449:42–55.
27. Wang X, Breaks J, Loutzenhiser K, Loutzenhiser R. Effects of inhibition of the Na+/K+/2Cl- cotransporter on myogenic and angiotensin II responses of the rat afferent arteriole. *Am J Physiol Renal Physiol*. 2007;292:F999–F1006.
28. Akar F, Jiang G, Paul RJ, O'Neill WC. Contractile regulation of the Na(+)-K(+)-2Cl(-) cotransporter in vascular smooth muscle. *Am J Physiol, Cell Physiol*. 2001;281:C579–C584.
29. Moriguchi T, Urushiyama S, Hisamoto N, Iemura S, Uchida S, Natsume T, Matsumoto K, Shibuya H. WNK1 regulates phosphorylation of cation-chloride-coupled cotransporters via the STE20-related kinases, SPAK and OSR1. *J Biol Chem*. 2005;280:42685–42693.
30. Vitari AC, Deak M, Morrice NA, Alessi DR. The WNK1 and WNK4 protein kinases that are mutated in Gordon's hypertension syndrome phosphorylate and activate SPAK and OSR1 protein kinases. *Biochem J*. 2005;391(Pt 1):17–24.
31. Rinehart J, Kahle KT, de Los Heros P, Vazquez N, Meade P, Wilson FH, Hebert SC, Gimenez I, Gamba G, Lifton RP. WNK3 kinase is a positive regulator of NKCC2 and NCC, renal cation-Cl- cotransporters required for normal blood pressure homeostasis. *Proc Natl Acad Sci USA*. 2005;102:16777–16782.
32. Ponce-Coria J, San-Cristobal P, Kahle KT, Vazquez N, Pacheco-Alvarez D, de Los Heros P, Juárez P, Muñoz E, Michel G, Bobadilla NA, Gimenez I, Lifton RP, Hebert SC, Gamba G. Regulation of NKCC2 by a chloride-sensing mechanism involving the WNK3 and SPAK kinases. *Proc Natl Acad Sci USA*. 2008;105:8458–8463.
33. Kahle KT, Rinehart J, de Los Heros P, Louvi A, Meade P, Vazquez N, Hebert SC, Gamba G, Gimenez I, Lifton RP. WNK3 modulates transport of Cl- in and out of cells: implications for control of cell volume and neuronal excitability. *Proc Natl Acad Sci USA*. 2005;102:16783–16788.
34. Boyden LM, Choi M, Choate KA, et al. Mutations in kelch-like 3 and cullin 3 cause hypertension and electrolyte abnormalities. *Nature*. 2012;482:98–102.
35. Louis-Dit-Picard H, Barc J, Trujillano D, et al; International Consortium for Blood Pressure (ICBP). KLHL3 mutations cause familial hyperkalemic hypertension by impairing ion transport in the distal nephron. *Nat Genet*. 2012;44:456–60, S1.
36. Ohta A, Schumacher FR, Mehellou Y, Johnson C, Knebel A, Macartney TJ, Wood NT, Alessi DR, Kurz T. The CUL3-KLHL3 E3 ligase complex mutated in Gordon's hypertension syndrome interacts with and ubiquitylates WNK isoforms: disease-causing mutations in KLHL3 and WNK4 disrupt interaction. *Biochem J*. 2013;451:111–122.
37. Wakabayashi M, Mori T, Isobe K, et al. Impaired KLHL3-mediated ubiquitination of WNK4 causes human hypertension. *Cell Rep*. 2013;3:858–868.
38. Rozansky DJ, Cornwall T, Subramanya AR, Rogers S, Yang YF, David LL, Zhu X, Yang CL, Ellison DH. Aldosterone mediates activation of the thiazide-sensitive Na-Cl cotransporter through an SGK1 and WNK4 signaling pathway. *J Clin Invest*. 2009;119:2601–2612.
39. Lin SH, Yu IS, Jiang ST, Lin SW, Chu P, Chen A, Sytwu HK, Sohara E, Uchida S, Sasaki S, Yang SS. Impaired phosphorylation of Na(+)-K(+)-2Cl(-) cotransporter by oxidative stress-responsive kinase-1 deficiency manifests hypotension and Bartter-like syndrome. *Proc Natl Acad Sci USA*. 2011;108:17538–17543.
40. Grimm PR, Taneja TK, Liu J, Coleman R, Chen YY, Delpire E, Wade JB, Welling PA. SPAK isoforms and OSR1 regulate sodium-chloride co-transporters in a nephron-specific manner. *J Biol Chem*. 2012;287:37673–37690.
41. McCormick JA, Mutig K, Nelson JH, Saritas T, Hoorn EJ, Yang CL, Rogers S, Curry J, Delpire E, Bachmann S, Ellison DH. A SPAK isoform switch modulates renal salt transport and blood pressure. *Cell Metab*. 2011;14:352–364.

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.

ONLINE SUPPLEMENT

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

Moko Zeniya[1], Eisei Sohara[1], Satomi Kita[2], Takahiro Iwamoto[2], Koichiro Susa[1], Takayasu Mori[1], Katsuyuki Oi[1], Motoko Chiga[1], Daiei Takahashi[1], Sung-Sen Yang[3], Shih-Hua Lin[3], Tatemitsu Rai[1], Sei Sasaki[1] and Shinichi Uchida[1]

[1]Department of Nephrology, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University, Tokyo, Japan
[2]Department of Pharmacology, Faculty of Medicine, Fukuoka University, Fukuoka, Japan
[3]Division of Nephrology, Department of Medicine, Tri- Service General Hospital, National Defense Medical Center, Taipei, Taiwan

Correspondence to: Eisei Sohara, M.D., Ph.D.
1-5-45 Yushima, Bunkyo, Tokyo 113-8519, Japan
Phone: +81-3-5803-5214; Fax: +81-3-5803-5215
E-mail: esohara.kid@tmd.ac.jp

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.

References

1. Oi K, Sohara E, Rai T, et al. A minor role of WNK3 in regulating phosphorylation of renal NKCC2 and NCC co-transporters in vivo. *Biol Open.* Feb 2012;1(2):120-127.

Figure S1, Zeniya M, et al.

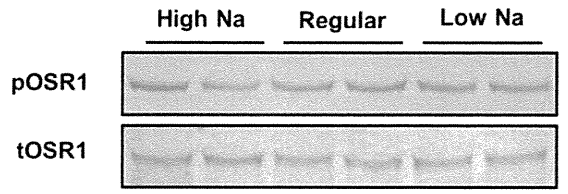

Figure S1. Phosphorylation of OSR1 was not regulated by dietary salt intake
(A) Immunoblots of total- and phosphorylated- OSR1 in aortas from mice fed high-, normal- and low-salt diets. Phosphorylation levels of OSR1 were not altered by dietary salt.

Figure S2, Zeniya M, et al.

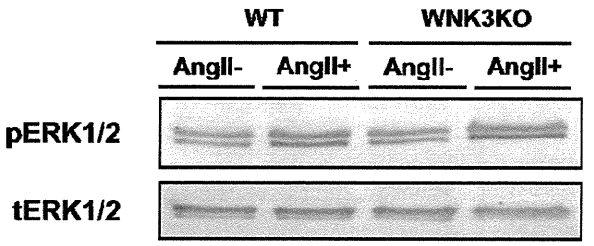

Figure S2. Phosphorylation of ERK1/2 in mouse aorta after AngII treatment. Phosphorylation of ERK1/2 increased in both of wild-type and WNK3 knockout mice aorta by AngII infusion, confirming that WNK3 knockout mice do not lack AngII response.

Figure S3, Zeniya M, et al.

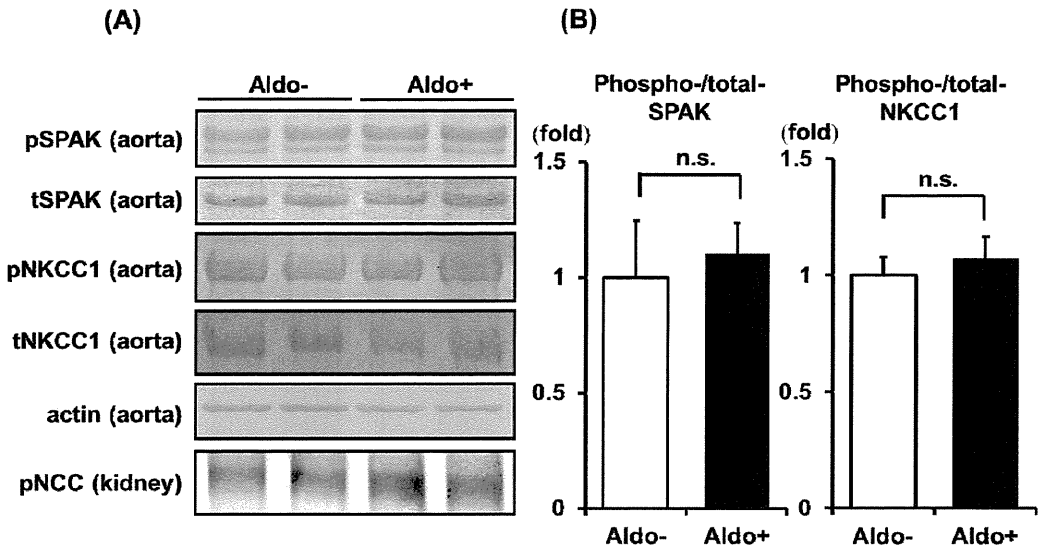

Figure S3. Aldosterone did not increase phosphorylation of SPAK and NKCC1 in mouse aorta

(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.

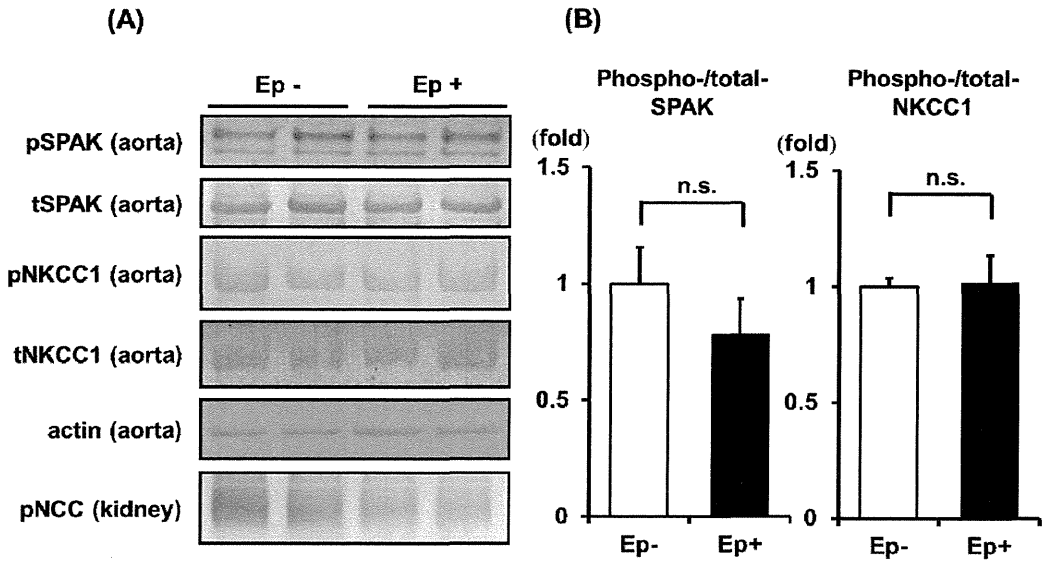

Figure S4. Eplerenone did not decrease phosphorylation of SPAK and NKCC1 in aorta

(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.

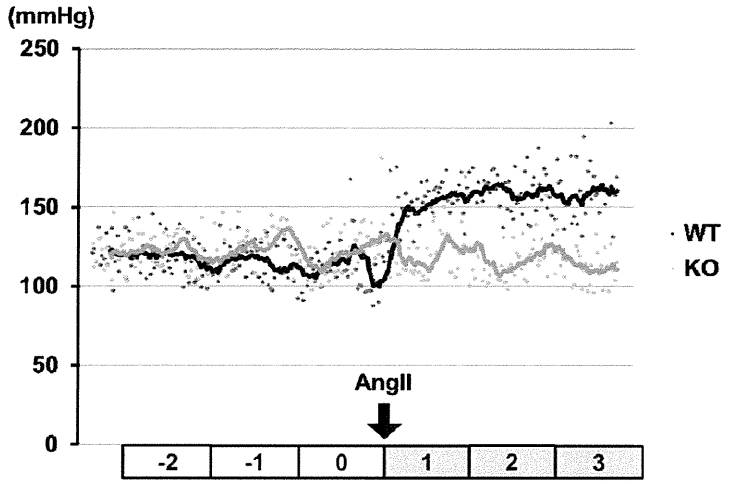

Figure S5. Radiotelemetry trace of systolic blood pressure in wild-type and WNK3 knockout mouse infused AngII

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