SPAK Deficiency Corrects Pseudohypoaldosteronism II Caused by WNK4 Mutation

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Abstract

Stimulation of the OSR1 (Oxidative stress-responsive kinase-1)/SPAK [STE20 (sterile 20)/SPS1-related proline/alanine-rich kinase]-NCC (Na+-Cl- cotransporter) signaling cascade plays an important role in the WNK [With-No-Lysine (K)] kinase 4 (Wnk4) signaling pathway. Here, mice crossed with kidney tubule-specific (KSP) Osr1 knockout (KSP-Osr1+/−) and SpaK knockout (Spak+/−) mice exhibited exaggerated salt excretion in response to thiazide diuretics while Wnk4+/− mice recapitulated the phenotypes of PHA II, Bartter-like syndrome, and Gitelman syndrome, respectively. Wnk4+/−/KSP-Osr1+/− mice became normotensive and lacked the PHA II phenotype. Phosphorylated Spak and Ncc were similarly increased in both Wnk4+/−/KSP-Osr1+/− and Wnk4+/−/KSP-Osr1−/− mice while phosphorylated Ncc normalized in Wnk4+/−/Spak+/− mice. Furthermore, Wnk4+/−/KSP-Osr1+/−/Spak+/− mice exhibited normal responses. Wnk4+/−/KSP-Osr1−/−/Spak+/− triple mutant mice had low blood pressure and diminished phosphorylated Ncc. Both SPAK and OSR1 are important in the maintenance of blood pressure but activation of SPAK-NCC plays the dominant role in PHA II. SPAK may be a therapeutic target for disorders with salt-sensitive hypertension related to WNK4 activation.

Introduction

The kidneys are vital to salt balance and blood pressure regulation. The thiazide-sensitive sodium chloride cotransporter (Na+-Cl- cotransporter, NCC) in the distal convoluted tubule (DCT) is responsible for 5-10% of total filtered Na+ reabsorption and regulates Na+ homeostasis and blood pressure.[1,2] Inactivating mutations of the NCC gene SLC12A3 cause autosomal recessive Gitelman syndrome (GS) characterized by renal salt wasting with secondary hypertension and hyperaldosteronism, renal potassium (K+) wasting with chronic hypokalemia, metabolic alkalosis, and hypocalciuria.[3-10] In contrast, autosomal dominant Gordon syndrome, also called familial hyperkalemic hypertension and pseudohypoaldosteronism type II (PHA II), is the mirror image of GS and featured with thiazide-responsive salt-sensitive hypertension with low plasma renin activity (PRA) and aldosterone levels and hyperkalemic metabolic acidosis.[11-14] It is caused by gain of NCC function from well-described mutations in the WNK [With-No-Lysine (K)] 1 and 4 kinase genes[15] and newly-reported mutations in Kelch-like 3 or cullin 3.[16-18] Previous in vitro studies have demonstrated SPAK [STE20 (sterile 20)/SPS1-related proline/alanine-rich kinase] and OSR1 (Oxidative stress-responsive kinase-1) as downstream substrates of WNK kinases.[19-22] They belong to the same germinal center kinase VI family and share high sequence homology in their N-terminal catalytic and C-terminal regulatory domains. In the kidneys, the distributions of SPAK and OSR1 virtually overlap in the distal nephron.[23-25] Activated SPAK/OSR1 subsequently phosphorylate and activate several cation-chloride cotransporters (CCG), including Na+-K+-2Cl− cotransporter isoform 1 and 2 (NKCC1, NKCC2), NCC, and potassium chloride cotransporter. [26-28] The WNK-SPAK/OSR1 signaling cascade plays a pivotal role in volume regulation and blood pressure control.[27,29-31] In the mutant Wnk4 D561A mouse model of PHA II, constitutively active Wnk4 increased phosphorylation of Spak/Osr1 and lead to Ncc overactivity in the DCT.[32] In contrast, Wnk4 hypomorphic or deficient mice exhibit decreased phosphorylated (p)-Spak/Osr1 and p-Ncc and hypotension. In the next step of the cascade, knock-out Spak and Osr1 mice exhibit hypotension with decreased abundance of p-Nkcc and p-Ncc.[24,29,33]

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### Table 1. Phenotype in Wnk4^{D561A/+}:Ksp-Osr1^{+/−} littermate mice.

| Genotype (n) | Systolic BP (mmHg) | Body Weight (g) | Plasma | Urine (ml/day) | FENa (%) | FEK (%) | FECl (%) | FESO4 (%) | Ca<sup>2+</sup>/Cr (mg/mg) |
|--------------|-------------------|----------------|--------|----------------|-----------|---------|----------|-----------|-------------------|
| WT (♂)      | 112.5±10.0        | 29.7±5.1       | Aldosterone (pg/ml) | 832±120.0 | 9.0±0.5  | 152±3   | 4.2±0.3  | 115±2   | 26±4.0             |
| Wnk4^{D561A/+} (♂) | 135.3±9.1<sup>a</sup> | 26.1±4.5  | PRAng ml h | 5.5±1.6<sup>a</sup> | 151±2.4  | 4.9±0.2<sup>a</sup> | 118±2<sup>a</sup> | 22±2.5<sup>a</sup> | 0.26±0.06          |
| Ksp-Osr1<sup>+/−</sup> (♂) | 108.0±8.1<sup>b</sup> | 26.1±4.6  | Na<sup>+</sup> (mmol/l) | 5.1±3.1<sup>b</sup> | 154±3   | 3.9±0.2<sup>b</sup> | 114±3<sup>b</sup> | 27±5.1<sup>b</sup> | 0.27±0.07          |
| Wnk4^{D561A/+}:Ksp-Osr1<sup>+/−</sup> (♂) | 133.6±11<sup>bc</sup> | 25.6±1.5  | Cl<sup>−</sup> (mmol/l) | 4.2±2.2<sup>c</sup> | 153±4   | 4.8±0.2<sup>c</sup> | 119±2<sup>c</sup> | 23±3.1<sup>c</sup> | 0.31±0.10          |

BP, blood pressure; PRA, plasma rennin activity; Cr, creatinine; FENa, FEK, FECl and FESO4 represent the fractional excretion of Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>−</sup> and Mg<sup>2+</sup> respectively. *p<0.05 vs. WT. **p<0.05 vs. Wnk4^{D561A/+}: Spak<sup>+/−</sup>. doi:10.1371/journal.pone.0072969.t001

### Table 2. Phenotype in Wnk4^{D561A/+}:Spak<sup>+/−</sup> littermate mice.

| Genotype (n) | Systolic BP (mmHg) | Body Weight (g) | Plasma | Urine (ml/day) | FENa (%) | FEK (%) | FECl (%) | FESO4 (%) | Ca<sup>2+</sup>/Cr (mg/mg) |
|--------------|-------------------|----------------|--------|----------------|-----------|---------|----------|-----------|-------------------|
| WT (♂)      | 109.8±2.9         | 213.3±3.2      | Aldosterone (pg/ml) | 831±262.7 | 5.5±1.0  | 149±5   | 4.2±0.2  | 114±2   | 25±5              |
| Wnk4^{D561A/+} (♂) | 128.3±2.2<sup>a</sup> | 239.3±3.1     | PRAng ml h | 2.2±0.4<sup>a</sup> | 152±2   | 4.9±0.2<sup>a</sup> | 117±3<sup>a</sup> | 21±2<sup>a</sup> | 0.26±0.04          |
| Spak<sup>+/−</sup> (♂) | 101.4±4.0<sup>b</sup> | 24.4±4.4      | Na<sup>+</sup> (mmol/l) | 2.2±0.4<sup>a</sup> | 148±3   | 3.6±0.2<sup>b</sup> | 109±2<sup>b</sup> | 30±4<sup>b</sup> | 0.32±0.04<sup>a</sup> |
| Wnk4^{D561A/+}:Spak<sup>+/−</sup> (♂) | 107.6±4.8<sup>bc</sup> | 22.4±4.3     | K<sup>+</sup> (mmol/l) | 1.0±3.5<sup>a</sup> | 151±2   | 4.3±0.3<sup>bc</sup> | 113±3<sup>bc</sup> | 24±3<sup>bc</sup> | 0.33±0.03<sup>a</sup> |

BP, blood pressure; PRA, plasma rennin activity; Cr, creatinine; FENa, FEK, FECl and FESO4 represent the fractional excretion of Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>−</sup> and Mg<sup>2+</sup> respectively. *p<0.05 vs. WT. **p<0.05 vs. Wnk4^{D561A/+}: Spak<sup>+/−</sup>. doi:10.1371/journal.pone.0072969.t002
The individual roles of Spak and Osr1 in the pathogenesis of PHA II in vivo have not been easily elucidated as they are coincidentally expressed in the DCT and thick ascending limb (TAL). [29] To study them individually, we abolished the expression of Spak or Osr1 specifically in the distal nephron in a mouse model of PHA II by crossing constitutively active Wnk4D561A/+ knockin (K) mice with kidney tube-specific (KSP−Osr1) knockout (KSP−Osr1−/−) or Spak knockout (Spak−/−) mice.[29,33] The results to be reported indicated that Wnk4D561A+/+KSP−Osr1−/− mice still exhibited the PHA II phenotype with an exaggerated response to thiazide diuretics while Wnk4D561A+/+Spak−/− mice exhibited a normal phenotype with normal p-NCC expression and response to thiazide. We also created Wnk4D561A+/+Spak−/−,KSP−Osr1−/− mice, which exhibited low blood pressure with decreased p-NCC, suggesting that SPANK may play the more dominant role in the pathogenesis of PHA II but Osr1 can compensate in its absence.

**Materials and Methods**

**Animals**

The experimental protocols used in the present study were approved by the Institutional Animal Care and Use Committee of the National Defense Medical Center, Taipei, Taiwan. Wnk4D561A+/+ (C57BL/6 x 129S4/SvJae background), Spak−/− (C57BL/6 and 129X1/SvJ x 129S1 background) and KSP−Osr1−/− (C57BL/6 and 129X1/SvJ x 129S1 background) mice were generated as we described previously.[29,32,33] Wnk4D561A+/+ mice were crossed with Spak−/− and KSP−Osr1−/− to obtain Wnk4D561A+/+Spak−/− and Wnk4D561A+/+KSP−Osr1−/− mice. Then Wnk4D561A+/+Spak−/− and Wnk4D561A+/+KSP−Osr1−/− mice were crossed with Spak−/− and KSP−Osr1−/− mice, respectively, to obtain the Wnk4D561A+/+Spak−/−,KSP−Osr1−/− double transgenic mice (Figure S1). Wnk4D561A+/+Spak−/−,KSP−Osr1−/− triple mutant mice were obtained by systemically breeding Wnk4−/−,Spak−/−,KSP−Osr1−/− and Wnk4D561A+/+KSP−Osr1−/−. Spak+/− mice. These mice were raised on a 12-hour day and night cycle and fed normal rodent chow diet [Na+: 0.4% (w/w); K+: 1.0% (w/w); Ca2+: 0.9% (w/w)] and plain drinking water ad libitum for 12–14 weeks. The phenotypes of male mice were evaluated at the age of 12–14 weeks.

**Blood and urine analysis and blood pressure measurement**

Blood was drawn from the submandibular venous plexus under light ether anesthesia. Mice were kept in metabolic cages for 24-h urine collection. Serum and urine biochemistries and plasma renin activity and aldosterone were measured as previously described.[29,33,34] The blood pressure of restrained conscious mice at steady state was measured with a programmable tail-cuff sphygmomanometer (MK-2000A, Muromachi, Tokyo).[34]

**Hydrochlorothiazide (HCTZ) and furosemide administration**

Hydrochlorothiazide (HCTZ, 12.5 mg/kg) and furosemide (15 mg/kg) were administered intraperitoneally in Wnk4D561A+/+,KSP−Osr1−/− and Wnk4D561A+/+Spak−/− littermates to determine the activity of Ncc and Ncc2 in vivo. Urine samples were collected for analysis after 4 hours.[29,33]

**Immunoblotting and immunofluorescence staining**

Immunoblotting and immunofluorescence staining were carried out as previously described.[29,33,35] The intensities of the resulting immunoblot bands were determined by UVP Bio-imaging system (Cambridge, UK) followed by densitometry (VisionWorksLS Image Acquisition and Analysis Software, Upland, CA). All densitometry data were normalized to the mean of wild type group. The antibodies used in this study include our previously-generated rabbit anti-p-NCC (T53, T58 and T71),[29,33] anti-p-Osr1(S325)/SPAK(S383)[21,36,37] and anti-p-KCC2 (T96)[29,33] antibodies, and other commercially available rabbit anti-SPA (Cell Signaling),[37] Na+−K+−2Cl− cotransporter 2 (NKCC2) (Alpha Diagnostic),[29,34] NCC (Millipore),[32] and mouse anti-OSR1 (Abnova).[29,33] All primary antibodies were used at 1:200 dilution for immunoblotting. Alkaline phosphatase-conjugated anti-IgG antibodies (1:3000 dilution, Promega) were used as secondary antibodies for immunoblotting and Alexa 488 or 546 dye-labeled (Molecular Probes) secondary antibodies were used for immunofluorescence staining. The immunofluorescence images were obtained by confocal microscopy (LSM510, Carl Zeiss).

**Statistical analysis**

All results are expressed as mean ± standard deviation (SD). The significance of differences between groups was examined by K-independent samples Kruskal-Wallis nonparametric test with SPSS 21.0 for Windows (SPSS, Chicago, IL), followed by Mann-Whitney two-sample test. The slope of Urine Na+ and Cl− excretion rates between before and after diuretics administering was analyzed by one-sample Kolmogorov-Smirnov test. A P-value less than 0.05 was considered to be statistically significant.

**Results**

**Phenotypes**

We have previously reported the phenotypes of Wnk4D561A+/+, KSP−Osr1−/−, and Spak−/− mice, which recapitulates PHA II, Barter syndrome and Gitelman syndrome respectively.[29,33,34]

**Wnk4D561A+/+,KSP−Osr1−/− mice**

As seen in Table 1, Wnk4D561A+/+ mice exhibited the typical phenotype of PHA II with significant hypervolemia reflected by higher systolic blood pressure and hyperreninemia, hyperkalemia with decreased fractional urinary K+ excretion (FEK), and hypercalciuria. KSP−Osr1−/− mice exhibited a Barter syndrome-like phenotype with significant hypokalemia due to excessive renal K+ secretion and hypercalciuria. Of note, Wnk4D561A+/+,KSP−Osr1−/− mice still preserved all the phenotypic indices of PHA II, which were not significantly different from those in Wnk4D561A+/+ mice, indicating that the renal phenotype of Wnk4D561A+/+ mice can not be corrected by the genetic deletion of Osr1 in the kidney.

**Wnk4D561A+/+,Spak−/− mice**

Spak−/− mice resembled the phenotype of Gitelman syndrome with relative hypotension, secondary hyperaldosteronism, low plasma K+ concentration with increased FEK, hypomagnesemia with increased fractional urinary Mg2+ excretion (FEgk),[37] and hypocalciuria (Table 2). Wnk4D561A+/+,Spak−/− mice became normotensive and exhibited similar plasma and urine indices as WT mice (except fractional urinary Cl− excretion) suggesting that the phenotype of Wnk4D561A+/+ mice can be corrected by Spak deficiency in the kidney.

**Wnk4D561A+/+,KSP−Osr1−/−,Spak−/− mice**

Out of 160 offsprings, we only obtained two Wnk4D561A+/+,KSP−Osr1−/−,Spak−/− mice (n = 2). These mice exhibited relative hypotension (97 ± 5.0 mmHg vs 110 ± 4.0 mmHg in WT) and
Figure 1. Osr1 gene deletion does not change phosphorylation of Ncc and Nkcc2 in PHA II. Shown is the representative immunoblot (top, n = 3/group) and densitometry (bottom, n = 6/group) of (A) total Ncc, (B) p-Ncc (T53), (C) p-Ncc (T58), (D) p-Ncc (S71), (E) total Nkcc2, and (F) p-Nkcc2 (T96) in whole kidneys of WT, Wnk4<sup>D561A/+</sup>, KSP-Osr1<sup>−/−</sup>, and Wnk4<sup>D561A/+</sup>.KSP-Osr1<sup>−/−</sup> mice, representively. Semiquantitative measurements of each
mild hypokalemia (3.7 ± 0.3 mmol/l vs 4.2 ± 0.4 mmol/l in WT) as compared to their WT littermates.

Protein expression and phosphorylation of Ncc, Nkcc2, Spak, and Osr1

Compared to WT controls, Wnk4^D561A/+ mice had increased expressions of total Ncc (433.0 ± 49.7%, \( p < 0.01 \)), p-NccT53 (223.2 ± 38.1%, \( p < 0.01 \)), p-NccT58 (275.0 ± 20.7%, \( p < 0.01 \)), p-NccS71 (548.3 ± 80.8%, \( p < 0.01 \)) (Figure 1A-D), and p-Nkcc2 (164.6 ± 16.6%, \( p < 0.01 \), Figure 1F) assessed by semi-quantitative immunoblotting of whole kidney homogenate. As shown in Figure S2, Nkcc2 was predominantly expressed in the medulla and p-Nkcc2 mostly expressed in the cortex of WT mice. In Wnk4^D561A/+ mice, the abundance of Nkcc2 in the medullar region was reduced but p-Nkcc2 in the cortical region was increased. KSP-Osr1^−/− mice had slightly increased total Ncc (101.4 ± 12.0%, Figure 1A) and p-NccT53, T58 and S71 (116.4 ± 4.0%, 121.8 ± 7.5%, 143.9 ± 16.5%, all \( p < 0.05 \), Figure 1B-D), but a significant

Figure 2. Immunofluorescence images of Ncc and Nkcc2 in kidneys. (Osr1 experiment series) Representative immunofluorescence images of (A) total Ncc, (B) p-Ncc (T53), (C) p-Ncc (T58), (D) p-Ncc (S71), (E) total Nkcc2, and (F) p-Nkcc2 (T96) in kidneys of WT, Wnk4^D561A/+, KSP-Osr1^−/−, and Wnk4^D561A/+;KSP-Osr1^−/− mice. The scale bars indicate 20 µm.

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decreased p-Nkcc2 (63.9±4.0%, p<0.01, Figure 1F) with unchanged total Nkcc2 (Figure 1E). Wnk4ΔD561A/+KSP-Osr1+/− mice still had significantly increased total Ncc (371.9±25.8%, p<0.01), p-NccT53 (131.4±6.6%, p<0.01, Figure 1B), p-NccT58 (211.8±23.6%, p<0.01), and p-NccS71 (424.2±49.4%, p<0.01) (Figure 1A-D), and p-Nkcc2 (179.2±12.3%, p<0.01, Figure 1F) with unchanged Nkcc2. Similar to Wnk4ΔD561A/+ mice, the expression of p-Spak was increased in KSP-Osr1+/− and Wnk4ΔD561A/+KSP-Osr1+/− mice (data not shown). The results measured by semi-quantitative immunofluorescence of Ncc and Nkcc2 in the kidney sections of WT, Wnk4ΔD561A/+, KSP-Osr1+/−, and Wnk4ΔD561A/+KSP-Osr1+/− mice were consistent with the immunoblotting findings (Figure 2 and Figure S3).

In Spak−/− mice, the expression of total Ncc (46.7±9.0%, p<0.01) and p-NccT53 (16.2±4.7%, p<0.01), p-NccT58 (39.5±2.6%, p<0.01) and p-NccS71 (23.8±8.7%, p<0.01) (Figure 3A-D) were markedly attenuated but total Nkcc2 (132.8±4.2%, p<0.01, Figure 3E) and p-Nkcc2 (308.6±24.6%, p<0.01, Figure 3F) were significantly increased. Despite increased p-Osr1 (data not shown), Wnk4ΔD561A/+Spak−/− mice had similar expressions of total Ncc, p-Ncc, total Nkcc2, p-Nkcc2 to wild type (Figure 3A-F). The immunofluorescence results of total and phosphorylated Ncc and Nkcc2 expression in the kidney sections of WT, Wnk4ΔD561A/+, Spak−/−, and Wnk4ΔD561A/+Spak−/− mice (Figure 4 and Figure S4) were also consistent with those immunoblotting observations.

In triple Wnk4ΔD561A/+Spak−/−KSP-Osr1+/− mice (n=2/each group), the expression of total Ncc (83.5±1.9%) and p-NccT53 (64.1±13.8%), p-NccT58 (80.9±2.1%) and p-NccS71 (78.2±1.9%) were markedly decreased compared to Wnk4ΔD561A/+ mice (Figure 5A-D). Total Nkcc2 (70.6±6.7%) (Figure 5E) and p-Nkcc2 (49.2±5.7%) (Figure 5F) were also decreased in Wnk4ΔD561A/+KSP-Osr1+/− triple mutant mice than WT and Wnk4ΔD561A/+ mice (Nkcc2: 87.3±10.8%; p-Nkcc2: 192.2±9.8%, Figure 5E-F).

Response to diuretics

Urinary Na+ and Cl− excretion rates (FENa and FECl2) were measured in mice before and after the administration of hydrochlorothiazide (HCTZ, a Ncc inhibitor) or furosemide (a Nkcc2 inhibitor) to assess the in vivo activities of Ncc and Nkcc2, respectively. Compared with WT and KSP-Osr1+/− mice, Wnk4ΔD561A/+ and Wnk4ΔD561A/+KSP-Osr1+/− mice exhibited exaggerated salt excretion in response to a single dose of HCTZ, indicating the Ncc overactivity in both sets of mice (Figure 6A). When challenged with furosemide, KSP-Osr1+/− mice showed blunted response in comparison with WT, suggesting lower Nkcc2 activity (Figure 6B). However, Wnk4ΔD561A/+ and Wnk4ΔD561A/+KSP-Osr1+/− micr responded similarly to WT.

The Spak−/− mice had blunted urine Na+ and K+ excretion compared to WT in response to HCTZ (Figure 7A), indicating lower Ncc activity. Interestingly, the response of Wnk4ΔD561A/+Spak−/− mice was between that of Spak−/− and Wnk4ΔD561A/+ mice and similar to WT controls, indicating that the Ncc function had normalized in these mice. In the furosemide challenge, Spak−/− and Wnk4ΔD561A/+Spak−/− mice had significantly increased Na+ and Cl− excretion, suggesting increased Nkcc2 function (Figure 7B).

Discussion

In this study, we crossed Wnk4ΔD561A/+ mice with KSP-Osr1+/− and Spak−/− mice to investigate the independent roles of Osr1 and Spak in the pathogenesis of Wnk4-PHA II. Wnk4ΔD561A/+KSP-Osr1+/− mice preserved the PHA II phenotype with increased abundance of p-Spak and p-Ncc and corresponding exaggerated response to thiazide diuretics. Wnk4ΔD561A/+Spak−/− mice exhibited normal blood pressure and blood/urine electrolytes with relatively normal abundance of p-Ncc despite enhanced p-Osr1 expression and a normal response to thiazides. These findings indicated that activation of Spak-Ncc plays the more dominant role in Wnk4-PHA II, which is affirmed by the decreased total expression and phosphorylation of Ncc in triple mutant Wnk4ΔD561A/+Spak−/−KSP-Osr1+/− mice.

Mutations in the Wnk4 kinase gene have been shown to cause many cases of PHA II.[15,38-40] Recent studies have implicated
the activation of downstream WNK4 substrates, SPAK and OSR1, in the pathogenesis of PHA II.[26,32,36,41,42] Although SPAK has been found to be predominantly expressed in the cortex and OSR1 in the medulla, both are expressed in the TAL and DCT.[29] Unlike the interchangeability of SPAK and OSR1 in peripheral neurons,[43] these two kinases seem to be differentially regulated and have different function in renal tubules. This study clarified the relative contribution of Spaek and Osr1 to PHA II in vivo. Wnk4+/+ mice had increased expression of total Ncc and p-Ncc in parallel with increased total and p-Spak. Their exaggerated response to thiazide diuretics indicated Ncc hyperactivity, similar to Wnk4+/+ mice, and in line with the immunoblotting. These findings suggest that Osr1 is not essential and can be fully compensated by the increased Spaek expression in this model of PHA II. However, the decreased p-Ncc expression found in heterozygous kinase-dead Osr1 knockin (Osrt151A/+ ) mice and Wnk4+/+ mice suggested that the rescue of Ncc activation in Osr1 deficient states depends on abnormal activation of the Spaek pathway.[41]

The roles of Osr1 and Spaek on Nkcc2 in TAL were also clarified by this study. Ksp-Osr1+/− mice exhibited reduced p-Nkcc2, indicating that Osr1 is an up-regulator of Nkcc2. Substantively, the Spaek−/−, Wnk4+/+ , and Wnk4+/+ .Spak−/− mice all exhibited increased p-Nkcc2. These three sets of mice share the commonality of preserved or increased Osr1. However, the Wnk4+/+ .Ksp-Osr1+/− mice also exhibited increased p-Nkcc2 suggesting that mutant Wnk4 can activate Nkcc2 through activated Spaek. This is corroborated by our finding of decreased p-Nkcc2 when Spaek is abolished in the triple mutant Wnk4+/+ ,Ksp-Osr1+/−.Spak−/− mice. It would appear that Osr1 is the major activator of Nkcc2 but Spaek may play a role in abnormally activated states. Recently, it has reported that the kinase-deficient SPAK variant, so-called kidney specific SPAK (KSp-SPAK), functions as an antagonist of OSR/SPAK-NKCC2 pathway and is the major SPAK isoform in renal medulla.[27] Since WNK4 expression in the Henle’s loop is primarily in the cortical TAL,[44] the role of KS-SPAK in PHA II with WNK 4 mutation is still questionable.

The furosemide challenge studies generally correlate with the densitometry studies except in the Wnk4+D561A/+ and Wnk4+D561A/+ .Ksp-Osr1+/− mice, which had normal responses to furosemide despite increased phosphorylated Nkcc2. It is important to note that both of these mice have hyperactive downstream Ncc, which may attenuate the observable response to furosemide. Supporting this theory is the observation that Wnk4+D561A/+ .Spak−/− mice, with their increased activated Osr1 and p-Nkcc2 but relatively normal p-Ncc expression, showed an exaggerated response to furosemide, providing direct evidence...
linking increased Nkcc2 activity through Osr1. Besides Wnk4, other upstream regulator, such as WNK1 or calcium-binding protein 39 (Cab39), also regulate OSR1-NKCC2 pathway.\[19,21,45–47\].

Regarding the Ncc in the distal nephron, Spak appears to be the dominant player as \(Wnk4^{D561A/+}\).\(\text{Spak}^{2/2}\) mice became virtually normal phenotype with expression levels of total and p-Ncc similar to WT littermates, indicating that the PHA II phenotype could be effectively corrected by Spak deficiency. The response to thiazide diuretics in \(Wnk4^{D561A/+}\).\(\text{Spak}^{2/2}\) mice was similar to WT mice (and higher than \(\text{Spak}^{2/2}\) mice) suggesting that Spak is a major but not sole activator of Ncc in \(Wnk4\)-PHA II. Osr1 is a likely accomplice and increased Osr1 activity through activated Wnk4 may compensate enough to sustain normal Ncc expression and activity. This borne out by the finding of decreased expression and phosphorylation of Ncc in triple mutant \(Wnk4^{D561A/+}\).\(KSP^{-}\).\(\text{Spak}^{2/2}\) mice, where Osr1 has been abolished.

The phenotype and Ncc phosphorylation level of our \(Wnk4^{D561A/+}\).\(\text{Spak}^{2/2}\) mice resembled those of the recently-reported \(Wnk4^{D561A/+}\).\(\text{Spak}^{T243A/T243A}\) mice [kinase-dead knock-in],[41] which also support the importance of SPAK kinase activity in PHA II. Because both Spak deficiency and inhibition of Spak kinase activity corrected the phenotype of PHA II due to \(Wnk4\) mutation, specific inhibition of SPAK may be a plausible therapy for patients with salt-sensitive hypertension related to \(Wnk4\) activation. Since human PHA II is also linked to the mutations in WNK1, Kelch-like 3 or cullin 3 genes, the SPAK in those gene mutations will need to be clarified first.\[16,17,20,58–60\].

In conclusion, \(Wnk4^{D561A/+}\).\(\text{Spak}^{2/2}\) mice exhibited normal phenotype with relatively normal Ncc activity while \(Wnk4^{D561A/+}\).\(\text{Spak}^{+/+}\) mice overexpressed Ncc activity. Spak appears to be the major activator of Ncc while Osr1 is the major activator of Nkcc2. However, they may be overdriven to compensate for the other’s absence in PHA II conditions. Disruption of Spak-Ncc cascade can efficiently correct hypertension and hyperkalemia in the \(Wnk4\)-PHA II mouse model. This study suggests that inhibition of Spak can be a promising therapy for salt-sensitive hypertension with \(Wnk4\)-SPAK-dependent NCC activation.

Figure 7. Spak gene deletion normalizes the response to HCTZ and exaggerates the response to furosemide in PHA II mice. Responses of FENa and FECl in WT (■), \(Wnk4^{D561A/+}\) (○), \(\text{Spak}^{+/+}\) (▲), and \(Wnk4^{D561A/+}\).\(\text{Spak}^{−/−}\) (●) littermate mice (n = 6/group) to (A) HCTZ and to (B) furosemide. *denotes \(p<0.05\) vs. WT. **\(p<0.05\) vs. \(Wnk4^{D561A/+}\).

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Historically, WNK4 was reported to inhibit membrane trafficking of NCC based on oocyte experiments.[52–54] However, this Ncc inhibitory mechanism has not been found in vivo. Similarly, in vitro studies proposing various mechanisms WNK4-related for Ncc degradation are equally suspect.[55,56] How WNK4 directly affects NCC in vivo merits further study.

Thiazide diuretics are commonly and effectively used to treat human PHA II disease. However, the chronic use of thiazide also cause several side effects, such as insulin resistance with hyperglycemia, hyperlipidemia, hyperuricemia with gout, chronic kidney injury and even renal failure. These side effects can be independent of volume status and plasma \(K^+\) concentration.[57]

Because both Spak deficiency and inhibition of Spak kinase activity corrected the phenotype of PHA II due to Wnk4 mutation, specific inhibition of SPAK may be a plausible therapy for patients with salt-sensitive hypertension related to WNK4 activation. Since human PHA II is also linked to the mutations in WNK1, Kelch-like 3 or cullin 3 genes, the SPAK in those gene mutations will need to be clarified first.[16,17,20,58–60].
Supporting Information

Figure S1 Expression of Osr1 and Spak in kidneys of Wnk4/D561A/+ KSP-Or1−/− and Wnk4/D561A/+ Spak−/− mice. Representative immunoblots from three separate experiments of (A) Osr1 (top) and phosphorylated (p-)Osr1 (bottom) abundance in the kidneys of WT, Wnk4/D561A/+ KSP-Or1−/−, and Wnk4/D561A/+ KSP-Or1−/− mice and (B) Spak (top) and p-Spak (bottom) abundance in the kidneys of WT, Wnk4/D561A/+ Spak−/−, and Wnk4/D561A/+ Spak−/− mice. The scale bars indicate 100 μm. (PPT)

Figure S2 Immunofluorescence images of Nkcc2 and p-Nkcc2(T96) in kidneys of WT and Wnk4/D561A/+ mice. In WT mice, Nkcc2 (red) was dominantly expressed in the medullar (M) region and p-Nkcc2 (green) was mostly expressed in the cortical (C) region. In Wnk4/D561A/+ mice, the abundance of Nkcc2 (red) in the medullar region was reduced but p-Nkcc2 (green) in the cortical region was enhanced. The scale bars indicate 100 μm. (PPT)

Figure S3 Low-power immunofluorescence of renal Ncc and Nkcc2. (Osrl experiment series) Representative (A) total Ncc, (B) total Nkcc2(T96), (C) p-Nkcc2(T96), (D) p-Ncc(S71), (E) total Nkcc2, and (F) p-Nkcc2(T96) in kidneys of WT, Wnk4/D561A/+ KSP-Or1−/−, and Wnk4/D561A/+ KSP-Or1−/− mice. The scale bars indicate 100 μm. (PPT)

Figure S4 Low-power immunofluorescence of renal Ncc and Nkcc2. (Spak experiment series) Representative (A) total Ncc, (B) p-Ncc(T53), (C) p-Ncc(T58), (D) p-Ncc(S71), (E) total Nkcc2, and (F) p-Nkcc2(T96) in kidneys of WT, Wnk4/D561A/+ Spak−/−, and Wnk4/D561A/+ Spak−/− mice. The scale bars indicate 100 μm. (PPT)

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Author Contributions
Conceived and designed the experiments: PYC SSY SHL. Performed the experiments: PYC CJC TC SSY SHL. Analyzed the data: PYC CJC YFW SSY SHL. Contributed reagents/materials/analysis tools: YCW SU SS. Wrote the paper: PYC CJC TC SSY SHL.

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