Differential roles of WNK4 in regulation of NCC in vivo

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INTRODUCTION

The thiazide-sensitive Na⁺-Cl⁻ cotransporter (NCC) in the distal convoluted tubule (DCT) is responsible for reabsorbing 5–10% of the filtered Na⁺ load and plays an important role in regulating total body Na⁺ and extracellular fluid homeostasis. Recent studies show that NCC-mediated NaCl reabsorption in DCT also plays a critical role in renal K⁺ excretion and the total body K⁺ homeostasis (16, 25). Renal K⁺ excretion occurs predominantly via transcellular K⁺ secretion in the late DCT, CNT, and early CCD (28). The processes include K⁺ efflux from cell to lumen through apical K⁺ channels, Na⁺ reabsorption via the epithelial Na⁺ channel ENaC depolarizes the apical membrane potential and provides the electrical driving force for cellular K⁺ exit. The activity of NCC in DCT thus regulates K⁺ secretion by altering Na⁺ delivery to ENaC in the downstream DCT, CNT, and CCD.

To maintain K⁺ homeostasis, the kidney adjusts urinary K⁺ excretion to match dietary intake. Dietary K⁺ intake alters distal K⁺ secretion via both aldosterone-dependent and -independent mechanisms (29, 31). With respect to aldosterone-dependent mechanisms, K⁺ loading increases circulating levels of aldosterone to increase the activity of ENaC and Na⁺, K⁺-ATPase. These effects increase the electrical and chemical driving force for K⁺ secretion through K⁺ channels. The effect of dietary K⁺ on NCC, however, is independent of aldosterone. Dietary K⁺ loading downregulates NCC to increase Na⁺ delivery to ENaC to enhance K⁺ secretion (8, 26, 27). Unlike the effect on ENaC and Na⁺, K⁺-ATPase, downregulation of NCC by K⁺ loading is not mediated by aldosterone. Downregulation of NCC by K⁺ loading persists in mice with defective synthesis of aldosterone and in mice with knockout of SGK1, a downstream mediator of aldosterone (21, 26). Conversely to the effect of K⁺ loading, dietary K⁺ restriction stimulates NCC to diminish Na⁺ entry via ENaC and to decrease K⁺ secretion.

Recent studies have suggested that WNK (with-no-lysine [K]) kinases likely mediate aldosterone-independent mechanism of regulation of NCC by dietary K⁺. The serine-threonine WNK kinase family consists of four members in mammals (32). Mutations of two members, WNK1 and WNK4, in humans cause a hyperkalemic hypertension disease (30). WNK1 and WNK4 activate intermediate kinases, ste20-related proline/alanine-rich kinase (SPAK) or oxidative-stress response kinase-1 (OSR1), to phosphorylate NCC to increase its phosphorylation and functional activity in wild-type mice, but not in Wnk4-KO mice. Increased luminal NaCl delivery similarly upregulates NCC, which, contrary to low K⁺ intake, is not abolished in Wnk4-KO mice. The results reveal that modulation of WNK4 activity by [Cl⁻] is not the sole mechanism for regulating NCC. Increased luminal NaCl delivery upregulates NCC via unknown mechanism(s) that may override inhibition of WNK4 by high [Cl⁻].

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to NCC, such as in the setting of inhibition of Na\(^+\) reabsorption in the thick ascending limb by furosemide, induces hypertrophy of DCT and upregulation of NCC. Increased luminal NaCl delivery, unlike low K\(^+\) intake, would be expected to increase [Cl\(^-\)]. Conversely, inhibition of NCC activity by thiazide results in atrophy of DCT and downregulation of NCC transporter. How activity causes adaptive changes of NCC and DCT is unknown.

It should be emphasized that in DCT cells with active transcellular NaCl flux, a decrease in [Cl\(^-\)], under low-K\(^+\) diet would create a favoring driving force to increase NaCl entry via NCC, which would mitigate the magnitude of fall in [Cl\(^-\)]. Whether the level of [Cl\(^-\)], under low-K\(^+\) diet is sufficiently low to activate WNK4 to stimulate NCC is unknown. Conceivably, the upregulation of NCC under low-K\(^+\) diet could be an adaptive response to increased activity from favorable driving force. Thus the hypothesis that modulation of WNK4 activity by [Cl\(^-\)], is important for regulation of NCC in vivo remains unclear. Furthermore, increased luminal NaCl delivery and low K\(^+\) intake, conditions expected to increase and decrease [Cl\(^-\)], respectively, both cause upregulation of NCC. These issues prompt us to conduct the current study to ask whether WNK4-[Cl\(^-\)], is the sole mechanism of regulation of NCC.

**MATERIALS AND METHODS**

**Animals and genotyping.** All animal care, maintenance, and experiments were conducted in accordance with the Guide for the Use and Care of Laboratory Animals and approved by IACUC of the University of Texas Southwestern Medical Center at Dallas. Wild-type (WT), Ncc-KO (obtained from mutant mouse regional center) (20), and Wnk4-KO mice used in these studies are in a C57BL/6 and 129 mixed background. For genotyping, mice tail clips were digested overnight in Viagen DirectPCR reagents with 0.2 mg/ml proteinase K at 55°C, heat-inactivated by boiling in water bath for 5 min or at 85°C for 45 min. Genomic DNA was analyzed by using PCR using primers as described below. All genotyping PCR were performed using GoTaq Green Master Mix (Promega, Madison, WI) and with the setting of hot start at 95°C for 4 min, followed with 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 45 s. For genotyping of Wnk4-KO mice, forward and reverse primer sequences were 5'-ACATAGCTTGGGGACAGGC-3' and 5'-ATAATGCAGCTGTTGCGAGAAAATCT-3', respectively. For genotyping of Ncc-KO mice, forward primer sequence was 5'-AGGCTCAGGGGACCGGT-3' and reverse primer sequences were 5'-CTGCTCCCGAGCTG-3' and 5'-GGTAAAGGGGACCGGTCAGG-3' for mutant and wild-type allele, respectively.

**Metabolic cage studies.** Before setting up for physiological studies, mice had ad libitum access to water and normal rodent chow (control K diet, CK; Harlan TD.88238) containing 0.3% (g/100 g food) NaCl. Mice had ad libitum access to water and normal rodent chow (control diet). Metabolic cage studies. Each mouse was removed from the metabolic cage and placed individually in a clean shoebox-sized plastic container for at least 5–10 min until a spontaneous urination occurred or were given a bladder massage to ensure an empty bladder at the end of each collection point. Urine Na\(^+\) and K\(^+\) concentration was measured using flame photometer (Jenway, PFP 7). In NaCl loading studies, 0.9% NaCl was injected to mice subcutaneously twice daily for 3 consecutive days before mice were subjected to experimentation. To avoid potential K\(^+\) depletion kaliuresis, drinking water for mice during the NaCl loading period contains 0.1% KCl.

**Western blot analysis.** Kidneys were harvested from mice at indicated time points. Total kidney protein extracts were obtained by homogenizing kidney in chilled lysis buffer containing 250 mM sucrose, 10 mM triethanolamine (Sigma-Aldrich), 50 mM NaF, 1 mM EDTA, 1 mM EGTA, complete protease inhibitor cocktail (Roche, Indianapolis, IN), and PhosSTOP (Roche, Indianapolis, IN). Homogenates were centrifuged at 16,000 g at 4°C for 20 min to remove nuclei and cell debris. Protein concentration in supernatant was determined using Bio-Rad DC protein assay (Bio-Rad, Hercules, CA). Total 40 μg lysates in Laemmli buffer were heated at 60°C for 15 min before loading onto a 6–8% SDS-PAGE gradient gel. After electrophoresis, separated proteins were transferred to polyvinylidene fluoride (PVDF) membranes (Thermo Scientific, Rockford, IL) for Western blot analysis. The membrane was blocked with 10% BSA in TBS buffer containing 0.1% Tween-20 before probing with various antibodies. Antibodies used in Western blot studies were: T-NCC (EMD Millipore, Temecula, CA), p-NCC (T533) (33), and β-actin (Santa Cruz Biotechnology).

**Quantitative real-time PCR analysis.** Mouse kidney mRNAs were extracted and then subjected to cDNA synthesis using iScript Reverse Transcription Supermix (Bio-Rad). Real-time PCR was performed with iTaq SYBR Green Supermix on CFX Connect Real-Time System machine (Bio-Rad). PCR cycles were 95°C for 10 s, 60°C for 30 s, and repeated cycles for 39 times. GAPDH was used as internal control for all samples. The primer sequences used for mouse renin analysis were as described (15). Both GAPDH and renin primers were checked for their amplification efficiency, and a single melting curve was observed to ensure a specific amplification in each reaction. The expression of renin in samples was normalized to the internal control GAPDH gene expression and their relative ratio of expression to GAPDH was analyzed and graphed.

**Statistical analysis.** All data are presented as means ± SE. Statistical comparisons between two groups of data were made using a two-tailed unpaired Student’s t-test. Multiple comparisons were determined using one-way ANOVA followed by Tukey’s multiple-comparison tests. P values of <0.05 and <0.01 were considered significant for single and multiple comparisons, respectively.

**RESULTS**

**Validation of thiazide-sensitive increases in urinary Na\(^+\) excretion as readout of NCC transporter activity in vivo.** The most direct way to assess NCC activity is to measure thiazide-sensitive Na\(^+\) reabsorption by in vivo microperfusion of DCT. Unfortunately, it is virtually impossible to perform in vivo microperfusion in mice due to the short length of the surface DCT loop accessible for perfusion. An increase in urinary Na\(^+\) excretion in response to thiazide has been established as an alternative measurement for functional NCC activity in vivo. Many of these studies have employed intraperitoneal injection of thiazide (3, 33). However, we found that intraperitoneal injection of hydrochlorothiazide (HCTZ, 50 mg/kg body wt) induces a brisk diuresis and natriuresis as expected, but does not lead to kaliuresis (not shown). A recent paper reported a similar finding of lack of kaliuresis following intraperitoneal delivery of HCTZ (10). Because K\(^+\) reabsorption in proximal tubules parallels Na\(^+\) and water reabsorption, we considered
the possibility that thiazide-induced volume loss may cause compensatory $K^+$ reabsorption in the proximal tubules. Our pilot experiments revealed that HCTZ induced a net loss of $Na^+$ and water equivalent to that in 0.5 ml normal saline. We thus administered HCTZ along with 0.5 ml normal saline. Despite repletion of volume loss, we found that lack of kaliuresis persists (Fig. 1A). This lack of kaliuresis is not unique to HCTZ: it also occurs during intraperitoneal injection of saline vehicle have a rapid rise of an injury marker NGAL (human neutrophil gelatinase-associated lipocalin) (5), and to avoid this potentially untoward effect as the cause of lack of kaliuresis, we designed a protocol for administration of HCTZ along with volume replacement via subcutaneous route.

As shown in Fig. 1C (set of 3 open bars on the left), subcutaneous administration of vehicle (0.5 ml NS + DMSO) caused a sustained increase in urinary $Na^+$ excretion over 24 h postadministration. The total $Na^+$ excretion was 257 $\mu$mol for the 0–4 h period and 4–24 h period combined. The 24-h $Na^+$ excretion before vehicle (labeled “Pre”) was 149 $\mu$mol. Note that $Na^+$ contained in the 0.5 ml NS (77 $\mu$mol) was essentially excreted over 24 h ($149 + 77 = 226$ $\mu$mol, roughly $\approx 257$ $\mu$mol). In contrast, administration of HCTZ in 0.5 ml NS induced a brisk natriuresis within 4 h (0–4 h period), which subsided to the pre-HCTZ level during 4–24 h period (Fig. 1C, set of 3 gray bars on the right). Interestingly, the total 24-h $Na^+$ excretion with HCTZ (165 $\mu$mol for period 0–4 h plus 80 $\mu$mol for period 4–24 h = 245 $\mu$mol) was essentially identical to that with vehicle (257 $\mu$mol). Thus these results indicate that coadministration with 0.5 ml NS prevented HCTZ-induced volume contraction; HCTZ induced natriuresis by excreting coadministered NaCl within the first 4 h instead of slowly excreting over 24 h as in vehicle-treated. Using this protocol, we found that subcutaneously administered HCTZ stimulated kaliuresis alone with natriuresis reproducibly (Fig. 1D).

A previous paper by Leviel et al. (13) reported that HCTZ also inhibits $Na^+$-driven $Cl^-$/HCO$_3^-$ exchanger (NDCBE/SLC4A8). In the paper, authors demonstrated that HCTZ (given by ip injection at 50 mg/kg) caused an increase in urinary $Na^+$ excretion in Ncc-KO mice. The effect, however, occurs in 6–12 h after HCTZ, but not in the period 0–6 h after HCTZ, indicating that the effect on NCC is separate from on NBCDE. To validate the fidelity of HCTZ-sensitive urinary $Na^+$ excretion truly reflecting NCC activity in vivo, we examined urinary $Na^+$ excretion rate during the acute phase (0–4 h) of HCTZ administration in wild-type vs. Ncc-KO mice (Fig. 2) (20). Genotyping and Western blot analysis confirmed deletion of Ncc and lack of NCC protein expression in Ncc-KO mice (Fig. 2, A–C). As shown in Fig. 2D, HCTZ administrated using our protocol did not cause natriuresis in Ncc-KO mice within 0–4 h while inducing brisk natriuresis in wild type. Thus HCTZ-induced $Na^+$ excretion within 4 h after HCTZ is a good assessment of functional NCC activity in our experimental system.

NCC activity is markedly reduced in Wnk4-null mice. To examine the role of WNK4 in the regulation of NCC, we generated global Wnk4-KO (Wnk4$^{-/-}$) mice by deleting exons...
1 and 2 of Wnk4. We removed both exons because exon 2 contains an in-frame ATG. We generated ES cells containing the Wnk4-floxed allele using standard recombinase techniques (14) and a BAC clone containing the mouse Wnk4 gene (Fig. 3A). Global Wnk4-KO mice were generated using ES cells in which floxed regions were excised in the ES cell stage, and confirmed by genotyping using primer sets that detect mutant allele (“CD-IJ”) or wild-type allele (“GH-IJ”) (Fig. 3B). The HCTZ-induced increase in Na+ excretion was mostly eliminated in Wnk4-KO mice. Whole kidney WNK1 abundance (analyzed by Western blot) was not significantly different between WT and Wnk4-KO mice (measured by densitometry and normalized to WT: 1.01 ± 0.05 vs. 1.15 ± 0.09, n = 4 each; NS). Susa et al. (22) found a slight compensatory upregulation of WNK1 abundance in Wnk4-KO mice, but concluded that it does not contribute significantly to NCC activities. Of note, despite the marked reduction of NCC activity in Wnk4-KO mice, baseline urinary Na+ excretion rate was not significantly different between WT and Wnk4-KO mice (*Pre* in WT vs. *Pre* in Wnk4-KO, Fig. 3D). This finding is likely due to compensatory upregulation of Na+ transporters in upstream and downstream nephron segments, such as NKCC2 and ENaC, in the knockout mice (2). Overall, serum Na+ levels are slightly lower and BUN higher in Wnk4-KO mice relative to WT mice (Table 1), consistent with the notion of mild and compensated hypovolemia in Wnk4-KO mice.

Fig. 2. Blunting of HCTZ-induced Na+ excretion in Ncc-knockout mice. A: primer position for genotyping for the targeted Ncc (Na+ - Cl- cotransporter) gene. A Neo cassette was inserted into exon 12 of Ncc to disrupt gene function. B: genotyping of WT vs. Ncc-KO mice. C: Western blot analysis of NCC protein in kidney extracts from WT and Ncc-KO mice. D: HCTZ-induced Na+ excretion during the acute phase (HCTZ, from 0 to 4 h) after the drug administration was detected in WT (n = 5) mice, but not in the Ncc-KO (n = 5) mice. In Ncc-KO mice, the level of Na+ excretion after HCTZ was equivalent to vehicle-treated WT mice (n = 6) (indicated by the horizontal line).

Fig. 3. Blunting of HCTZ-induced Na+ excretion in Wnk4-KO mice. A: gene targeting strategy and primer position for genotyping. B: genotyping of Wnk4-KO mice. The “CD-II” primer set generates ~500 bp PCR product from the mutant allele, in which the large (>5 kb) DNA fragment flanked by the loxP sites is deleted. This primer set does not generate PCR product from WT allele due to its size. The “GH-II” primer set generates PCR product in WT and KO mice compared. HCTZ induced a large increase in urinary Na+ excretion in WT mice above the level of that observed in vehicle-treated controls (Fig. 3D). The HCTZ-induced increase in Na+ excretion was mostly eliminated in Wnk4-KO mice. Whole kidney WNK1 abundance (analyzed by Western blot) was not significantly different between WT and Wnk4-KO mice (measured by densitometry and normalized to WT: 1.01 ± 0.05 vs. 1.15 ± 0.09, n = 4 each; NS). Susa et al. (22) found a slight compensatory upregulation of WNK1 abundance in Wnk4-KO mice, but concluded that it does not contribute significantly to NCC activities. Of note, despite the marked reduction of NCC activity in Wnk4-KO mice, baseline urinary Na+ excretion rate was not significantly different between WT and Wnk4-KO mice (*Pre* in WT vs. *Pre* in Wnk4-KO, Fig. 3D). This finding is likely due to compensatory upregulation of Na+ transporters in upstream and downstream nephron segments, such as NKCC2 and ENaC, in the knockout mice (2). Overall, serum Na+ levels are slightly lower and BUN higher in Wnk4-KO mice relative to WT mice (Table 1), consistent with the notion of mild and compensated hypovolemia in Wnk4-KO mice.
mice. These results agree with previous studies using Wnk4-null mice reporting that WNK4 stimulates NCC by increased transporter abundance and phosphorylation (23).

WNK4 is essential for upregulation of NCC stimulated by low dietary potassium intake. Mice fed a K⁺-deficient diet have increased abundance of the total and phosphorylated NCC in DCT (24). We examined the role of WNK4 in the upregulation of NCC by low K⁺ intake. Mice were pair-fed on a control K⁺ diet (“CK”; 1% K⁺ in g/100 g food) or K⁺-deficient diet (“LK”; ~0% K⁺) for 7 days. Feeding a K⁺-deficient diet caused hypokalemia in WT mice (Fig. 4A; 5.03 ± 0.14 mM control-K⁺ vs. 4.44 ± 0.21 mM low-K⁺), which was more pronounced in Wnk4-KO mice (4.76 ± 0.13 vs. 4.01 ± 0.24). Serum Na⁺ levels were not different in WT and Wnk4-KO mice under either control-K⁺ or K⁺-deficient diet (Fig. 4B). K⁺-deficient diets increased the abundance of both T-NCC and p-NCC (T53) in WT mice (Fig. 4C). The K⁺ deficiency-induced increase in abundance of T-NCC and p-NCC was absent in Wnk4-KO mice (Fig. 4D). Consistent with the results of analysis of protein abundance, K⁺-deficient diets enhanced functional NCC activity reflected by HCTZ-sensitive increases in urinary Na⁺ excretion in WT, but not in Wnk4-KO mice (Fig. 4E).

WNK4 is not required for upregulation of NCC stimulated by increasing luminal NaCl delivery. Sustained increases in luminal NaCl delivery to NCC leads to adaptive hypertrophy of DCT and upregulation of NCC (6, 11, 12). One experimental approach to increase luminal NaCl delivery to NCC is inhibition of NaCl reabsorption in the thick ascending limb by furosemide. This approach requires careful volume replacement to prevent potential compensatory upregulation of NCC resulting from furosemide-induced diuresis and volume loss. To simplify the experimental approach for increasing luminal NaCl delivery to NCC, we experimented with a protocol that involves injection of 0.5 ml normal saline subcutaneously twice daily for 3 days. We found that by the end of 3 days of mild NaCl loading, mice reached a new steady-state Na⁺ balance, and excreted the entire injected Na⁺ in urine (Fig. 5A; 0.76 ± 0.59 vs. 0.93 ± 0.53 in WT and Wnk4-KO mice, respectively). HCTZ-sensitive Na⁺ excretion in WT, but not in Wnk4-KO mice. HCTZ-sensitive Na⁺ excretion was increased by LK diet in WT, but not in Wnk4-KO mice (Fig. 4E).

**Table 1. Blood chemistry in WT vs. Wnk4-KO mice**

|       | Na, mmol/l | K, mmol/l | Cl, mmol/l | Ca, mmol/l | Mg, mmol/l | BUN, mg/dl |
|-------|------------|-----------|------------|------------|------------|------------|
| WT (n = 10) | 141.5 ± 1.3 | 5.62 ± 0.29 | 117.2 ± 0.76 | 0.59 ± 0.04 | 0.28 ± 0.01 | 13.8 ± 1.4 |
| Wnk4-KO (n = 11) | 137.4 ± 0.9 | 5.02 ± 0.20 | 109.7 ± 0.93* | 0.53 ± 0.01 | 0.24 ± 0.01* | 19.1 ± 2.09 |

Values are means ± SE. *P < 0.05 WT vs. Wnk4-KO.

Fig. 4. Potassium-deficient (LK) diet induces NCC upregulation in WT mice, but not in Wnk4-KO mice. A and B: serum K⁺ and Na⁺ levels in WT and Wnk4-KO mice under control K⁺ (CK) or low-K⁺ (LK) diet. Mice on normal rodent chow were placed on CK or LK diet under pair-feeding for 7 days. *P < 0.05 LK vs. CK. # P < 0.05 between indicated. C and D: kidney extracts were analyzed for the abundance of T-NCC and p-NCC (T53) by Western blotting. Please note that cropped images of control K (CK) and Low K (LK) shown in C are from Western blotting of kidney tissues analyzed in the same SDS-PAGE gel electrophoresis. E: HCTZ-sensitive Na⁺ excretion was increased by LK diet in WT, but not in Wnk4-KO mice. HCTZ-sensitive Na⁺ excretion is calculated by subtracting excretion at “Pre” period from excretion at 0–4 h period after HCTZ. n = 16 for WT mice on CK diet, n = 19 for WT on LK diet, n = 17 for Wnk4-KO on CK, n = 19 for Wnk4-KO on LK. *P < 0.05.
7.4 μmol/h more Na+ excretion in bar 3 vs. bar 1; 7.4 × 24 h = 177 μmol/day ≈ 155 μmol contained in 1 ml normal saline). At this new steady state, no significant volume expansion occurs as evidenced by comparable levels of renin expression between control and NaCl-loaded mice (Fig. 6). Hematocrit was not significantly different between vehicle and NaCl-loaded mice (47 ± 1 vs. 46.1 ± 0.9, n = 15 each; NS), further supporting that no significant volume expansion by mild NaCl loading. Please note that the amount of NaCl in 1 ml of normal saline per 25 g body wt via subcutaneous injection, twice a day for 3 consecutive days). Number inside bars indicates number of mice for each condition.

Indeed, we found that the NaCl loading protocol induced increases in HCTZ-sensitive Na+ excretion (Fig. 5, A and D). Interestingly, NaCl loading also induced a large increase in HCTZ-sensitive Na+ excretion in Wnk4-KO mice (Fig. 5, D and E). Because of a lower baseline HCTZ-sensitive Na+ excretion in Wnk4-KO, the level of HCTZ-sensitive Na+ excretion during NaCl loading was slightly lower in the Wnk4-KO than in WT mice (Fig. 5E). However, NaCl loading-induced increases in HCTZ-sensitive Na+ excretion were comparable between WT and Wnk4-KO mice. The fact that NaCl loading could enhance HCTZ-sensitive Na+ excretion in Wnk4-KO mice suggests that the lack of stimulation of NCC by low K+ intake in KO mice is due to a specific requirement for the WNK4 signaling cascade, rather than intrinsic defects in NCC preventing it from upregulation. The upregulation of functional NCC activity was parallel with an increase in the abundance of the activated form of NCC, p-NCC (T53), in both WT and Wnk4-KO mice (Fig. 7A). Semiquantitative analysis of the p-NCC (T53) signal intensity showed significant increases upon NaCl loading in both strains of mice (Fig. 7B).
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stimulating NCC activity as lowering basolateral [K+] will decrease [Cl^-], (Fig. 8, B and C). These facts, together with that changes in [Cl^-] under active transport conditions may be too small to affect WNK kinase activity, beg the question whether mechanism(s) other than [Cl^-], regulates WNK kinase activity to affect NCC activity in vivo. Therefore, we set out to examine the effect of increasing luminal NaCl delivery on NCC in Wnk4-KO mice. These experiments allow us to investigate the role of WNK4 in mediating the general mechanism of use hypertrophy as well as to investigate whether low baseline NCC activity in Wnk4-KO mice prevents detection of upregulation of NCC by other stimulations. Our results show that deletion of Wnk4 in mice eliminated NCC upregulation by dietary K^+ restriction, but that WNK4 is dispensable for upregulation of NCC stimulated by increasing luminal NaCl delivery. The results provide compelling support for the notion that WNK4 is critical for upregulation of NCC by low K^+ intake. The results appear to support the notion that low dietary K^+ intake decreases [Cl^-], to activate WNK4. Yet, the fact that increasing luminal NaCl delivery, which if anything is expected to increase [Cl^-], also induces upregulation of NCC indicates that other additional, WNK4- and [Cl^-]-independent mechanism(s) regulates NCC as well. The additional mechanism can override the effect of inhibition of WNK4 by high [Cl^-]. The result that Wnk4-KO does not affect the additional mechanism of regulation is consistent with the notion.

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Changes in NCC activity in DCT is frequently accompanied by reciprocal changes in epithelial Na^+ channel (ENaC) activity in the downstream CNT and CCD segments (20). Consistent with this notion, we found that mild NaCl loading induced a decrease in amiloride-sensitive Na^+ excretion rate (Fig. 5F). This adaptive decrease in ENaC activity probably contributes to the overall outcome of minimal volume in mildly NaCl loaded mice.

DISCUSSION

Downregulation and upregulation of NCC are important renal adaptive responses to high K^+ intake and deficiency, respectively (16, 25). How dietary K^+ intake regulates NCC remains incompletely understood. Recent studies strongly suggest that WNK kinases are involved. Piala et al. (18) reported that two key leucine residues, L369 and L371 in the DLG motif within the kinase domain of WNK1, form a chloride-binding site, and that WNK1 kinase activity is inhibited when chloride is bound to the site. Subsequently, Terker et al. (24) and Bazúa-Valenti et al. (1) demonstrated that changes in extracellular [K^+] alter membrane potential and Cl^- efflux, leading to changes in [Cl^-], WNK kinase activity, and phosphorylation and activity of NCC. These studies used cultured cells under nontransporting conditions. Whether changes in [Cl^-], in DCT cells under the low- or high-K^+ diet are sufficient to account for observed differences in WNK kinase activity is unknown. Under active transcellular NaCl transport in DCT, coupling between apical NaCl entry to basolateral exit will almost certainly blunt the changes in [Cl^-], compared with nontransporting conditions.

DCT and NCC undergo adaptation in response to changes in NCC transport activity. Sustained activation of NCC-mediated NaCl reabsorption leads to hypertrophy of DCT and upregulation of NCC whereas decreased activity has the opposite effect (6, 11, 12, 17). The molecular mechanism of the activity-associated adaptation of DCT and NCC is unknown. The model of transcellular NaCl reabsorption in DCT predicts that increasing luminal Na^+ delivery will have a similar effect of stimulating NCC activity as lowering basolateral [K^+] by low-K^+ diet (Fig. 8A). Interestingly, although both stimulations increase NCC activity, they have opposite effects on DCT [Cl^-]. Enhanced NaCl entry by increasing luminal NaCl delivery will increase [Cl^-], whereas lowering basolateral [K^+] will decrease [Cl^-], (Fig. 8, B and C). The facts, together with that changes in [Cl^-], under active transport conditions may be too small to affect WNK kinase activity, beg the question whether mechanism(s) other than [Cl^-], regulates WNK kinase activity to affect NCC activity in vivo. Therefore, we set out to examine the effect of increasing luminal NaCl delivery on NCC in Wnk4-KO mice. These experiments allow us to investigate the role of WNK4 in mediating the general mechanism of use hypertrophy as well as to investigate whether low baseline NCC activity in Wnk4-KO mice prevents detection of upregulation of NCC by other stimulations. Our results show that deletion of Wnk4 in mice eliminated NCC upregulation by dietary K^+ restriction, but that WNK4 is dispensable for upregulation of NCC stimulated by increasing luminal NaCl delivery. The results provide compelling support for the notion that WNK4 is critical for upregulation of NCC by low K^+ intake. The results appear to support the notion that low dietary K^+ intake decreases [Cl^-], to activate WNK4. Yet, the fact that increasing luminal NaCl delivery, which if anything is expected to increase [Cl^-], also induces upregulation of NCC indicates that other additional, WNK4- and [Cl^-]-independent mechanism(s) regulates NCC as well. The additional mechanism can override the effect of inhibition of WNK4 by high [Cl^-]. The result that Wnk4-KO does not affect the additional mechanism of regulation is consistent with the notion.

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DISCUSSION

Downregulation and upregulation of NCC are important renal adaptive responses to high K^+ intake and deficiency, respectively (16, 25). How dietary K^+ intake regulates NCC remains incompletely understood. Recent studies strongly suggest that WNK kinases are involved. Piala et al. (18) reported that two key leucine residues, L369 and L371 in the DLG motif within the kinase domain of WNK1, form a chloride-binding site, and that WNK1 kinase activity is inhibited when chloride is bound to the site. Subsequently, Terker et al. (24) and Bazúa-Valenti et al. (1) demonstrated that changes in extracellular [K^+] alter membrane potential and Cl^- efflux, leading to changes in [Cl^-], WNK kinase activity, and phosphorylation and activity of NCC. These studies used cultured cells under nontransporting conditions. Whether changes in [Cl^-], in DCT cells under the low- or high-K^+ diet are sufficient to account for observed differences in WNK kinase activity is unknown. Under active transcellular NaCl transport in DCT, coupling between apical NaCl entry to basolateral exit will almost certainly blunt the changes in [Cl^-], compared with nontransporting conditions.

DCT and NCC undergo adaptation in response to changes in NCC transport activity. Sustained activation of NCC-mediated NaCl reabsorption leads to hypertrophy of DCT and upregulation of NCC whereas decreased activity has the opposite effect (6, 11, 12, 17). The molecular mechanism of the activity-associated adaptation of DCT and NCC is unknown. The model of transcellular NaCl reabsorption in DCT predicts that increasing luminal Na^+ delivery will have a similar effect of stimulating NCC activity as lowering basolateral [K^+] by low-K^+ diet (Fig. 8A). Interestingly, although both stimulations increase NCC activity, they have opposite effects on DCT [Cl^-]. Enhanced NaCl entry by increasing luminal NaCl delivery will increase [Cl^-], whereas lowering basolateral [K^+] will decrease [Cl^-], (Fig. 8, B and C). These facts, together with that changes in [Cl^-], under active transport conditions may be too small to affect WNK kinase activity, beg the question whether mechanism(s) other than [Cl^-], regulates WNK kinase activity to affect NCC activity in vivo. Therefore, we set out to examine the effect of increasing luminal NaCl delivery on NCC in Wnk4-KO mice. These experiments allow us to investigate the role of WNK4 in mediating the general mechanism of use hypertrophy as well as to investigate whether low baseline NCC activity in Wnk4-KO mice prevents detection of upregulation of NCC by other stimulations. Our results show that deletion of Wnk4 in mice eliminated NCC upregulation by dietary K^+ restriction, but that WNK4 is dispensable for upregulation of NCC stimulated by increasing luminal NaCl delivery. The results provide compelling support for the notion that WNK4 is critical for upregulation of NCC by low K^+ intake. The results appear to support the notion that low dietary K^+ intake decreases [Cl^-], to activate WNK4. Yet, the fact that increasing luminal NaCl delivery, which if anything is expected to increase [Cl^-], also induces upregulation of NCC indicates that other additional, WNK4- and [Cl^-]-independent mechanism(s) regulates NCC as well. The additional mechanism can override the effect of inhibition of WNK4 by high [Cl^-]. The result that Wnk4-KO does not affect the additional mechanism of regulation is consistent with the notion.

Previously, Castañeda-Bueno et al. (4) showed that dietary K^+-intake induced regulation of NCC (based on analysis of phospho-NCC) is absent in Wnk4-KO mice. However, they found that phosphorylation of SPAK is unaltered in Wnk4-KO.
Because of a very low baseline NCC protein in Wnk4-KO mice, they reasoned that phosphorylation of SPAK, the downstream target of WNK4, might be a better readout for WNK4 activity and concluded that WNK4 is not required for dietary K⁺ regulation of NCC. To address the question whether low baseline NCC activity may confound the interpretation of results, we examined the upregulation of NCC in Wnk4-KO mice by increasing luminal NaCl delivery. We show that increasing luminal NaCl induces upregulation of NCC in Wnk4-KO mice, suggesting that failure for upregulation of NCC by dietary K⁺ restriction in these mice is specific and due to defects in the WNK4 kinase cascade. Of note, double knockout of Spak and Osrl in mice abolishes dietary K⁺ regulation of NCC (7). Phospho-SPAK from the whole kidney extract likely does not reliably reflect the activity of the WNK4 kinase cascade in DCT.

With respect to the identity of WNK kinases in mediating [Cl⁻]-sensitive regulation of NCC, Terker et al. (25) showed that in vitro WNK1 is less sensitive to [Cl⁻] than WNK4, and suggested that WNK4, but not WNK1, is the principal mediator of regulation of NCC by low-K⁺ diet. Our finding is consistent with the hypothesis. WNK1 is also present in DCT, particularly the alternative splice variant lacking exon 11 (19, 28). The role of WNK1 and splice variants in the regulation of NCC remains largely unknown. WNK1 splice variants may mediate upregulation of NCC stimulated by increasing luminal NaCl delivery via [Cl⁻]-dependent mechanism(s) and/or by participating in the regulation by dietary K⁺ in formation of heteromers with WNK4. In the case of obligatory WNK1 and WNK4 heteromers, deletion of WNK4 will result in a complete loss of function of heteromers. Overall, our present findings support the hypothesis that WNK4 is essential for regulation of NCC by dietary K⁺, potentially through the mechanism of [Cl⁻]-dependent regulation of kinase activity. Definitive evidence for the role of [Cl⁻]-dependent regulation of WNK kinases in vivo requires direct measurement of DCT [Cl⁻], under active transport condition and/or studies using knockin mice carrying Cl⁻-insensitive WNK mutants. In vitro, WNK kinase activity is also modulated by changes in [K⁺], although appears less so when compared with modulation with changes in [Cl⁻] (18). The possibility that [K⁺] contributes to WNK kinase regulation of NCC by K⁺ diets or activity remains to be investigated. Finally, our study indicates additional aldosterone- and [Cl⁻]-independent mechanism(s) for regulating NCC yet to be uncovered.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Y.-S.Y., J.X., S.-S.Y., and C.-L.H. conceived and designed research; Y.-S.Y., J.X., and S.-S.Y. performed experiments; Y.-S.Y., J.X., S.-S.Y., and C.-L.H. analyzed data; Y.-S.Y., J.X., S.-S.Y., S.-H.L., and C.-L.H. interpreted results of experiments; Y.-S.Y., J.X., and C.-L.H. prepared figures; Y.-S.Y., J.X., and S.-S.Y. drafted manuscript; Y.-S.Y., J.X., S.-S.Y., S.-H.L., and C.-L.H. approved final version of manuscript; S.-H.L. and C.-L.H. edited and revised manuscript.
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