the kidney (Holden et al., 2004). Therefore, although WNK3 mutation has not been observed in PHAII, WNK3 could be an important component of WNK kinase-mediated signal cascade in kidney. Previous in vitro data found that WNK3 regulates SLC12A cotransporters. WNK3 was shown to be an activator of Na-K-Cl cotransporter (NKCC1 and 2) and NCC (Kahle et al., 2005; Rinehart et al., 2005; Yang, C. L. et al., 2007; San-Cristobal et al., 2008; Ponce-Coria et al., 2008; Glover et al., 2009; Cruz-Rangel et al., 2011), and a repressor of K-Cl cotransporters (KCC 1-4) (Kahle et al., 2005; de Los Heros et al., 2006), when co-expressed in *Xenopus laevis* oocytes. Similar to WNK1 and WNK4, WNK3 was found to phosphorylate SPAK in *Xenopus laevis* oocytes (Ponce-Coria et al., 2008).

Previously, WNK4 hypomorphic mice and WNK1 heterozygous mice reportedly showed low blood pressure (Ohta et al., 2009; Zambrowicz et al., 2003). Therefore, we aimed to determine the contribution of WNK3 to WNK-mediated kidney functions by generating WNK3 knockout mice. The data obtained suggest that WNK3 may not play a major role in the WNK kinase cascade in the kidney.

Results

Generation of WNK3 knockout mice

In order to generate WNK3 knockout mice, we planned to delete exon 2 (Fig. 1A), as exon 2 contains the catalytic domain of mouse WNK3 (Holden et al., 2004; Verissimo et al., 2006). We crossed chimeric mice from recombinant ES clones with C57BL/6 mice to produce WNK3 (flox/+) mice. The generation of WNK3 (flox/+) mice was verified by PCR (Fig. 1B). Next, to delete exon 2 from the *Wnk3* gene, we crossed WNK3 (flox/+) female mice with Cre recombinase transgenic male mice. The Cre-mediated excision of exon 2 and Neo cassette was verified by PCR, as shown in Fig. 1C. The absence of WNK3 protein was confirmed by immunoblotting in brain and testis (Fig. 1D). However, due to the low level of WNK3 protein expression in the kidney, WNK3 was not detected by immunoblotting, even in wild-type mouse kidney. To verify that WNK3 is also disrupted in the kidney, we performed RT-PCR of WNK3 and confirmed the absence of WNK3 mRNA in the kidneys of WNK3 knockout mice (Fig. 1E).

Segmental expression of WNK3 along mouse nephron

First, we aimed to determine where WNK3 is expressed along the mouse nephron, as we had to identify the transporters present in the segment where WNK3 is expressed. It was previously reported that, on immunofluorescence, WNK3 is present in all nephron segments (Rinehart et al., 2005). However, because the same antibody did not work both in immunofluorescence and immunoblotting in our hand, we performed laser capture microdissection (LCM) and RT-PCR in order to confirm segmental expression of WNK3 along nephron. As shown in Fig. 2, we confirmed that WNK3 is expressed in proximal tubules, thick ascending limb of Henle's loop (TAL), distal convoluted tubules (DCT) and collecting ducts, where Na/H exchanger 3 (NHE3), NKCC2, NCC and epithelial Na channel (ENaC) are expressed, respectively.

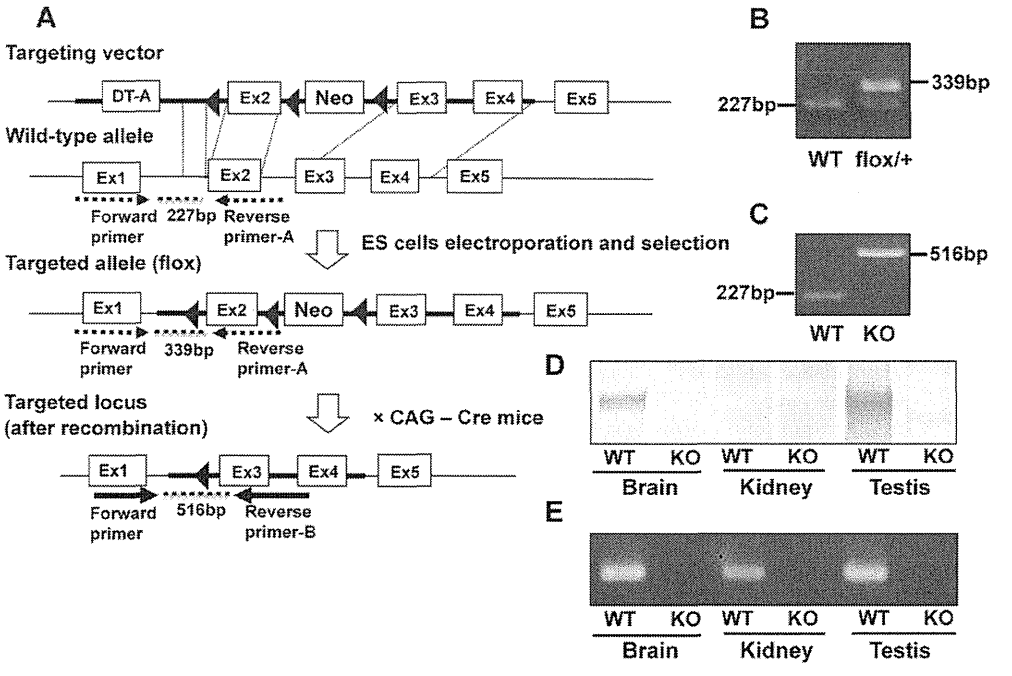

Fig. 1. Generation of WNK3 knockout mice. (A) Targeting strategy for *Wnk3* gene interruption. The diagram shows the targeting construct, the wild-type WNK3 locus, and the targeted locus before and after Cre recombination. Three loxPs were inserted to flank exon 2 and the LacZ-Neo-selective marker. Exon 2 was deleted by mating the flox mice with CAG promoter Cre recombinase mice. **(B)** Verification of homologous recombination by PCR of genomic DNA derived from tails of mice. The primer set is indicated by dotted arrows in A. The 227-bp band and 339-bp band represent the wild-type allele and flox allele, respectively. **(C)** Genotyping PCR after Cre recombination, using a primer set indicated by solid arrows in A. A 516-bp PCR product was specific to the mutant allele. **(D)** Immunoblot of brain, kidney and testis homogenates probed with anti-WNK3 antibody. Absence of WNK3 protein in WNK3 knockout mouse was confirmed by immunoblotting in brain and testis. WNK3 was not detected by immunoblotting, even in wild-type mouse kidney, due to the low level of WNK3 protein expression in the kidney. **(E)** RT-PCR of brain, kidney and testis of wild-type and WNK3 knockout mice.

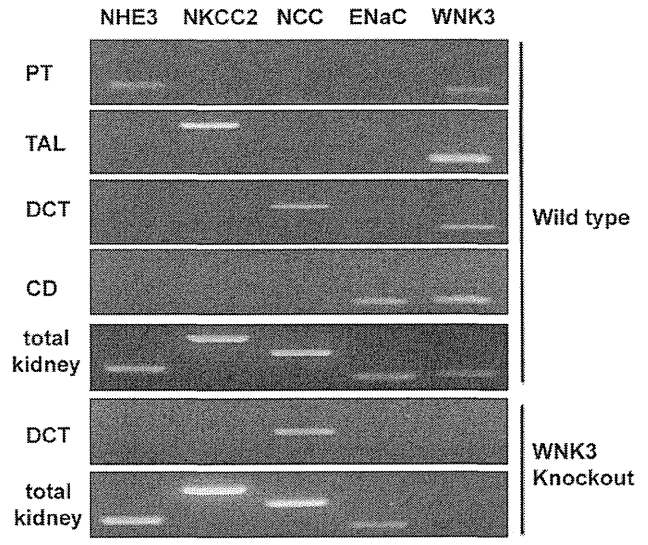

Fig. 2. Segmental expression of WNK3 along mouse nephron. Primers for NHE3, NKCC2, NCC and ENaC were used as markers for each segment. WNK3 was positive in proximal tubules, thick ascending limb of Henle's loop, distal tubules and collecting ducts of wild type mouse. As a negative control, distal tubules and total kidney homogenate from WNK3 knockout mouse were used. PT, proximal tubule; TAL, thick ascending limb of Henle's loop; DCT, distal convoluted tubule; CD, collecting duct.

Blood and urine analysis

We observed no obvious differences between the WNK3 knockout mice and wild-type littermates in survival, gross physical appearance and organ morphology. There were no significant differences in the plasma K^+ and HCO_3^- levels (Table 1). Urine volume and urinary excretion of Na^+ and K^+ under normal conditions were not significantly affected in WNK3 knockout mice. To more thoroughly characterize the phenotypes, we fed WNK3 knockout and wild-type mice low-salt (0.01% NaCl) and high-salt (4% NaCl) diets, and monitored urinary Na^+ and K^+ excretion, particularly focusing on the transition periods while changing diets. However, as shown in Fig. 3A–C, sodium excretion in the WNK3 knockout mice did not change during the experimental period.

Blood pressure

We used a tail-cuff system to measure blood pressure. As shown in Fig. 4A, blood pressure in WNK3 knockout mice did not show any significant differences when compared with wild-type mice under normal diet (109.7±1.6 vs. 111.2±1.6 mmHg, knockout: n=11, wild-type: n=14). However, when mice were fed with low-salt diet, blood pressure in WNK3 knockout mice was lower compared with wild-type mice (105.8±1.1 vs. 110.7±0.7 mmHg, knockout: n=11, wild-type: n=9, P<0.01) (Fig. 4B).

Table 1. Biochemical analysis of blood from wild-type and WNK3 knockout mice

	Wild type (n=9)	WNK3 knockout (n=10)	P Value
Na^+ (mmol/l)	145.8±1.7	144.9±2.0	0.34
K^+ (mmol/l)	4.7±0.4	4.9±0.3	0.17
Cl^- (mmol/l)	113.1±1.1	111.9±1.4	0.07
HCO_3^- (mmol/l)	23.6±1.4	23.8±1.1	0.78
pH (venous)	7.36±0.02	7.35±0.03	0.41

Values in mean ± SD.

Expression and phosphorylation of NCC and NKCC2 were not affected in WNK3 knockout mouse kidney

WNK3 reportedly activates NCC and NKCC2 function when co-expressed in *Xenopus* oocytes. Therefore, we investigated whether expression and phosphorylation of NCC and NKCC2 were decreased in the kidneys of WNK3 knockout mice. However, as shown in Fig. 5A–B, we could not see any significant difference in the protein abundance or the magnitude of phosphorylation of NKCC2 and NCC between WNK3 knockout and wild-type mice under a normal diet. Next, to investigate whether phosphorylation of OSR1 and SPAK was lower due to an absence of WNK3, we examined phosphorylation of OSR1 at 325S and SPAK at 380S, phosphorylation sites for WNK kinases, using phospho-specific antibodies. As shown in Fig. 5C–D, phosphorylation of OSR1 and SPAK at their WNK phosphorylation sites was not lower in kidneys from WNK3 knockout mice.

Furthermore, we examined the phosphorylation status of NCC and NKCC2 in WNK3 knockout mice under low-salt diet, since blood pressure in WNK3 knockout mice was lower, when mice were fed with low-salt diet. However, even under low-salt diet, WNK3 knockout mice did not show decreased phosphorylation of either NCC or NKCC2, when compared with wild-type mice (Fig. 6A–B). These results indicated that WNK3 does not play a major role in regulation of NCC and NKCC2 in vivo mouse kidney, in contrast to several over-expression studies in *Xenopus* oocytes. Phosphorylation of OSR1 and SPAK at their WNK phosphorylation sites was not lower in kidneys from WNK3 knockout mice, even under low-salt diet (Fig. 6C–D).

Expression of NHE3 and γ-ENaC in the kidneys of WNK3 knockout mice was not significantly different from that in wild-type mice (supplementary material Fig. S1).

Expression levels of WNK4 were elevated in kidneys of WNK3 knockout mice

The WNK kinase family phosphorylates and activates OSR1 and SPAK, and activated OSR1 and SPAK kinases phosphorylate NCC. We hypothesized that other WNKs could compensate for the absence of WNK3 in the kidney. To examine this hypothesis, we examine the expression levels of WNK1 and WNK4 in the kidneys of WNK3 knockout mice. As shown in Fig. 7A–B, we found that WNK4 expression was slightly but significantly elevated in kidneys from WNK3 knockout mice, as compared to those from wild-type mice. We also examined WNK1 and WNK4 expression in WNK3 knockout mice fed low-salt diet, as compensation would become clearer when the WNK-OSR1/SPAK-NCC phosphorylation cascade is activated (Chiga et al., 2008). As expected, under low-salt diet, both WNK1 and WNK4 expression increased significantly in kidneys from WNK3 knockout mice, as compared to wild-type mice (Fig. 7C–D). These results indicate that increased expression of WNK1 and WNK4 compensate for the absence of WNK3 in kidneys from WNK3 knockout mice.

Discussion

One of the major issues in hypertension research is regulation of renal sodium transporters that control sodium reabsorption in the

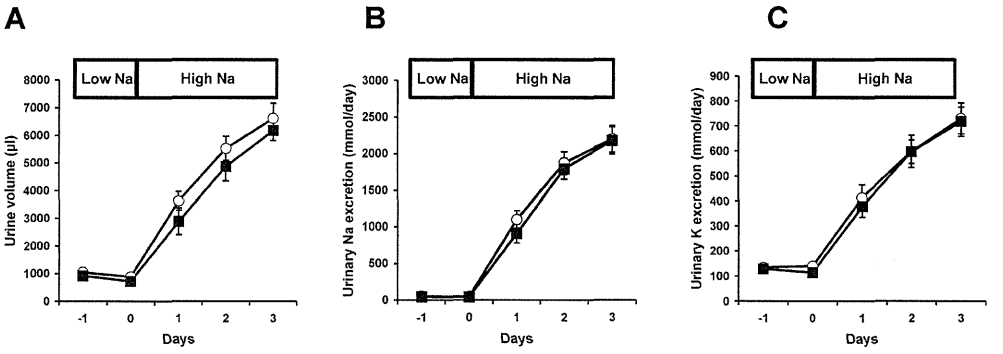

Fig. 3. Urinary excretion of Na^+ and K^+ in WNK3 knockout mice. There were no significant differences in urine volume **(A)**, urinary Na^+ excretion **(B)** or K^+ excretion **(C)** between WNK3 knockout mice (squares, $n=8$) and their wild-type littermates (circles, $n=8$). On day 0, mice were switched from low-salt diet (0.01% NaCl) to high-salt diet (4.0% NaCl). Before switching diet, mice were fed low-salt diet for 1 week. n.s. not significant.

kidney. NKCC2 and NCC are kidney-specific members of the SLC12A family of electroneutral cation-chloride co-transporters (Haas and Forbush, 1998; Gamba, 2005). They have been shown to play an important role in regulation of blood pressure and sodium reabsorption in the kidney.

Through analyses using genetically engineered mice, we have established the existence of the WNK-OSR1/SPAK-NCC kinase cascade in the in vivo kidney (Yang, S. S. et al., 2007; Chiga et al., 2008; Chiga et al., 2011; Ohta et al., 2009). To date, several physiological regulators of WNK-OSR1/SPAK-NCC phosphorylation cascade have been reported. We have reported that the WNK-OSR1/SPAK-NCC cascade is activated by low-salt diet and inhibited by high-salt diet, mainly via the action of aldosterone (Chiga et al., 2008). Angiotensin II has also been shown to be a regulator of their phosphorylation (San-Cristobal et al., 2009; Talati et al., 2010; van der Lubbe et al., 2011). Thus, WNK-OSR1/SPAK-NCC is a regulator of NaCl homeostasis in the kidney through the renin-angiotensin-aldosterone system. Another vasoactive hormone, vasopressin, was shown to regulate this cascade (Pedersen et al., 2010). We recently demonstrated that insulin is another potent regulator of the WNK-OSR1/SPAK-NCC phosphorylation cascade in the kidney (Sohara et al., 2011). This discovery is important when considering mechanisms of salt-sensitive hypertension in hyperinsulinemic patients, such as those with metabolic syndrome. Moreover, Vallon et al. and Naito et al. reported that potassium intake and extracellular potassium ions, respectively, regulate this cascade (Vallon et al., 2009; Naito et al., 2010). Taken together, these reports indicate that the WNK-OSR1/SPAK-NCC phosphorylation cascade is very important in kidney, not only for the pathogenesis of PHAII, but also for the homeostatic regulation of sodium, potassium, and blood pressure under various pathophysiological conditions.

Similar to NCC, WNKs, OSR1 and SPAK reportedly regulate NKCC2. OSR1 and SPAK kinases interact with an RFQV motif on NKCC2 and directly phosphorylate NKCC2 in in vitro kinase assays (Moriguchi et al., 2005; Richardson et al., 2008; Richardson et al., 2011). In addition to OSR1 and SPAK, reduced expression of WNK1 by siRNA inhibits endogenous NKCC1 activity in HeLa cells, measured by ^{86}Rb influx assays (Anselmo et al., 2006). WNK3 is another WNK kinase that is reported to regulate NKCC2 as well as NCC. Rinehart et al. reported that WNK3 increases NKCC2 phosphorylation at Thr-184 and Thr-189 residues, which had been identified to be necessary for vasopressin-mediated plasma membrane translocation and activation of NKCC2 in *Xenopus laevis* oocytes (Rinehart et al., 2005). In addition, overexpression of WNK3 in *Xenopus laevis* oocytes leads to the activation of NKCC2, which is dependent upon the interaction of SPAK and OSR1 with one of the three RFx[V/I]-motifs that are present in WNK3 (Ponce-Coria et al., 2008; Richardson and Alessi, 2008). Several other groups have reported that co-expression of NCC with WNK3 in *Xenopus laevis* oocytes increased its transport activity (Rinehart et al., 2005; Yang, C. L. et al., 2007; San-Cristobal et al., 2008; Glover et al., 2009; Cruz-Rangel et al., 2011). Therefore, in the kidney, it was expected that WNK3 could regulate sodium reabsorption along the nephron by activating NCC and NKCC2 via phosphorylation of OSR1 and SPAK.

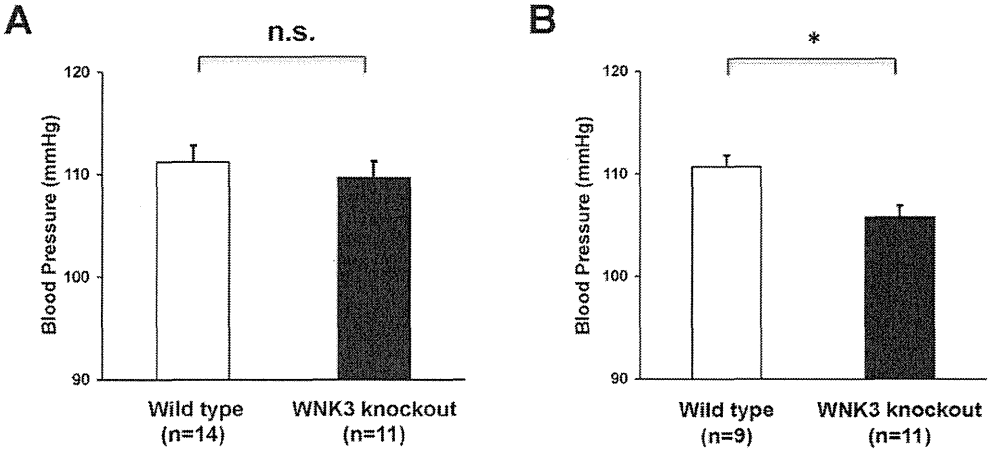

Fig. 4. Lower blood pressure in WNK3 knockout mice fed low-salt diet. (A) Blood pressure in WNK3 knockout mice under normal diet. Blood pressure was measured using a tail-cuff system. WNK3 knockout mice ($n=9$) did not show significantly decreased systolic blood pressure, as compared to their wild-type littermates ($n=14$). **(B)** Blood pressure in WNK3 knockout mice ($n=11$) under low-salt diet. WNK3 knockout mice showed lower blood pressure, as compared to their wild-type littermates ($n=9$). *$P<0.05$. n.s. not significant.

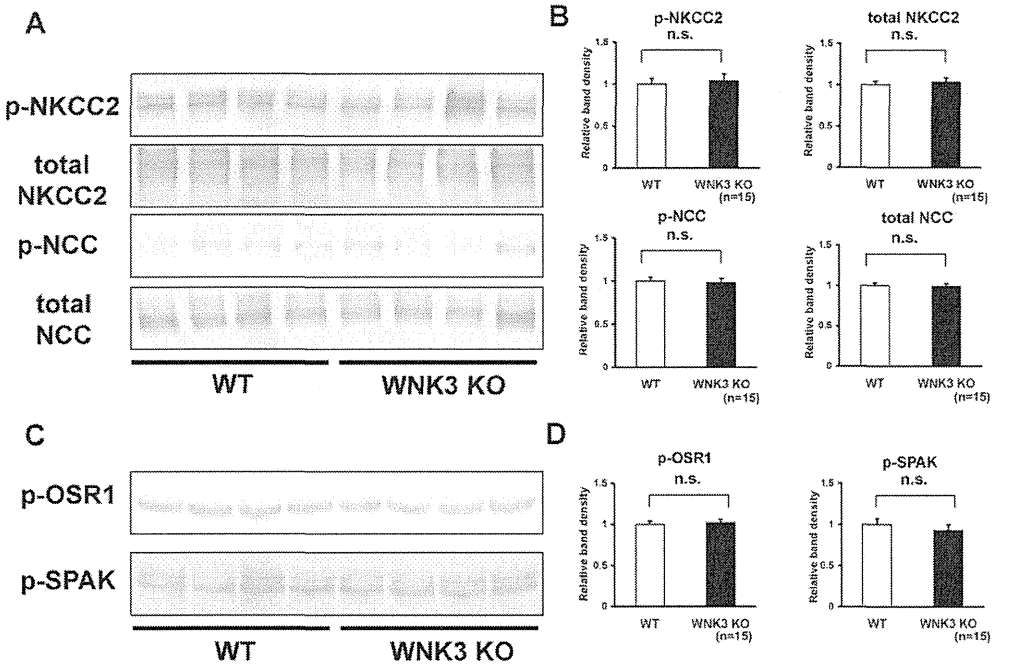

Fig. 5. Expression and phosphorylation of NKCC2 and NCC in kidneys from WNK3 knockout mice under normal diet. (A) Representative immunoblots of total- and phosphorylated- NKCC2 and NCC in kidneys from wild-type and WNK3 knockout mice. **(B)** Densitometry analyses of expression and phosphorylation of NKCC2 and NCC in kidney from wild-type and WNK3 knockout mice. For densitometry analysis, values ($n=15$) are expressed as ratios against the average of signals in the wild-type group. There were no significant decreases in the expression and phosphorylation of NKCC2 and NCC in kidneys from WNK3 knockout mice, as compared to wild-type littermates. **(C)** Representative immunoblots of p-OSR1 and p-SPAK in kidneys from wild-type and WNK3 knockout mice. **(D)** Densitometry analyses of p-OSR1 and p-SPAK in kidneys from wild-type and WNK3 knockout mice. For densitometry analysis, values ($n=15$) are expressed as ratios against the average of signals in the wild-type group. There were no significant decreases in phosphorylation of OSR1 and SPAK in kidneys from WNK3 knockout mice, as compared to wild-type littermates. n.s. not significant.

Recently, we re-evaluated the immunolocalization of WNK4 along the mouse nephron. In this study, we confirmed that WNK4 is present in DCT, collecting duct and other segments of the nephron, but not in TAL, where NKCC2 and SPAK are present (Ohno et al., 2011). The lack of WNK4 protein in TAL is consistent with our observation that increased phosphorylation of NKCC2 is not observed in WNK4 knock-in mice (manuscript in preparation), and the fact that PHAII is sensitive to thiazide, but not to furosemide. This indicates that activation of furosemide-sensitive NKCC2 is not observed in PHAII caused by WNK1 or WNK4 mutation, indicating that WNK1 and WNK4 may not play a major role in regulation of NKCC2 in the kidney, although WNK1 phosphorylates NKCC2 in in vitro kinase assays (Anselmo et al., 2006). Therefore, another WNK kinase could be present in TAL to regulate SPAK and NKCC2. This suggests that WNK3 is a regulator of NKCC2 in the kidney.

In this study, we successfully generated WNK3 knockout mice and analyzed their renal phenotype. However, in contrast to data obtained in the *Xenopus* oocytes system and our expectation that WNK3 is a major regulator of NKCC2, WNK3 knockout mice did not show any decrease in expression and phosphorylation of NKCC2 and NCC. Indeed, urinary excretion of Na^+ and K^+ was not significantly affected in WNK3 knockout mice, even though we focused on the transition periods when changing sodium diets. These results suggest that WNK3 is not a powerful regulator of NKCC2 and NCC in the kidney, unlike in *Xenopus* oocytes.

There are several possible explanations as to why expression and phosphorylation of NKCC2 and NCC were not lower in WNK3 knockout mouse kidney. It is possible that the expression level of WNK3 in individual tubular cells was too low to contribute to the overall WNK kinase activity. Our immunoblot data clearly showed that WNK3 protein abundance in kidney was below the detection limit. RT-PCR using dissected nephron segments did not show the presence of preferential expression sites for WNK3 along the nephron. These results suggest that WNK3 does not have a specific role in the kidney, in contrast to the role of WNK4 and kidney-specific WNK1 as regulators of NCC in DCT. Another possibility is that the other WNKs, WNK1 and WNK4, compensated for the absence of WNK3 in the NKCC2- and NCC-expressing nephrons. Considering that 1) phosphorylation of OSR1 and SPAK at their WNK phosphorylation sites was not lower in the kidneys of WNK3 knockout mice, and 2) WNK1 and WNK4 expression levels were slightly higher in the kidneys of WNK3 knockout mice, it is possible that WNK1 and WNK4 compensated for the absence of WNK3. Therefore, the lack of phenotype in the absence of WNK3 in the mouse kidney does not necessarily exclude a potential role for WNK3 in activation of NCC and NKCC2 in vivo. On the other hand, WNK4 hypomorphic mice showed decreased phosphorylation of NCC and lower blood pressure, and other WNKs could not compensate (Ohta et al., 2009). Therefore, as the absence of WNK3 is compensated for by other WNKs, the role of WNK3 in the regulation of NKCC2 and NCC in vivo may not be substantial. To clarify this issue further, WNK3 and WNK4 double-knockout mouse would be required; however, we have not obtained conditional WNK4 knockout mice to date.

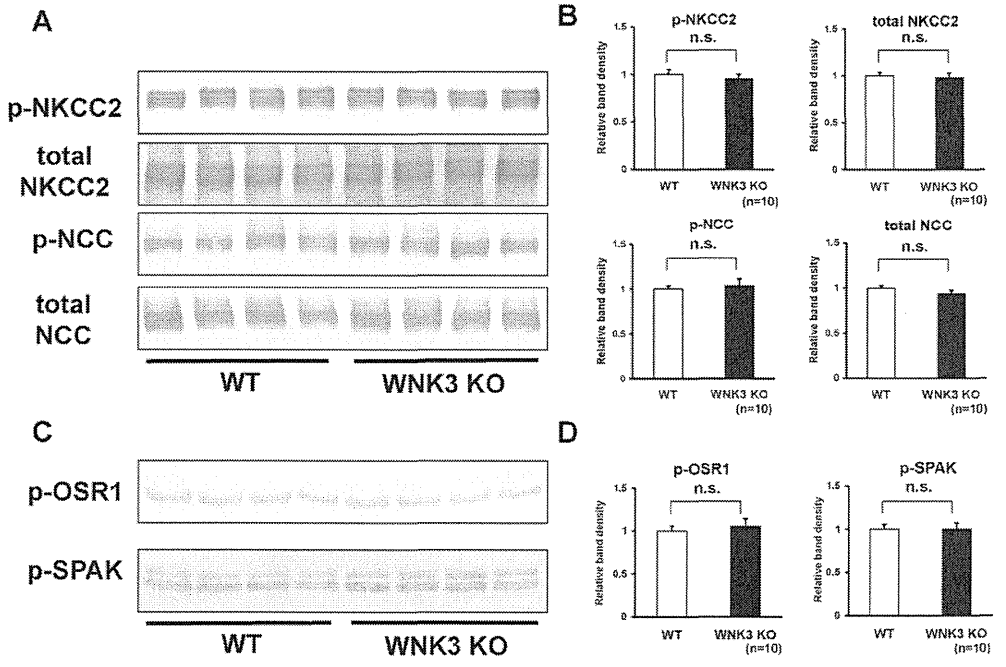

Fig. 6. Expression and phosphorylation of NKCC2 and NCC in kidneys from WNK3 knockout mice fed low-salt diet. (A) Representative immunoblots of total and phosphorylated NKCC2 and NCC in kidneys from wild-type and WNK3 knockout mice fed with low-salt diet. **(B)** Densitometry analyses of expression and phosphorylation of NKCC2 and NCC in kidneys from wild-type and WNK3 knockout mice fed low-salt diet. For densitometry analysis, values ($n=10$) are expressed as ratios against the average of signals in the wild-type group. There were no significant decreases in the expression and phosphorylation of NKCC2 and NCC in kidneys from WNK3 knockout mice, as compared to wild-type littermates, even under low-salt diet. **(C)** Representative immunoblots of p-OSR1 and p-SPAK in kidneys from wild-type and WNK3 knockout mice fed low-salt diet. **(D)** Densitometry analyses of p-OSR1 and p-SPAK in kidneys from wild-type and WNK3 knockout mice fed low-salt diet. For densitometry analysis, values ($n=10$) are expressed as ratios against the average of signals in the wild-type group. There were no significant decreases in phosphorylation of OSR1 and SPAK in kidneys from WNK3 knockout mice, as compared to wild-type littermates. n.s. not significant.

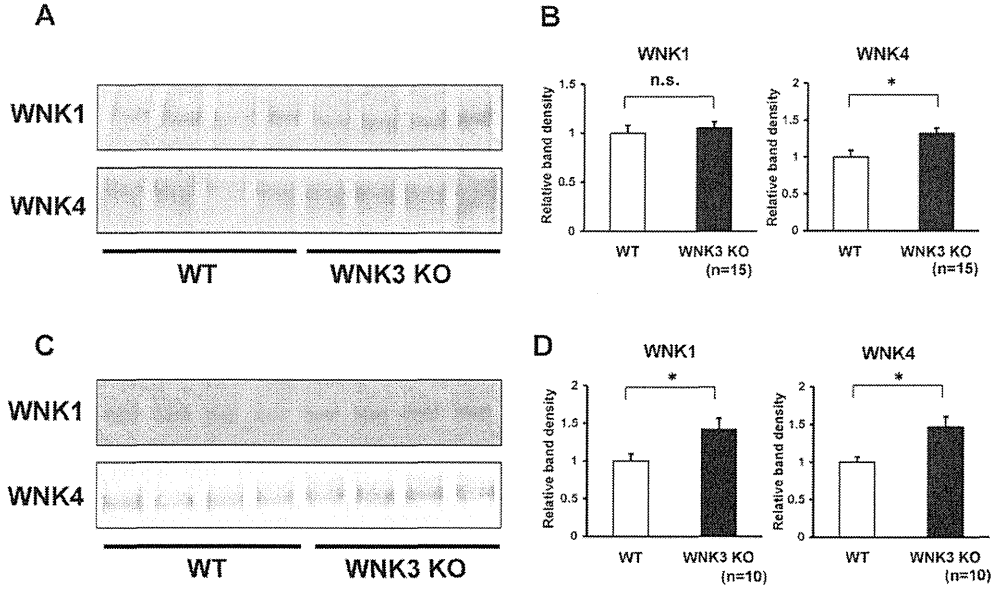

Fig. 7. WNK1 and WNK4 were elevated in kidneys from WNK3 knockout mice fed low-salt diet. (A) Representative immunoblots of WNK1 and WNK4 in kidneys from wild-type and WNK3 knockout mice fed normal diet. **(B)** Densitometry analyses of WNK1 and WNK4 in kidneys from wild-type and WNK3 knockout mice fed normal diet. For densitometry analysis, values ($n=15$) are expressed as ratios against the average of signals in the wild-type group. Expression of WNK4 was elevated in kidneys from WNK3 knockout mice, as compared to wild-type littermates. **(C)** Representative immunoblots of WNK1 and WNK4 in kidneys from wild-type and WNK3 knockout mice fed low-salt diet. **(D)** Densitometry analyses of WNK1 and WNK4 in kidneys from wild-type and WNK3 knockout mice fed low-salt diet. For densitometry analysis, values ($n=10$) are expressed as ratios against the average of signals in the wild-type group. Expression of WNK1 and WNK4 was elevated in kidneys from WNK3 knockout mice, as compared to wild-type littermates. $^*P<0.05$. n.s. not significant.

WNK3 knockout mice showed lower blood pressure only when mice were fed low-salt diet, although urinary excretion of Na^+ was not significantly affected in WNK3 knockout mice. Indeed, consistent with urinary data, WNK3 knockout mice did not show any decrease in expression and phosphorylation of NKCC2 and NCC in the kidney even under low-salt diet. These results clearly indicated that lower blood pressure in WNK3 knockout mice fed low-salt diet is not due to decreased sodium reabsorption in the kidney, but due to the other mechanism(s). Since WNK3 is abundantly expressed in brain, it is possible that the absence of WNK3 in brain might affect neuronal regulatory mechanisms of blood pressure, such as secretion of vasopressin, control of sympathetic nerve activity, etc., although the involvement of WNK3 in such processes has not been investigated. It is also possible that WNK3 knockout mice might have reduced vascular resistance since NKCC1, a substrate of SPAK/OSR1, reportedly regulated tonus of vascular smooth muscles, and decreased blood pressure was in fact in the NKCC1 knockout mouse (Akar et al., 2001; Meyer et al., 2002; Garg et al., 2007). Interestingly, similar to WNK3 knockout mice, NKCC1 knockout mice also showed lower blood pressure only when mice were fed with low-salt diet (Kim et al., 2008). Moreover, we recently found that SPAK knockout mice showed the reduced aortic contractility with decreased phosphorylation of NKCC1 (Yang et al., 2010). Accordingly, we tried to evaluate the phosphorylation status of NKCC1 in aorta of WNK3 knockout mice. Unfortunately, we have not yet detected an apparent decrease of NKCC1 phosphorylation in the aorta of WNK3 knockout mice (data not shown). Since the exact quantification of NKCC1 phosphorylation in tiny mouse aortic tissues by immunoblot is in fact very tricky, further investigation must be required to clarify this issue.

In this study, we generated and analyzed WNK3 knockout mice, focusing on their renal phenotypes. However, we did not observe any significant decreases in expression and phosphorylation of NKCC2 and NCC in the WNK3 knockout mouse kidney. Our results suggest that WNK3 only has a minor role in the regulation of NKCC2 and NCC in the in vivo mouse kidney.

Materials and Methods

Targeted disruption of the *Wnk3* gene

For generation of WNK3 knockout mice, we prepared the targeting vector using PCR-amplified segments of the *Wnk3* gene after verifying sequences. The targeting vector was transfected into J1 ES cells by electroporation, as described previously (Sohara et al., 2006). After selection with 150 μg/ml G418 and 2 μM ganciclovir, correctly targeted ES cell clones were selected by PCR and Southern blotting. Chimeric male mice were bred with C57BL/6 female mice to produce the heterozygous floxed mice, and the neo cassette was then deleted by crossing the mice with CAG-Cre recombinase-expressing transgenic mice (Sakai and Miyazaki, 1997). Wild-type controls and WNK3 knockout mice were bred and tail genomic DNA was applied for genotyping by PCR (forward primer; 5′-GATATGTAAGCACTACTACC-3′, reverse primer-A; 5′-TCTAATAGCT-CAACTGAGTG-3′, reverse primer-B; 5′-GTTCTCAAGTTCTACATCTC-3′). The mice were raised in a 12-hour day and night cycle, fed with normal rodent diet and plain drinking water. The phenotype of male mice was evaluated at the age of 8–14 weeks. The experiment was approved by the Animal Care and Use Committee of Tokyo Medical and Dental University.

Laser capture microdissection (LCM) and RT-PCR

For LCM, mouse kidneys were cut along the long axis and embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and immediately frozen in dry ice. Tissue samples were then immersed in nitrogen oxide. Frozen tissue blocks were cut into 10-μm sections, and were mounted on uncoated, uncharged glass slides. Sections were stained for 20 s in Histogene Staining Solution (Arcturus, Mountain View, CA, USA), subjected to dehydration in a graded alcohol series, cleared for 5 min in fresh xylene, and air-dried for 5 min. LCM was performed using a Pixcell II laser capture system (Arcturus). Tubular cells in each nephron were visualized and captured using CapSure LCM macrocaps (Arcturus). Laser setting ranged between 70 to 90 mW in power and 0.6 to 1.0 ms in duration. Total RNA from LCM samples was extracted from captured cells using a PicoPure RNA Isolation kit (Arcturus) and total RNA from mouse kidneys was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA was reverse-transcribed using Omniscript reverse transcriptase (Qiagen, Hilden, Germany). We confirmed the presence of nephron cells using region-specific primers; NHE3 (sense; 5′-GCTGTCATTGGCACTATATGG-3′ (exon 2) and antisense; 5′-GAGGACTTCATTGACATGGAC-3′ (exon 3)), NKCC2 ((sense; 5′-AACTCA-GTGCCCAGTAGTGC-3′ (exon 2) and antisense; 5′-AGGCATCCCATCT-CCATTAG -3′ (exon 3)), NCC ((sense; 5′- GTCATCATGGTCTCCTTTGC -3′ (exon 7) and antisense; 5′-TAGCTGAGATGGCAAGGTAG-3′ (exon 9)), and ENaC α subunit (sense; 5′-TCAACATTCTGTCCAGACTGC-3′ (exon 3) and antisense; 5′-GTAGCATGGCCCATACATGG-3′ (exon 4)). Finally, we investigated the presence of WNK3 in these tissues; WNK3 (sense; 5′-GCTGTTGCAACTTCCCCTAGT-3′ (exon 1) and antisense; 5′-CCGTTGCTG-CTCAGCTTTAG-3′ (exon 2)).

Blood and urine analysis and blood pressure measurement

Blood for electrolyte analyses was obtained from the submandibular venous plexus under light ether anesthesia. Electrolyte levels were determined with an i-STAT analyzer (Fuso, Osaka, Japan). Mice were kept in metabolic cages for urine collection. Low-salt (0.01% NaCl) and high-salt (4% NaCl) diets were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Urine samples were analyzed by DRI-CHEM (Fujifilm, Tokyo, Japan). Blood pressure in restrained conscious mice at steady state was measured with a programmable tail-cuff sphygmomanometer (MK-2000A; Muromachi, Tokyo, Japan).

Antibodies

We prepared an affinity-purified sheep antibody raised against a fragment of recombinant mouse WNK3 (residues 1145 – 1508). Primary antibodies used in this study were: rabbit anti-WNK1 (1:250) (Bethyl Laboratories, Montgomery, TX, USA); rabbit anti-WNK4 (1:250) (Ohno et al., 2011); rabbit anti-phosphorylated OSR1 (1:3000) (Ohta et al., 2009); rabbit anti-phosphorylated SPAK (1:500) (Yang et al., 2010); rabbit anti-NHE3 (1:200) (Alpha Diagnostic, San Antonio, TX, USA); rabbit anti-phosphorylated NCC (1:250) (Yang, S. S. et al., 2007); guinea pig anti-NCC (1:500) (Nomura et al., 2011), rabbit anti-phosphorylated NKCC2 (1:500) (Yang et al., 2010); rabbit anti-NKCC2 (1:1000) (kindly provided by K. Mutig, Charité-Universitätsmedizin Berlin, Campus Charité-Mitte, Germany); and rabbit anti-ENaC γ subunit (1:250) (kindly provided by M. Knepper, National Institutes of Health, USA). Alkaline-phosphatase-conjugated anti-IgG antibodies (Promega, Madison, WI, USA) were used as secondary antibodies for immunoblotting.

Immunoblotting

Semiquantitative immunoblotting was performed as described previously using whole kidney homogenates without the nuclear fraction (600 × g) or the crude membrane fraction (17000 × g) (Yang, S. S. et al., 2007). Band intensity was analyzed using Image J (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Statistical significance was evaluated using unpaired *t*-test. P-values <0.05 were considered to be significant.

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Competing Interests

The authors declare no competing interests.

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ORIGINAL ARTICLE

Effect of heterozygous deletion of WNK1 on the WNK-OSR1/SPAK-NCC/NKCC1/NKCC2 signal cascade in the kidney and blood vessels

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Abstract
Background We found that a mechanism of hypertension in pseudohypoaldosteronism type II (PHAII) caused by a WNK4 missense mutation (D561A) was activation of the WNK-OSR1/SPAK-NCC signal cascade. However, the pathogenic effect of intronic deletions in WNK1 genes also observed in PHAII patients remains unclear. To understand the pathophysiological roles of WNK1 in vivo, $WNK1^{+/-}$ mice have been analyzed, because homozygous WNK1 knockout is embryonic lethal. Although $WNK1^{+/-}$ mice have been reported to have hypotension, detailed analyses of the WNK signal cascade in the kidney and other organs of $WNK1^{+/-}$ mice have not been performed.
Method We assess the effect of heterozygous deletion of WNK1 on the WNK-OSR1/SPAK-NCC/NKCC1/NKCC2 signal cascade in the kidney and blood vessels.
Results Contrary to the previous report, the blood pressure of $WNK1^{+/-}$ mice was not decreased, even under a low-salt diet. Under a $WNK4^{D561A/+}$ background, the heterozygous deletion of the WNK1 gene did not reduce the high blood pressure either. We then evaluated the phosphorylation status of OSR1, SPAK, NCC, NKCC1, and NKCC2 in the kidney, but no significant decrease in the phosphorylation was observed in $WNK1^{+/-}$ mice or $WNK1^{+/-}WNK4^{D561A/+}$ mice. In contrast, a significant decrease in NKCC1 phosphorylation in the aorta and a decreased pressure-induced myogenic response in the mesenteric arteries were observed in $WNK1^{+/-}$ mice.
Conclusion The contribution of WNK1 to total WNK kinase activity in the kidney may be small, but that WNK1 may play a substantial role in the regulation of blood pressure in the arteries.

Keywords WNK kinase · Blood pressure · Na–K–2Cl cotransporter · Vascular smooth muscle cell · Myogenic tone

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Introduction

Pseudohypoaldosteronism type II (PHAII), also known as Gordon's syndrome, is an autosomal dominant disease characterized by hypertension, hyperkalemia, and metabolic acidosis [1]. In 2001, PHAII was shown to be caused by mutations in the WNK1 and WNK4 genes [2], whose physiological roles were not clarified at that time. The PHAII-causing mutations are large deletions of the first intron in the WNK1 gene that cause increased expression and missense mutations outside the protein kinase domain (E562K, D564A, Q565E, and R1185C) of the WNK4 gene [2]. Since then, the pathophysiological roles of WNK kinases in blood pressure regulation and renal Na and K transport have been investigated [3, 4].

Oxidative stress responsive kinase 1 (OSR1) and Ste20-related proline-alanine-rich kinase (SPAK), which are serine-threonine kinases, were identified as substrates of WNK kinases [5–7] and are known to phosphorylate and regulate Na–K–2Cl cotransporter 1 (NKCC1) [8]. Therefore, we speculated that Na–Cl cotransporter (NCC) could also be a substrate of OSR1 and SPAK. To verify this hypothesis, we generated and analyzed $WNK4^{D561A/+}$ knockin mice corresponding to human PHAII patients with a D564A mutation. In the knockin mice, phosphorylation of OSR1 and SPAK at their phosphorylation sites by WNK kinases was increased, and NCC phosphorylation at phosphorylation sites by OSR1 and SPAK was also highly increased [8]. Furthermore, we confirmed by analyzing OSR1 and SPAK knockin mice and SPAK knockout mice [9–11] that the NCC phosphorylation in the kidney is completely dependent on OSR1 and SPAK kinase activation by WNK kinases, not only in the PHAII mice but also in wild-type mice. Thus, we established a novel kinase cascade in the kidney that is important for regulating salt balance in the body and blood pressure. We have also shown that this signal cascade is regulated by various upstream stimuli, including vasoactive hormones such as angiotensin II and vasopressin, insulin, and extracellular K concentration and K intake [12–14].

Although the constitutive activation of this cascade by a disease-causing WNK4 mutant is a major cause of PHAII [8], the pathogenic mechanism of PHAII by the mutation of the WNK1 gene remains to be determined. In addition, even in the $WNK4^{D561A/+}$ knockin mice, a clear mechanism of how the WNK4 mutation increased total WNK kinase activity in the kidney remains to be determined. Yang et al. [15] previously suggested the possibility that WNK kinases might function by forming a kinase complex, rather than each isoform working independently. Therefore, WNK1 could also be involved in the pathogenesis of PHAII in $WNK4^{D561A/+}$ knockin mice.

To determine the pathophysiological roles of WNK1 in vivo, an analysis of the WNK1 null mouse is necessary. However, the WNK1 null mouse is lethal due to developmental abnormalities, mainly in the cardiovascular system, including the heart, arteries, and veins [16]. Accordingly, $WNK1^{+/-}$ mice have been analyzed and shown to have lower blood pressure than wild-type mice [17]. However, detailed analyses of the WNK-OSR1/SPAK-NCC signal cascade in the kidney and other organs of $WNK1^{+/-}$ mice have not been performed.

The purpose of this study is to determine the effect of the heterozygous deletion of WNK1 on the WNK-OSR1/SPAK signal cascade. In addition, we examined the myogenic tone of mesenteric arteries in $WNK1^{+/-}$ mice, since WNK1 is expressed in endothelial cells and vascular smooth muscle cells [18] and is involved in cardiovascular development.

Materials and methods

Animals

WNK1 heterozygous knockout mice ($WNK1^{+/-}$ mice) were generated on a C57BL/6 background by gene trap mutation. Gene trap vector pGT0lxf containing a β-geo cassette, which is a fusion of β-galactosidase and neomycin transferase, was inserted into intron 1 of the WNK1 gene. The ES cells were bought from the International Gene Trap Consortium. The mutant cell line RRI372 (detailed information is available online at http://www.genetrap.org/cgi-bin/annotation.py?cellline=RRI372) was used by the University of Dundee mouse transgenic service to create heterozygous mice. The offspring were genotyped by PCR (94°C for 30 s, 61°C for 2 min, 30 cycles) (Fig. 1a, b) with sense primers F1 (5′-AACGTTAACTCAGCATGTGGAAGCA-3′) and F2 (5′-TGAAACTTCCCAAGAGTCCGAGGGA-3′) within intron1 and antisense primers R1 (5′-GACTGCCCAGTAGGAGGAGAGGC-3′) in intron1 and R2 (5′-CCCGGCGCTCTTACCAAAGGG-3′) in pGT0lxf. F1/R1 amplified the wild-type allele (772 bp), and F2/R2 amplified the β-geo-inserted allele (1498 bp). $WNK1^{+/-}$ $WNK4^{D561A/+}$ mice were obtained by mating $WNK1^{+/-}$ mice with $WNK4^{D561A/+}$ mice, which were generated as described previously [8]. Mice of each genotype were placed on a normal-salt diet (NaCl 0.4% w/w) or a low-salt diet (0.01% w/w) for 1 week. All experiments were performed 1 week after changing diets. The Animal Care and Use Committee of Tokyo Medical and Dental University approved the experiment.

Measurement of blood pressure

The systolic blood pressure of restrained conscious mice in the steady state was measured with a programmable tail cuff sphygmomanometer (MK-2000A; Muromachi, Tokyo, Japan). Five preliminary blood pressure measurements were performed to acclimate the mice to the apparatus during the first 3 days. Five measurements each day were performed during the following 6 days. These experiments were performed under a normal-salt (0.4%) or low-salt diet (0.01%).

Immunoblot analysis

Kidneys and aortas were dissected from mice. Whole-kidney homogenate without the nuclear fraction (600*g*) was prepared to measure the levels of WNK4, OSR1, and SPAK, and the crude membrane fraction (17000*g*) was prepared to measure the levels of NCC, NKCC1, and NKCC2. Kidney cortex homogenate without the nuclear fraction (600*g*) was prepared to measure the level of

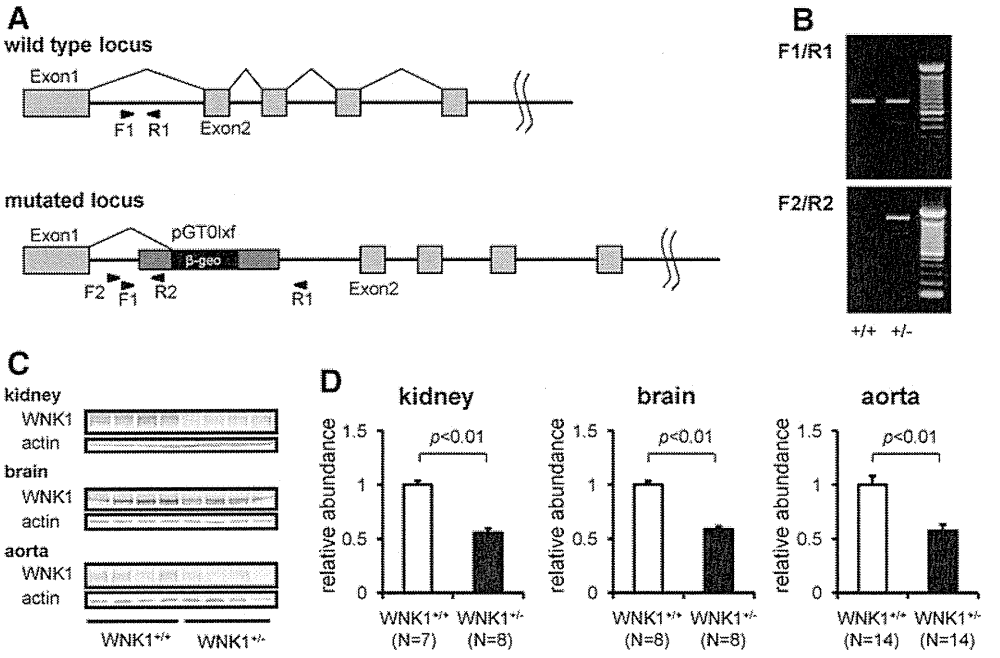

Fig. 1 Generation of WNK1 heterozygous knockout ($WNK1^{+/-}$) mice. **a** Targeting strategy for the WNK1 gene based on gene-trap technology. Primers for genotyping are shown. **b** Results of genotype PCR. Both primers (F1 and F2) are located between exon1 and the insertion site of pGT0lxf. Primers F1 and R1 amplified the wild-type allele (772 bp). Primers F2 and R2 flanking the gene-trap vector pGT0lxf in intron 1 of the WNK1 gene specifically amplified the mutated allele (1498 bp). Primers F1 and R1 did not amplify the mutated allele due to the insertion of pGT0lxf containing a β-geo cassette of 3876 bp. **c** Immunoblots of WNK1 in kidney, brain, and aorta homogenates of $WNK1^{+/+}$ and $WNK1^{+/-}$ mice. **d** In all three tissues, $WNK1^{+/-}$ mice showed decreased WNK1 protein levels, which were about half of the levels in the control $WNK1^{+/+}$ mice by densitometry analysis. *N* number of mice

WNK1. Aorta homogenate without the nuclear or mitochondrial fraction (6000*g*) was also prepared. Semiquantitative immunoblotting was performed as described previously [8]. The relative intensities of immunoblot bands were determined by laser scanning (GT-X970 scanner, EPSON, Nagano, Japan) followed by densitometry with YabGelImage (free software). The commercially available primary antibodies used were anti-WNK1 (A301-516A; BETHYL, Montgomery, TX, USA), anti-OSR1 (M09; Abnova, Taipei, Taiwan), anti-SPAK (Cell Signaling Technology, Danvers, MA, USA), anti-NKCC2 (Alpha Diagnostic, San Antonio, TX, USA), anti-total NCC (Chemicon, Billerica, MA, USA), and anti-actin (Cytoskeleton, Denver, CO, USA) antibodies. Other antibodies used were anti-pOSR1(S325)/SPAK(S383) [19], anti-pNCC (T53, T58, and S71) [8], anti-NKCC1 (T4) [20], and anti-pNKCC1 (T206) and anti-pNKCC2 (T96) antibodies [10]. Alkaline-phosphatase-conjugated anti-IgG antibodies (Promega, Madison, WI, USA) and WesternBlue (Promega) were used to detect the signals.

Immunofluorescence

Mesenteric arteries were dissected, embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan), and snap frozen in liquid nitrogen. The primary antibodies used were anti-WNK1 (A301-516A; BETHYL) and anti-α-smooth muscle actin (C6198; Sigma–Aldrich, St. Louis, MO, USA). Alexa 488 or 546 dye-labeled (Molecular Probes; Invitrogen, Carlsbad, CA, USA) secondary antibodies were used for immunofluorescence. Immunofluorescence images were obtained by LSM510 Meta (Carl Zeiss, Oberkochen, Germany).

Measurements of arterial diameter and myogenic tone

$WNK1^{+/+}$ mice and $WNK1^{+/-}$ mice (11–13 weeks old) were sacrificed by cervical dislocation. Small arteries from the superior mesenteric artery arcade were isolated and cannulated with small pipettes at both ends. Arterial diameter, myogenic tone, and evoked vasoconstriction were measured in the isolated, pressurized arteries in physiological salt solution (PSS) as described [21]. At the end of each experiment, the myogenic tone in 120 mmHg was measured in Ca-free physiological salt solution (Ca-free PSS). Myogenic tone is shown as the steady-state arterial diameter in PSS as a function of intravascular pressure.

Statistics

Data are presented as the mean ± SE. Student's *t* test was used to compare the data from control mice and mutant

mice. The Mann–Whitney U test was used for the analysis shown in Fig. 5c.

Results

Confirmation of WNK1 heterozygous deletion

As shown in Fig. 1, we genotyped the targeted WNK1 allele. To confirm the decreased WNK1 protein level in the $WNK1^{+/-}$ mice, we performed immunoblots of kidney, brain, and aorta homogenates. The expression level of WNK1 in the kidney was lower than the level in the brain, and we could not detect the WNK1 band in whole-kidney samples. When we divided the kidney into the cortex and medulla, we could detect the WNK1 signal in cortex samples. In all three tissues, the $WNK1^{+/-}$ mice showed decreased WNK1 protein levels that were about half of the levels in the control $WNK1^{+/+}$ mice.

Measurement of blood pressure

Previously, the blood pressure of $WNK1^{+/-}$ mice measured by the tail-cuff method was lower than that of $WNK^{+/+}$ mice [17]. To confirm this result, we first measured the mean systolic blood pressure of 8 male $WNK1^{+/+}$ mice and that of 7 male $WNK1^{+/-}$ mice under a normal-salt diet (0.4%). There was no significant difference ($p = 0.800$) between the systolic blood pressure of $WNK1^{+/+}$ mice (mean ± SE 101.9 ± 1.1 mmHg) and that of $WNK1^{+/-}$ mice (101.3 ± 2.2 mmHg) (Fig. 2a). Even under a low-salt diet (0.01%), the blood pressure of $WNK1^{+/-}$ mice was not lower than that of $WNK1^{+/+}$ mice (101.1 ± 1.4 and 96.5 ± 2.1 mmHg, respectively; $N = 8$ and 8, $p = 0.101$).

Next, the systolic blood pressures of $WNK4^{D561A/+}WNK1^{+/+}$ mice and $WNK4^{D561A/+}WNK1^{+/-}$ mice were measured. As we reported previously, $WNK4^{D561A/+}WNK1^{+/+}$ mice, a model of PHAII, showed hypertension (135.1 ± 5.5 mmHg, $N = 4$). Under this hypertensive PHAII genetic background, the heterozygous deletion of the WNK1 gene did not reduce the high blood pressure (133.0 ± 4.5 mmHg, $N = 4$).

Immunoblot analysis of $WNK1^{+/-}$ mouse kidney

Although lower blood pressure was not confirmed in the $WNK1^{+/-}$ mice in this study, we sought to determine whether the WNK-OSR1/SPAK-NCC/NKCC1/NKCC2 phosphorylation cascade was affected in the $WNK1^{+/-}$ mouse kidney in order to explore the contribution of WNK1 to this cascade in the kidney. As shown in Fig. 3a and b, no difference was detected in both the total expression levels and phosphorylation levels of OSR1, SPAK, NCC, NKCC1, and NKCC2 between $WNK1^{+/+}$ and $WNK1^{+/-}$ mice fed a normal-salt diet. There was no compensatory increase of WNK4 expression in $WNK1^{+/-}$ mice. Even under a low-salt diet, there was no evident difference. Under a $WNK4^{D561A/+}$ background, where the WNK-OSR1/SPAK-NCC cascade is constitutively activated, the heterozygous deletion of WNK1 did not affect the phosphorylation status of these proteins.

Role of WNK1 in blood vessels

NKCC1 is localized in vascular smooth muscle cells and regulates vascular contractility [22]. We showed in SPAK knockout mice that the phosphorylation of NKCC1 and the vascular responsiveness to the selective α1-adrenergic agonist phenylephrine and the NKCC1 inhibitor bumetanide in the aorta were decreased [10]. To investigate the involvement of WNK1 in the regulation of vascular contractility, we investigated the phosphorylation status of NKCC1 in the aorta and measured the myogenic response in the mesenteric artery in $WNK1^{+/-}$ mice. As shown in Fig. 4, phosphorylation of NKCC1 in the aorta of $WNK1^{+/-}$ mice was significantly decreased, suggesting the involvement of WNK1 in the regulation of vascular tone. Accordingly, to determine the contractile properties of the resistance vessels from $WNK1^{+/-}$ mice, we measured the diameter changes in isolated, pressurized mesenteric small arteries. As shown in Fig. 5a, WNK1 immunofluorescence that partially co-localized with α-smooth muscle actin was confirmed in the mesenteric artery. Mesenteric arteries constrict spontaneously when intravascular pressure is increased to more than 40 or 50 mmHg during perfusion with PSS at 35–37°C. This constriction reflects myogenic tone. Figure 5b shows the changes of arterial diameter in $WNK1^{+/+}$ and $WNK1^{+/-}$ mice as a function of intravascular pressure. The diameters of the arteries of $WNK1^{+/+}$ and $WNK1^{+/-}$ mice at 0 mmHg in the presence of Ca (2.5 mM) were 103.1 ± 2.9 mmHg ($N = 5$) and 88.8 ± 6.8 mmHg ($N = 5$), respectively (no significant difference). The diameters of the arteries of $WNK1^{+/+}$ and $WNK1^{+/-}$ mice at 120 mmHg in the absence of Ca were 150.6 ± 6.9 mmHg ($N = 5$) and 173.4 ± 12.6 mmHg ($N = 5$), respectively (no significant difference). On the other hand, the dilatation of arteries was significantly increased in $WNK1^{+/-}$ mice at pressures greater than 60 mm Hg, a physiological arterial pressure in mesenteric arteries (Fig. 5b). We also found that the arteries from $WNK1^{+/+}$ mice started to constrict when intravascular pressure was around 50.0 mmHg, while the arteries from $WNK1^{+/-}$ mice started to constrict when intravascular pressure was around 70.0 mmHg. As shown in Fig. 5c, this difference exhibited statistical significance ($p = 0.0374$). The data suggest that sensitivity to intravascular pressure

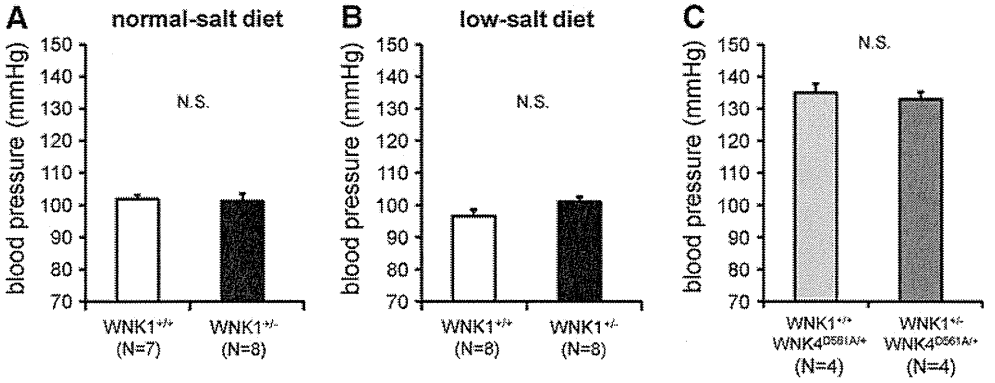

Fig. 2 Mean systolic blood pressures in $WNK1^{+/+}$ and $WNK1^{+/-}$ mice under normal and $WNK4^{D561A/+}$ backgrounds. **a** Blood pressures of $WNK1^{+/+}$ and $WNK1^{+/-}$ mice fed a normal-salt diet (NaCl 0.4% w/w). **b** Blood pressures of $WNK1^{+/+}$ and $WNK1^{+/-}$ mice fed a low-salt diet (NaCl 0.01% w/w). **c** Blood pressures of $WNK1^{+/+}$ $WNK4^{D561A/+}$ and $WNK1^{+/-}$ $WNK4^{D561A/+}$ mice fed a normal-salt diet. Mean blood pressure in $WNK^{+/-}$ mice was not statistically different from that of their control littermates ($WNK1^{+/+}$) under normal and $WNK4^{D561A/+}$ backgrounds. Blood pressures were measured 5 times per day for 6 consecutive days, and a mean value was calculated for each individual mouse. *N* number of mice. *N.S.* not significantly different

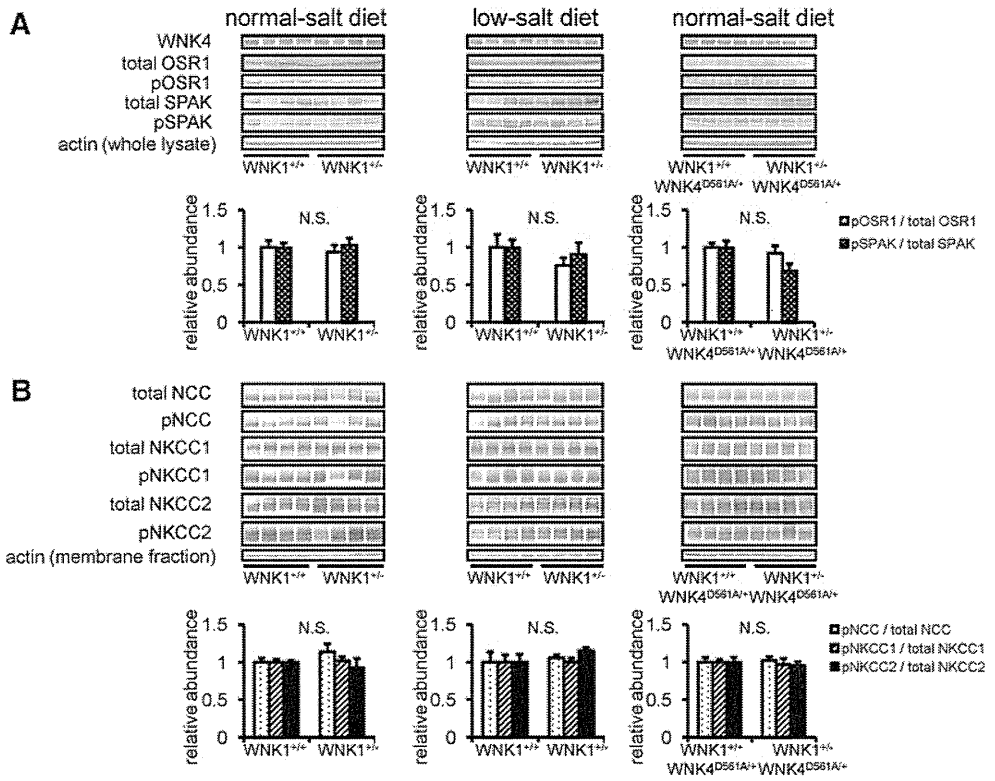

Fig. 3 Status of the WNK-OSR1/SPAK-NCC/NKCC1/NKCC2 signal cascade in kidneys of $WNK1^{+/+}$ mice and $WNK1^{+/-}$ mice. **a** Representative immunoblots of WNK4, OSR1, phosphorylated OSR1, SPAK, and phosphorylated SPAK in the kidneys of $WNK1^{+/+}$ and $WNK1^{+/-}$ mice fed a normal diet or a low-salt diet, and of $WNK1^{+/+}$ $WNK4^{D561A/+}$ and $WNK1^{+/-}$ $WNK4^{D561A/+}$ mice fed a normal diet. No significant difference between the two genotypes was detected in any immunoblot ($WNK1^{+/+}$ and $WNK1^{+/-}$ mice fed a normal diet: WNK4, $N = 4$; OSR1, phosphorylated OSR1, SPAK, and phosphorylated SPAK, $N = 10$; $WNK1^{+/+}$ and $WNK1^{+/-}$ mice fed a low-salt diet: $N = 4$; $WNK1^{+/+}$ $WNK4^{D561A/+}$ and $WNK1^{+/-}$ $WNK4^{D561A/+}$ mice fed a normal diet: $N = 4$). **b** Representative immunoblots of NCC, phosphorylated NCC, NKCC1, phosphorylated NKCC1, NKCC2, and phosphorylated NKCC2 in the kidneys of $WNK1^{+/+}$ and $WNK1^{+/-}$ mice fed a normal diet or a low-salt diet, and of $WNK1^{+/+}$ $WNK4^{D561A/+}$ and $WNK1^{+/-}$ $WNK4^{D561A/+}$ mice fed a normal diet. No significant difference between the two genotypes was detected in any immunoblot ($WNK1^{+/+}$ and $WNK1^{+/-}$ mice fed a normal diet: NCC and phosphorylated NCC, $N = 14$; NKCC1 and phosphorylated NKCC1, $N = 11$; NKCC2 and phosphorylated NKCC2, $N = 8$; $WNK1^{+/+}$ and $WNK1^{+/-}$ mice fed a low-salt diet: $N = 4$; $WNK1^{+/+}$ $WNK4^{D561A/+}$ and $WNK1^{+/-}$ $WNK4^{D561A/+}$ mice fed a normal diet: $N = 4$)

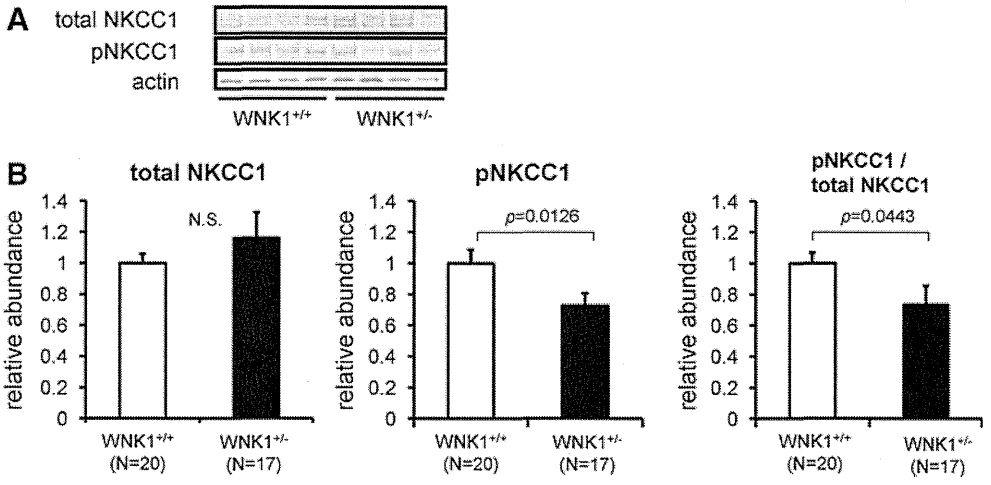

Fig. 4 Phosphorylation status of NKCC1 in the aortas of $WNK1^{+/+}$ and $WNK1^{+/-}$ mice. **a** Representative immunoblots of NKCC1 and phosphorylated NKCC1. **b** Results from quantifying the bands of immunoblots. Phosphorylation of NKCC1 in $WNK1^{+/-}$ mice was decreased. Total NKCC1 expression in $WNK1^{+/-}$ was not decreased. The ratio of phosphorylated NKCC1 to total NKCC1 was also significantly decreased

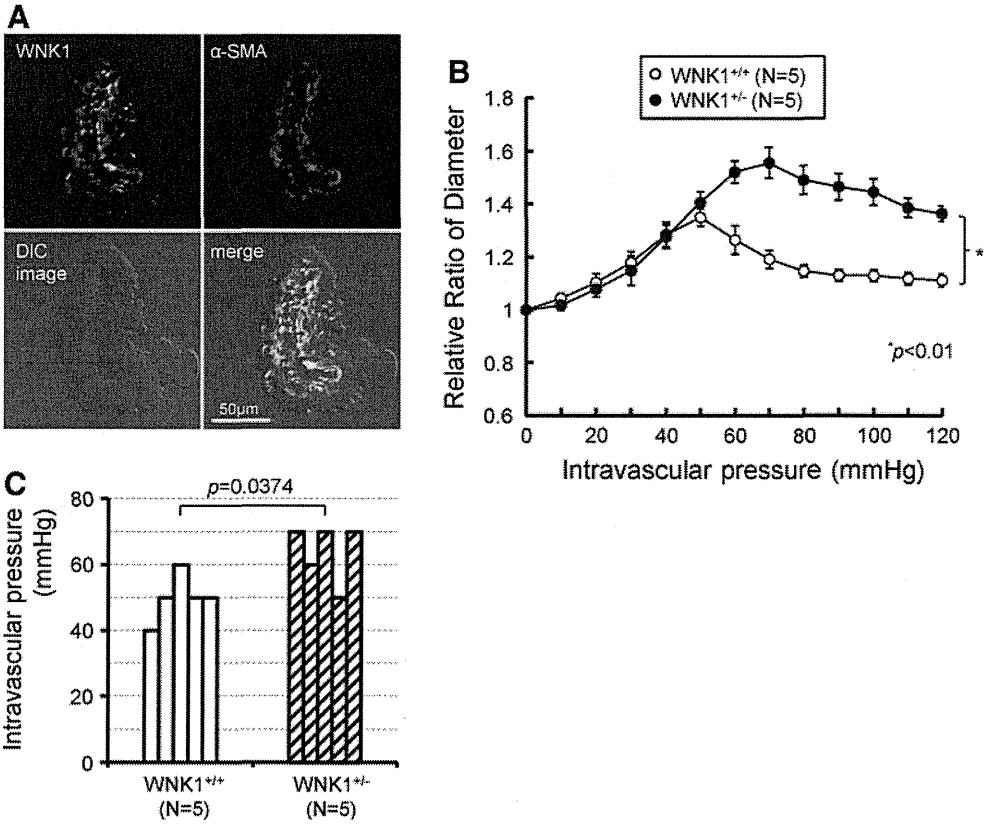

Fig. 5 Effect of heterozygous deletion of WNK1 on myogenic tone of small mesenteric arteries. **a** WNK1 immunofluorescence in mesenteric arteries. Most of the WNK1 signal (*green*) is co-localized with α-smooth muscle actin (α-SMA) (*red*). **b** Pressure-induced myogenic response in mesenteric arteries of $WNK1^{+/+}$ ($N = 5$) and $WNK1^{+/-}$ ($N = 5$) mice. The data are shown as the relative increase of arterial diameter at each pressure compared with those at 0 mmHg. Significantly attenuated contractile properties of arteries were observed in $WNK1^{+/-}$ mice ($^*p < 0.01$) at pressures of 60 mmHg or more. **c** The graph represents the intravascular pressure when mesenteric arteries from each mouse started to constrict. Significantly increased pressure was needed for the arteries of $WNK1^{+/-}$ mice to start to constrict ($p = 0.0374$) (color figure online)

might also be blunted in the resistance vessels of $WNK1^{+/-}$ mice.

Discussion

We previously elucidated through in vitro experiments that WNK1 as well as WNK4 phosphorylated and activated OSR1 and SPAK kinases, and that OSR1 and SPAK could phosphorylate threonine and serine residues in the N-terminal domain of NCC [5, 6, 23]. Then, we verified the existence of this WNK-OSR1/SPAK-NCC kinase cascade in the in vivo kidney through analyses of several genetically engineered mice we generated. We also clarified that this signal cascade was important not only for the pathogenesis of PHAII by WNK4 mutations but also for the physiological regulation of salt balance in the kidney. Salt intake regulates this cascade partially through aldosterone [24]. Potassium intake, extracellular potassium concentration, arginine vasopressin, and angiotensin II were identified as important regulators [12, 14, 25]. We recently found that insulin is also a powerful regulator of this cascade. We

speculate that this cascade may be responsible for salt-sensitive hypertension during hyperinsulinemia [13]. In most cases mentioned above, however, we have not yet determined which WNK kinase plays a dominant role in the kidney. We could not evaluate each WNK kinase activity separately, since each WNK phosphorylates the same phosphorylation sites within OSR1 and SPAK, and detecting the phosphorylation by phosphorylation-specific antibodies to OSR1 and SPAK is the sole method to measure WNK kinase activity in vivo. Furthermore, it has been very difficult to detect even total WNK1 and WNK3 proteins by immunoblot of kidney homogenates, although abundant expression could be detected in other organs such as the brain and testis [26, 27]. In contrast, WNK4 protein was most abundantly detected in the kidney [28], suggesting that WNK4 is the dominant WNK in the kidney.

Thus, the main purpose of this study was to clarify the involvement of WNK1 in the WNK-OSR1/SPAK-NCC/NKCC1/NKCC2 signal cascade in the kidney. Since mutations of the WNK1 gene cause PHAII, WNK1 may be somehow involved in the regulation of thiazide-sensitive NCC in the kidney, like WNK4. Furthermore, since $WNK1^{+/-}$ mice were reported to show lower blood pressure, we expected that WNK1 was also contributing to the total WNK kinase activity towards OSR1/SPAK in the kidney. However, the results obtained in this study were different from our expectations. First of all, the blood pressure of $WNK1^{+/-}$ mice was not decreased even under a low-salt diet. Very recently (while we were preparing this manuscript), Bergaya et al. reported the vascular phenotypes of $WNK1^{+/-}$ mice. They also could not detect a lower blood pressure in $WNK1^{+/-}$ mice, even using a telemetry system [29]. In the original report by Zambrowicz et al. [17], there was no difference in urine data, even in the presence of lower blood pressure, which may be consistent with our data on the phosphorylation of transporters. Thus, it may be true that there is no alteration in the status of the WNK-OSR1/SPAK-NCC/NKCC1/NKCC2 cascade in the kidneys of $WNK1^{+/-}$ mice. There may be two possible interpretations of this result. One explanation is that about 50% of the WNK1 protein may be sufficient to maintain the signal to each transporter. Complete deletion would show us some apparent phenotypes. To verify this idea, the kidney-specific WNK1 knockout mouse is absolutely necessary. Another interpretation is that WNK1 may not make a substantial contribution to the total WNK kinase activity in each type of cell expressing NCC, NKCC1, or NKCC2 in the kidney. This interpretation could be supported by the following data. First, we detected decreased NKCC1 phosphorylation in the aorta but not in the kidney of $WNK1^{+/-}$ mice. Heterozygous deletion was enough to induce a measurable decrease in NKCC1 phosphorylation, at least in the aorta. Second, in the case of WNK4, we could detect a salt-losing phenotype and the decreased phosphorylation of OSR1/SPAK and NCC in the kidneys of WNK4 mutant mice that were WNK4 hypomorphic rather than a total WNK4 knockout [19]. The same was true in the heterozygous inactive knockin mouse of OSR1 and SPAK, which exhibited a significant decrease in NCC phosphorylation in the kidney [9, 11]. Collectively, we suggest that WNK1 may not play a major role in the WNK-OSR1/SPAK signaling cascade in the kidney under physiological conditions. Even if the basal level of the WNK1 contribution to the total WNK kinase activity in the kidney is minimal (as observed in this study), a large increase in WNK1 gene transcription by the mutation found in PHAII could cause activation of the cascade. In fact, WNK1 transcription was increased in white blood cells of the patients carrying the WNK1 mutations [2]. Further investigation is necessary to clarify whether the WNK1 mutations are powerful enough to cause a robust increase in WNK1 transcription in kidney, thereby inducing the activation of the WNK-OSR1/SPAK-NCC signaling cascade.

This study also clarified that it is less likely that WNK1 is involved in the pathogenesis of PHAII caused by WNK4 mutations. Yang et al. [15] previously postulated (based on experiments in Xenopus oocytes) that WNK kinases might form hetero-oligomers and function as a WNK kinase complex. Considering this hypothesis, we did not attribute the increased WNK kinase activity in $WNK4^{D561A/+}$ mice only to the mutant WNK4 in our recent paper clarifying the pathogenesis of PHAII [11]. We postulated that the mutant WNK4 could somehow affect the function of the WNK complex and eventually enhance the total WNK kinase activity in the kidney. However, absolutely no difference in NCC, NKCC1, and NKCC2 phosphorylation was detected in the $WNK1^{+/-}$ mice, even under a $WNK4^{D561A/+}$ background, suggesting that the involvement of WNK1 in this setting may be unlikely. Rather, the mutant WNK4 itself may have a dominant function in activating the WNK-OSR1/SPAK-NCC cascade.

In contrast to the kidney, $WNK1^{+/-}$ mice presented vascular phenotypes characterized by decreased NKCC1 phosphorylation in the aorta and attenuated myogenic tone in the mesenteric arteries, where we confirmed the existence of WNK1 by immunofluorescence (Fig. 5a). Although mechanisms of the vascular myogenic response have remained elusive, the involvement of K channels, Ca channels, and mechanosensitive channels, such as the transient receptor potential (TRP) channels, has been postulated [30–33]. In vascular smooth muscle cells, external stimuli such as vasoactive hormones and mechanical stretch induce the release of Ca from intracellular stores, open Ca-dependent Cl channels in plasma membranes, and lead to depolarization. Then, voltage-dependent Ca

channels in plasma membranes opened by depolarization induce further Ca entry and contraction of smooth muscles. In this scheme, NKCC1 is thought to be important for accumulating Cl within cells and enabling depolarization by the opening of a Ca-dependent Cl channel. Bergaya et al. [29] recently—while we were preparing this manuscript—showed the vascular phenotypes in the $WNK1^{+/-}$ mice. Although there are some differences in details, the conclusion that the pressure-induced myogenic response was decreased in the $WNK1^{+/-}$ mice was the same as ours (Fig. 5b). Under our experimental conditions, we could clearly see the decreased sensitivity of arteries in $WNK1^{+/-}$ mice to luminal pressure. As shown in Fig. 5c, more pressure was necessary to induce contraction in the arteries of $WNK1^{+/-}$ mice. These data may imply that WNK1 may also be involved in the process of mechanosensation in resistance vessels, and that protein(s) other than NKCC1 may also be regulated by WNK1.

In summary, the involvement of WNK1 in the WNK-OSR1/SPAK-NCC/NKCC1/NKCC2 phosphorylation cascade in the kidney in normal mice and PHAII model mice may be minimal. On the other hand, WNK1 may be a major WNK kinase in arteries that is important for regulating vascular tone.

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WNK-OSR1/SPAK-NCC signal cascade has circadian rhythm dependent on aldosterone

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ABSTRACT

Blood pressure and renal salt excretion show circadian rhythms. Recently, it has been clarified that clock genes regulate circadian rhythms of renal transporter expression in the kidney. Since we discovered the WNK-OSR1/SPAK-NaCl cotransporter (NCC) signal cascade, which is important for regulating salt balance and blood pressure, we have sought to determine whether NCC protein expression or phosphorylation shows diurnal rhythms in the mouse kidneys. Male C57BL/6J mice were sacrificed every 4 h (at 20:00, 0:00, 4:00, 8:00, 12:00, and 16:00), and the expression and phosphorylation of WNK4, OSR1, SPAK, and NCC were determined by immunoblot. (Lights were turned on at 8:00, which was the start of the rest period, and turned off at 20:00, which was the start of the active period, since mice are nocturnal.) Although expression levels of each protein did not show diurnal rhythm, the phosphorylation levels of OSR1, SPAK, and NCC were increased around the start of the active period and decreased around the start of the rest period. Oral administration of eplerenone (10 mg/day) attenuated the phosphorylation levels of these proteins and also diminished the diurnal rhythm of NCC phosphorylation. Thus, the activity of the WNK4-OSR1/SPAK-NCC cascade was shown to have a diurnal rhythm in the kidney that may be governed by aldosterone.

1. Introduction

The endogenous circadian clock in mammals is hierarchically organized by molecular oscillators. The central pacemaker is located in the suprachiasmatic nucleus (SCN) of the brain and is entrained by light signals transmitted from the retina through the retinohypothalamic tract [1]. The core clock machinery in peripheral tissues includes clock genes such as Bmal1, Clock, Period (Per1, 2, and 3), and Cryptochrome (Cry1 and 2), which function mainly as transcription factors [2–6]. The central pacemaker in the SCN synchronizes the functions of these peripheral clocks through neuronal and hormonal signaling [7].

The role of circadian rhythms has recently been reviewed for physiological systems including renal functions. Kidneys in healthy human subjects have circadian rhythms for urinary sodium, potassium, and chloride excretion, under which they excrete more electrolytes and produce more urine during daytime than nighttime [6,8]. The daily blood pressure profile also falls during nighttime in healthy subjects and in patients with the dipper pattern of essential hypertension. Essential hypertension in which blood pressure fails to fall during the night (non-dipper pattern) has been associated with more serious target organ damage, such as left ventricular hypertrophy, albuminuria, and cerebrovascular disease, than hypertension with a dipper pattern [9–11]. In addition, it has been shown that blood pressure failed to fall during the night in patients with salt-sensitive essential hypertension [12], and the circadian rhythm of renal sodium excretion was disturbed in the non-dipper type of essential hypertension [13]. Salt restriction and diuretics modify the diurnal variation of blood pressure from a non-dipper to a dipper pattern in salt-sensitive essential hypertension [14,15].

In 2001, mutation in the WNK1 and WNK4 genes was shown to cause pseudohypoaldosteronism type II (PHA II) [16], which is an autosomal dominant disease characterized by hypertension, hyperkalemia, and metabolic acidosis [17]. Since then, the pathophysiological roles of WNK kinases in blood pressure regulation and renal Na and K transport have been investigated [18]. Oxidative stress responsive kinase 1 (OSR1) and Ste20-related proline–alanine-rich kinase (SPAK), which were already known to be serine–threonine kinases that phosphorylate and regulate Na–K–2Cl cotransporter 1 (NKCC1) [19], were identified as substrates of WNK kinases [20–22]. We found increased phosphorylation of OSR1 and SPAK at their phosphorylation sites by WNK kinases and increased NCC phosphorylation at phosphorylation sites by OSR1 and SPAK in the WNK4$^{D561A/+}$ knockin mice, a mouse model of human PHAII having a D564A mutation [19]. In addition, we confirmed by analyzing OSR1 and SPAK knockin mice and SPAK

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knockout mice [23–25] that NCC phosphorylation in the kidney is completely dependent on OSR1 and SPAK kinase activation by WNK kinases, not only in PHAII mice but also in wild-type mice. Furthermore, we have also shown that salt intake regulates this cascade partially through aldosterone [26]. Thus, this novel kinase cascade in the kidney is important for regulating salt balance and blood pressure, and it is suggested to be involved in the onset of salt-sensitive hypertension.

Recently, a number of clock-controlled genes that have been identified in the kidney through either gene expression profiling or candidate gene approaches have been implicated in the diurnal rhythms for urinary electrolyte excretion. WNK4 and NCC transcripts in the distal convoluted tubule, connecting tubule, and cortical collecting duct in the mouse kidney are also reported to show circadian oscillation [27]. However, the function of the WNK-OSR1/SPAK-NCC cascade cannot be evaluated only by the profile of transcription since its phosphorylation status, which plays a key role in the cascade, is not considered. In this study, we sought to determine the diurnal rhythm of the WNK-OSR1/SPAK-NCC signal cascade in the kidney by examining total protein expression and phosphorylation status of the proteins in this cascade.

2. Materials and methods

2.1. Animals

Studies were performed on 8-week-old male C57BL/6J mice that had free access to food and water. They were adapted to a 12-h light/12-h dark cycle. Eplerenone (10 mg/day) was given to mice with mouse chow during the administration period. The immunoblot analysis was performed on kidney homogenate prepared from at least four mice at each time point. The Animal Care and Use Committee of Tokyo Medical and Dental University approved the experiment.

2.2. Immunoblot analysis

Kidneys were dissected from mice. The homogenates of cortex, medulla, and whole kidney without the nuclear fraction (600 g) was prepared to measure the levels of WNK4, OSR1, and SPAK, and the crude membrane fraction (17,000 g) was prepared to measure the levels of NCC. Semi-quantitative immunoblotting was performed as described previously [19]. The relative intensities of immunoblot bands were determined by densitometry with YabGelImage free software. The commercially available primary antibodies used were anti-OSR1 (M09; Abnova, Taipei, Taiwan), anti-SPAK (Cell Signaling Technology, Danvers, MA), anti-total NCC (Chemicon, Billerica, MA), and anti-actin (Cytoskeleton, Denver, CO) antibodies. Other antibodies used were anti-pOSR1(S325)/SPAK(S383) [28] and anti-pNCC (T53, T58, and S71) antibodies [19]. Alkaline-phosphatase-conjugated anti-IgG antibodies (Promega, Madison, WI) and WesternBlue (Promega) were used to detect the signals.

2.3. Measurement of plasma aldosterone concentration

To determine plasma aldosterone concentration (PAC), venous blood from the mice was collected into heparinized tubes. Plasma was immediately separated from blood samples by centrifugation at 8000 rpm for 5 min at 4 °C and stored at −30 °C. PAC was measured by solid-phase RIA by SRL (Tokyo, Japan).

2.4. Statistics

Data are presented as means ± SE. ANOVA and Tukey's test were used to compare the groups.

3. Results

3.1. The diurnal rhythm of phosphorylation status of the WNK-OSR1/SPAK-NCC signal cascade

To clarify whether the WNK-OSR1/SPAK-NCC signal cascade in the kidney has a diurnal rhythm, the protein expression and the phosphorylation status were evaluated by immunoblot analysis. C57BL/6J mice were adapted to a 12-h light/12-h dark cycle (lights on at 8:00 and lights off at 20:00) for 2 weeks. Then, mice in the group were sacrificed every 4 h (at 20:00, 0:00, 4:00, 8:00, 12:00, and 16:00) by cervical dislocation. The left kidney from each mouse was immediately dissected and homogenized. Start of the active period and start of the rest period corresponded to 20:00 and 8:00, respectively, since C57BL/6J mice are nocturnal.

The immunoblot analysis performed on the whole kidney homogenates is shown in Fig. 1A. Densitometry was used to compare the protein expression levels between time points (Fig. 1B). No apparent diurnal changes in the total expression level of each protein (i.e., WNK4, OSR1, SPAK, and NCC) were found. However, the phosphorylation status of OSR1, SPAK and NCC was increased at 20:00 and was significantly decreased at 8:00; there was a 2.1-fold change between the highest and the lowest phosphorylation levels. Diurnal distributions of acrophases of the phosphorylation status generally corresponded to the start of the active period, while those of bathyphases generally corresponded to the start of the rest period.

Considering the heterogeneity of diurnal oscillation in different nephron segments in the kidney, we also performed the immunoblots of total WNK4, OSR1, SPAK, and NCC in the cortex and medulla separately, focusing on 20:00 and 8:00 (Fig. 1C). Even in either cortex or medulla samples, we could not observe apparent changes in the total protein levels of WNK4, OSR1, SPAK, and NCC between 20:00 and 8:00, further supporting the idea that the difference in phosphorylation of each protein might not be caused by its change in total protein level.

3.2. The effect of aldosterone on the diurnal rhythm of the WNK-OSR1/SPAK-NCC signal cascade

The plasma aldosterone concentration (PAC) has circadian fluctuations in humans [29] as well as in rodents [30,31]. In rodents, the PAC is elevated at the end of the inactive phase and falls at the end of the active phase; this pattern is similar to the pattern we observed in the diurnal rhythm of the WNK-OSR1/SPAK-NCC signal cascade. Although the detail of signal transduction from aldosterone to WNK is not known, we previously showed that this cascade is regulated by aldosterone [26,32]. Therefore, we hypothesized that the circadian fluctuations of PAC could be an upstream regulator for the diurnal rhythm of the WNK-OSR1/SPAK-NCC cascade. To examine this hypothesis, we investigated whether inhibition of aldosterone by eplerenone affected the diurnal rhythm of this kinase cascade in the kidney.

The C57BL/6J mice were divided into two groups, the control group and the eplerenone group. All mice were sacrificed at 20:00 (beginning of active period) or 8:00 (beginning of rest period) by cervical dislocation after adaptation to a 12-h light/12-h dark cycle for 2 weeks. Eplerenone (10 mg/day) was administered to the mice during the adaptation period.

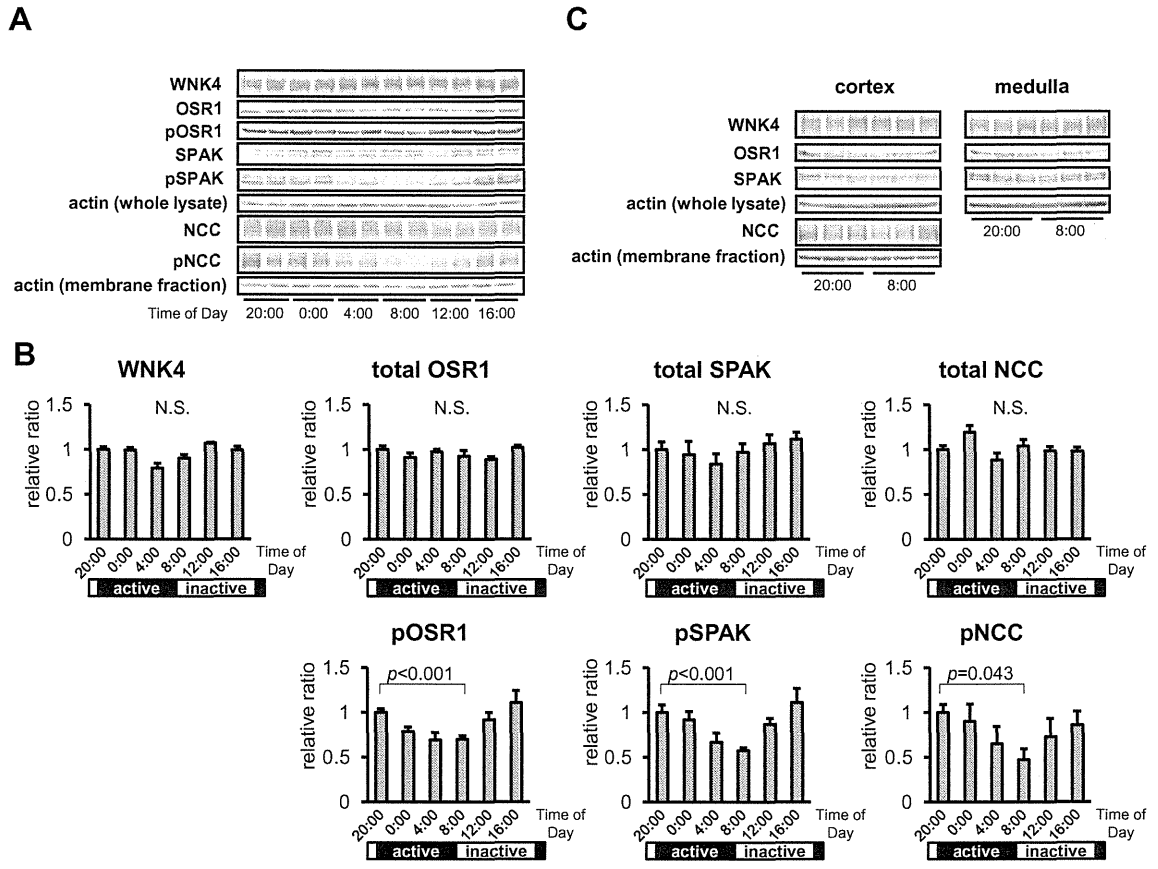

Fig. 1. The diurnal rhythm of phosphorylation status of the WNK-OSR1/SPAK-NCC signal cascade in the kidneys of C57BL/6J mice. (A) Representative immunoblots of WNK4, OSR1, phosphorylated OSR1, SPAK, phosphorylated SPAK, NCC, and phosphorylated NCC in the kidney at each time point. (B) Relative abundance of total and phosphorylated proteins. The total expression level of each protein (WNK4, OSR1, SPAK, and NCC) did not present an apparent diurnal rhythm, whereas phosphorylated OSR1, phosphorylated SPAK, and phosphorylated NCC exhibited acrophase around 20:00 and bathyphase around 8:00. N.S., not significantly different. (C) The total expression levels of WNK4, OSR1, SPAK, and NCC in the kidney cortex and medulla at 20:00 and 8:00. They did not show significant difference between 20:00 and 8:00 in the cortex and medulla. Immunoblot of NCC in the medulla is not shown since NCC was not detected in the medulla.

In our experiments, the PAC of the control group at 20:00 was significantly higher than that at 8:00 (349.50 ± 61.28 pg/ml at 20:00 and 179.40 ± 40.20 pg/ml at 8:00, $n = 12$, $p = 0.031$), which was consistent with previous studies [30,31]. On the other hand, the PAC of the eplerenone group at 20:00 and 8:00 was elevated to 1270.0 ± 64.9 pg/ml and 1337.5 ± 58.6 pg/ml, respectively ($p = 0.469$). These values were markedly higher than the corresponding values in the control group and indicated that the diurnal change in PAC was lost in the eplerenone group.

The immunoblot analysis of NCC and phosphorylated NCC in kidney homogenates is presented in Fig. 2. In the eplerenone group, the phosphorylation of NCC was decreased both at 20:00 and 8:00 as compared to the control group, and its diurnal fluctuation was remarkably decreased. The ratio of the phosphorylation status of NCC at 20:00 to that at 8:00 in the control group was 2.1, whereas the ratio in the eplerenone group was 1.1.

4. Discussion

The central pacemaker in the SCN functions autonomously but is reset each day by light signal. The strict scientific definition of circadian rhythm is a process that exhibits a 24-h pattern of oscillation under constant condition in the absence of timing cues. Therefore, we used the term "diurnal" instead of "circadian" in this study to describe the data obtained in the animal experiments because the animals were entrained on 12-h light–dark cycles.

What is the physiological role of the diurnal rhythm of the WNK-OSR1/SPAK-NCC signal cascade? Blood pressure and sodium excretion of humans increase during the daytime and decrease during the nighttime [33]. Considering this fact, one possible interpretation of our data would be that the WNK-OSR1/SPAK-NCC signal cascade may start to be suppressed upon awakening to prepare for adequate sodium excretion during the day. After adequate sodium excretion, this cascade may start to accelerate at bedtime to decrease sodium excretion during the night.

In this respect, we hypothesized that dysregulation of this cascade might cause hypertension with a non-dipping pattern, since volume expansion by dysregulated salt excretion is the major cause of this type of hypertension [34]. Although there have been no clear reports as to whether PHAII patients have non-dipping hypertension, the hypertension of PHAII patients is salt-sensitive and can be turned to normotensive by sodium restriction or diuretics [17]. In addition, polymorphisms of WNK genes are associated with essential hypertension in the general population [35–37]. Although the pathophysiological roles of these polymorphisms are not clear, these findings suggest that some cases of essential hypertension with a non-dipping pattern might be caused by dysregulation of this cascade. To confirm this hypothesis, it is necessary to establish methods to evaluate the activity of this cascade in patients. Quantification of urinary excretion of phosphorylated NCC could be a candidate marker to estimate the activity of this cascade in human patients.

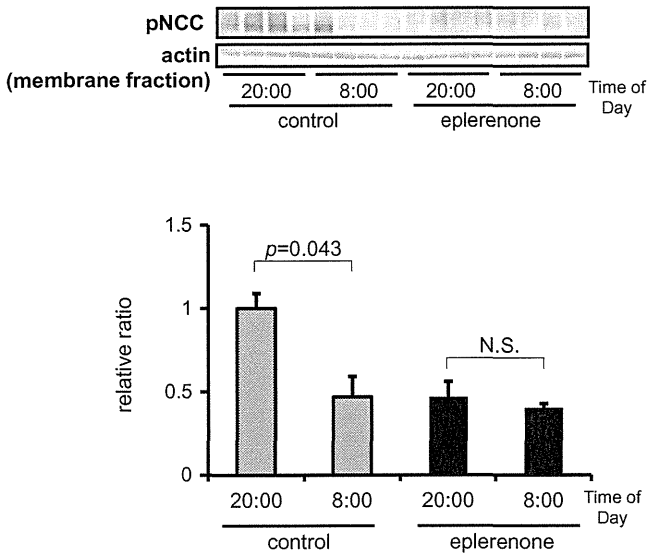

Fig. 2. The effect of aldosterone on the diurnal rhythm of the WNK-OSR1/SPAK-NCC signal cascade. The immunoblot analysis of NCC and phosphorylated NCC in the control group and the eplerenone group at 20:00 and 8:00 is exhibited. The graph shows a comparison of the immunoblot band densities of phosphorylated NCC. In the eplerenone group, the phosphorylation status of NCC was decreased both at 20:00 and at 8:00, and its diurnal rhythm was remarkably diminished. The ratio of the phosphorylation level of NCC at 20:00 to that at 8:00 in the control group was 2.1, whereas the ratio in the eplerenone group was 1.1. N.S., not significantly different.

Na–H exchanger (NHE3), epithelial sodium channel (ENaC) and Na–K–2Cl cotransporter 2 (NKCC2) also play major roles for sodium reabsorption in the kidney [38]. Nishinaga showed by real-time PCR that the level of NHE3 mRNA in the thin descending limbs and thick ascending limbs had a circadian rhythm with a peak at the rise time [39], which is similar to the diurnal rhythm of the WNK-OSR1/SPAK-NCC cascade. Furthermore, Gumz showed by real-time PCR that the expression of ENaC in the inner medullas, outer medullas, and cortex was increased around rise time and decreased around bedtime [40]. Zuber also showed similar results in microdissected distal tubules and collecting duct segments by microarray analysis [27]. These results indicate that the circadian rhythm of expression of NHE3 and ENaC is parallel to that of the WNK-OSR1/SPAK-NCC cascade. Since the regulation of NHE3 and ENaC is also highly dependent on aldosterone [41,42], NCC, NHE3, and ENaC may be involved in the circadian regulation of Na reabsorption as effectors of aldosterone in the kidney. On the other hand, the expression of NKCC2 in distal tubules was shown to have an acrophase around bedtime by the same microarray analysis. Since the distal tubules are not the main site of NKCC2 expression [43], these data do not necessarily exclude the involvement of NKCC2 in the coordinated regulation with NCC, NHE3, and ENaC. It may be necessary to study the circadian rhythm of NKCC2 expression in the thick ascending limb.

Although NCC transcripts had an acrophase around rise time in Zuber's microarray analysis [27], we did not observe a diurnal rhythm in the protein level of NCC by immunoblot analysis. Rather, we found that the level of NCC phosphorylation showed a diurnal rhythm. Phosphorylation is an important regulator of NCC activity and its plasma membrane localization [19] and could be a rapid way of regulating protein function since it does not require protein synthesis. In this respect, it may be reasonable to conclude that the diurnal rhythm of NCC activity was regulated not by its protein level but by its phosphorylation level. In this regard, mechanism(s) of circadian regulation of the WNK-OSR1/SPAK-NCC cascade may be different from those of NHE3 and ENaC. Both the NHE3 and ENaC genes contain E-box-binding elements (CANNTG) in their promoters [44,45], which are critical for the series of transcription-based feedback loops of clock genes [46]. Since Per1, Per2 and Bmal1 are induced by aldosterone [47], aldosterone might regulate NHE3 and ENaC via these clock genes. In addition, mRNA expression of the alpha subunit of ENaC (αENaC) was shown to be regulated by Per1 [40,44]. The expression of αENaC mRNA was attenuated in the renal medulla of the Per1 knockout mice, and these mice exhibited increased urinary sodium excretion. Furthermore, the circadian pattern of renal αENaC mRNA was dramatically altered in the knockout mice [40]. In contrast to these transcription-mediated regulations of transporters and channels, what we observed in the WNK-OSR1/SPAK-NCC cascade is not a change in the absolute abundance of these components, but the levels of phosphorylation (i.e., the activity of the kinases). Since we previously showed that exogenous aldosterone and spironolactone administration increased and decreased the phosphorylation of OSR1, SPAK and NCC in the kidney, respectively [26], it is certain that aldosterone is an upstream regulator of this cascade. However, we have not yet clarified how the signal from aldosterone to WNK kinases is transmitted. We could observe that the increased phosphorylation of OSR1, SPAK, and NCC by low salt intake, which was spironolactone-sensitive [26], was blunted in the Sgk1 knockout mice [48], suggesting that SGK1 might mediate the signal from aldosterone to WNK kinase. However, the detail of aldosterone action on WNK is still under investigation. Identification of the mechanism will also help to clarify how the activity of WNK-OSR1/SPAK-NCC signal cascade is regulated diurnally.

In summary, the WNK-OSR1/SPAK-NCC cascade has a circadian rhythm, which may regulate the diurnal rhythm of sodium excretion with other sodium transport proteins.

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