Mechanisms for Hypercalciuria in Pseudohypoaldosteronism Type II-Causing WNK4 Knock-In Mice

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The mechanisms underlying hypercalciuria in pseudohypoaldosteronism type II (PHAII) caused by WNK4 mutations remain unclear. In this study, we used Wnk4D561A/H11001 knock-in mice as a model of human PHAII for investigating the pathogenesis of hypercalciuria in PHAII. Serum and urine biochemistries were obtained from Wnk4/H11001/H11001 and Wnk4D561A/H11001 littermates. Expression of the epithelial Ca²⁺ channels [transient receptor potential channel vanilloid subtype 5 (TRPV5) and TRPV6] and calbindin-D28k (CBP-D28k) in the distal nephron and two upstream Na⁺ transporters, Na⁺-K⁺-2Cl⁻ cotransporter 2 involved in paracellular Ca²⁺ reabsorption, were examined by real-time PCR, immunofluorescent staining, and immunoblotting. Compared with Wnk4/H11001/H11001 littermate controls, Wnk4D561A/H11001 mice manifested hypercalciuria despite no significant differences in serum creatinine, ionized Ca²⁺, PTH, and 1,25 hydroxylvitamin D3 levels. There was no significant difference in TRPV5 expression, but a significant increase in TRPV6 and CBP-D28k was observed in Wnk4D561A/H11001 mice. Despite no significant change in Na⁺-K⁺-2Cl⁻ cotransporter 2 expression, Na⁺-K⁺-2Cl⁻ cotransporter 2 expression was significantly attenuated and urine Ca²⁺ excretion rate in response to furosemide was blunted in Wnk4D561A/H11001 mice. Decreased Ca²⁺ reabsorption in the upstream nephron, especially in the thick ascending loops of Henle, with a secondary adaptive increase in TRPV5 and CBP-D28k expression in the distal tubules might be involved in the hypercalciuria of PHAII. (Endocrinology 151: 1829–1836, 2010)

Pseudohypoaldosteronism type II (PHAII) is an autosomal dominant disorder characterized by salt-sensitive hypertension, hyperkalemia, and metabolic acidosis (1). Patients with PHAII are usually sensitive to thiazide treatment, suggesting gain-of-function in the thiazide-sensitive NaCl cotransporter (NCC) in the distal convoluted tubule as a major pathogenic mechanism (2). Despite no mutations identified in the SLC12A3 gene encoding the NCC, mutations in genes encoding two upstream regulatory kinases, with-no-lysine [K] kinase (WNK) 1 and 4, have been found in patients with PHAII. Mutations in the WNK1 gene are large deletions of the first intron resulting in increased expression of the gene. Mutations in the WNK4 gene are missense mutations outside the protein kinase domain (E562K, D564A, Q565E, and R1185C) (3).

Besides the described phenotypes, patients with PHAII due to WNK4 mutations also have hypercalciuria and low bone mineral density (4). The low bone mineral density in PHAII is thought secondary to renal hypercalciuria. In the kidneys, approximately 60% of filtered Ca²⁺ is passively reabsorbed in the proximal convoluted tubule, the ascending limb of the loop of Henle, and the distal convoluted tubule. Mutations in WNK4 lead to increased expression of TRPV6 in the distal convoluted tubule, which increases Ca²⁺ reabsorption, leading to hypercalciuria. This increased Ca²⁺ reabsorption is due to the increased expression of TRPV6, which is a Ca²⁺ channel that is activated by increased intracellular Ca²⁺ levels.
reabsorbed, mainly through paracellular junctions located in the proximal convoluted tubules (PCT), 20% is absorbed in the thick ascending loops of Henle (TAL) in parallel with Na⁺ reabsorption, and 10–15% of Ca²⁺ is actively reabsorbed in the distal nephron (5, 6). The final rate of Ca²⁺ excretion is regulated by calbindin, a cytosolic calcium facilitator, and epithelial Ca²⁺ channels in the late distal convoluted and collecting tubules, the transient receptor potential channel vanilloid subtype 5 (TRPV5) and 6 (TRPV6) (7). Thus, hypercalciuria may be caused by either increased glomerular Ca²⁺ filtration and/or reduced Ca²⁺ tubular reabsorption. Nonetheless, the molecular pathogenesis of hypercalciuria in WNK4-mutation causing PHAII remains unclear. Understanding the mechanisms behind the hypercalciuria in PHAII may provide insight into the accompanying low bone mineral density.

It has been shown through *Xenopus* oocyte expression that PHAII-mutant WNK4 activate TRPV5-mediated Ca²⁺ reabsorption less than wild-type WNK in the presence of NCC and was postulated as a mechanism for hypercalciuria in PHAII (8). However, these *in vitro* studies may not always directly apply to PHAII patients without further confirmatory *in vivo* studies. Disease-causing knock-in mouse models more accurately represent human genetic disorders caused by a specific mutation. We have successfully generated *Wnk4^D561A/+* knock-in mice (corresponding to human D564A) featuring typical phenotypes of human PHAII (9) and hypercalciuria. Using this model, we have uncovered new mechanisms of hypercalciuria in PHAII caused by WNK4 mutations.

**Materials and Methods**

**Preparation of Wnk4^D561A/+** knock-in mice

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. We had created *Wnk4^D561A/+* knock-in mice (9) to evaluate the expression of Na⁺ and Ca²⁺-absorption related transporters in renal tubules, *C57BL/6* background N2 offspring of *Wnk4^+/+* and *Wnk4^D561A/+* littermates were bred. The mice were housed in a 12-h light, 12-h dark cycle and were fed with normal raw chow [Na⁺: 0.4% (wt/wt); Ca²⁺: 0.9% (wt/wt) and plain drinking water *ad libitum* for 12–14 wk. Blood pressure was measured using a programmable tail-cuff sphygmomanometer (MK-2000A; Muromachi, Tokyo, Japan).

**Blood and urine biochemistry analysis**

Blood was collected from the retro-orbital sinus under light ether anesthesia. Mice were housed in metabolic cages for urine collection. Serum and urine biochemistry levels were determined using automated methods (AU 5000 chemistry analyzer; Olympus, Tokyo, Japan). Blood gas data were determined by i-STAT.

Serum PTH was measured with mouse PTH ELISA kits (Immuno-topics, San Clemente, CA). Serum 1,25 hydroxylvitamin D₃ [1,25(OH)₂D₃] concentrations were measured with RIA kits (ImmunoDiagnostic Systems Ltd., Boldon, England).

**Quantitative real-time PCR (Q-PCR)**

For assaying the gene expression of TRPV5 and TRPV6, total kidney RNA was isolated using TRizol reagent (Invitrogen, Carlsbad, CA) and first-strand cDNA synthesized using 2 μg of extracted RNA Oligo deoxynucleotide primers (Invitrogen) and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) following standard RT protocols. The forward (F) and reverse (R) primers used for mouse TRPV5, TRPV6, and β-actin gene expression (10) were as follows: TRPV5 (F) 5'-GAACTCTCTAATTGGGGTCAG-3', (R) 5'-TTTGCGCGCGAGTCACAGTTT-3'; TRPV6 (F) 5'-GTCATGCTAATTGGGTCAG-3', (R) 5'-ATAGAAAGCTGAACGTTCACTCTCACTTTGAC-3'; β-actin (F) 5'-ACCCCTCTTCAACTAGGACG-3’, (R) 5'-GGCAGCTTGGGTGGACC-3’.

Q-PCR was undertaken using an Applied Biosystems 7500 Real-Time PCR system and the fluorescent dye SYBR Green (Applied Biosystems, Foster City, CA) as a tracer. The β-actin gene was used as an internal control. For each target gene, the relative gene expression was performed in triplicate for obtaining a mean cycle threshold ( Ct ) value and determined as 2^(-ΔΔCt), where ΔCt was the first cycle at which the reporter signal significantly exceeded the baseline signal. Melting curve analysis was carried out after 40-cycles of amplification to validate the size of the PCR products. Amplified PCR products were analyzed using 7500 System SDS software version 1.3.1 (Applied Biosystems).

**TABLE 1. Blood pressure, serum, and urine biochemistry in Wnk4^+/+ and Wnk4^D561A/+ mice (n = 12)**

<table>
<thead>
<tr>
<th>Blood pressure</th>
<th>Wnk4^+/+</th>
<th>Wnk4^D561A/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic (mmHg)</td>
<td>116.3 ± 6.5</td>
<td>138.5 ± 8.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diastolic (mmHg)</td>
<td>70.3 ± 10.4</td>
<td>85.6 ± 9.5</td>
</tr>
<tr>
<td>Weight (grams)</td>
<td>22.8 ± 3.3</td>
<td>23.3 ± 1.8</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>49.2 ± 0.4</td>
<td>48.7 ± 1.1</td>
</tr>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>7.30 ± 0.04</td>
<td>7.27 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HCO₃⁻ (mmol/liter)</td>
<td>23.9 ± 1.9</td>
<td>20.3 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Na⁺ (mmol/liter)</td>
<td>146.0 ± 1.6</td>
<td>145.7 ± 1.5</td>
</tr>
<tr>
<td>K⁺ (mmol/liter)</td>
<td>4.0 ± 0.2</td>
<td>4.7 ± 0.3</td>
</tr>
<tr>
<td>Cl⁻ (mmol/liter)</td>
<td>112.6 ± 3.4</td>
<td>115.7 ± 3.4</td>
</tr>
<tr>
<td>Total Ca²⁺ (mg/dl)</td>
<td>8.8 ± 0.3</td>
<td>8.8 ± 1.1</td>
</tr>
<tr>
<td>Ionized Ca²⁺ (mg/dl)</td>
<td>4.7 ± 0.1</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>27.0 ± 5.7</td>
<td>24.7 ± 2.7</td>
</tr>
<tr>
<td>Cr (mg/dl)</td>
<td>0.33 ± 0.12</td>
<td>0.30 ± 0.10</td>
</tr>
<tr>
<td>1,25(OH)₂D₃ (pmg/ml)</td>
<td>44.3 ± 11.5</td>
<td>41.5 ± 10.2</td>
</tr>
<tr>
<td>PTH (pmg/ml)</td>
<td>27.4 ± 11.2</td>
<td>22.5 ± 10.4</td>
</tr>
</tbody>
</table>

**BUN, Blood urea nitrogen; Ca²⁺, calcium; Cr, creatinine; FEna and FEK represent the fractional excretion of Na⁺ and K⁺, respectively.**

<sup>a</sup> P < 0.05 in Wnk4^D561A/+. vs. Wnk4^+/+ comparison.
Immunoblotting (IB) and immunofluorescent (IF) staining

Semiquantitative IB and IF staining were carried out as per prior reports to assess the relative expression levels and cellular localization of proteins of interest (9). Proteins (10 μg) isolated from whole kidney homogenates without the nuclear fraction (600 × g) for calbindin-D28k (CBP-D28k) or crude membrane fractions (17,000 × g) for apically expressed channels/transporters were firstly loaded onto SDS-PAGE gels and protein samples verified by silver stain and IB of β-actin (data not shown) (9). The primary antibodies used for IB and IF were as follows: anti-TRPV5 (1:200) (for IB, Santa Cruz Biotechnology, Inc., Santa Cruz, CA; for IF, Alpha Diagnostic Intl., Inc., San Antonio, TX), TRPV6 (1:200) (for IB, Alomone Labs Ltd., Jerusalem, Israel; for IF, Alpha Diagnostic Intl., Inc.), Na⁺-K⁺-2Cl⁻ cotransporter 2 (NKCC2) (1:200) Alpha Diagnostic Intl., Inc.), Na⁺/H⁺ exchanger 3 (NHE3) (1:200) (Alpha Diagnostic Intl., Inc.), CBP-D28k (1:10,000) (Swant, Bellinzona, Switzerland), NCC (1:200) (Chemicon, Billerica, MA), epithelium Na⁺ channel β subunit [ENaC(β)] (1:200) (Alomone Labs Ltd.), and aquaporin 2 (1:200) (Santa Cruz Biotechnology, Inc.). Alkaline phosphatase-conjugated anti-IgG antibodies (Promega) (1:3000) were used as secondary antibodies for IB and Alexa 488- or 546-labeled (1:200) (Molecular Probes, Eugene, OR) secondary antibodies were used for IF. IF images were obtained using a LSM510 Meta camera attached to a fluorescent microscope (Carl Zeiss, Inc., Oberkochen, Germany).

Acute and chronic effects of furosemide treatment

Furosemide (15 mg/kg), a NKCC2 inhibitor, was ip administered to the Wnk4+/+ and Wnk4D561A/+ littermates twice per day for three consecutive days. The urine samples in the 8 h after the first dose (acute) and over 24 h on the third day (chronic) were collected for analysis (11).

Statistical analysis

Data are presented as mean ± sd. Results obtained for the knock-in Wnk4D561A/+ mice were compared with those from wild-type mice using Student’s t test or, if the data did not follow a normal distribution, by nonparametric Mann-Whitney test. P values less than or equal to 0.05 were deemed statistically significant.

FIG. 1. Expression of TRPV5, TRPV6, and CBP-D28k in the Wnk4D561A/+ mice kidney. A, Q-PCR analysis of TRPV5 and TRPV6 in Wnk4+/+ (white bars) and Wnk4D561A/+ (black bars) mice kidneys (n = 6/group) are presented as percentage of control (control, 100%). B, Specificity of the anti-TRPV5 antibody. Because TRPV5 expressed in MDCK cells was tagged with EGFP (32kD; right panel), the molecular weight of native TRPV5 in the mice kidney (dashed arrow) is smaller than that of mice EGFP-TRPV5 expressed in MDCK cells (solid arrow) (left panel). Anti-EGFP antibody clearly identifies the EGFP-TRPV5 protein expressed in MDCK cells but not the native TRPV5 present in mice kidney (right panel). C, IB of TRPV5, TRPV6, and CBP-D28k. IB of TRPV5 (upper panel), TRPV6 (middle panel) in crude membrane fractions, and CBP-D28k (lower panel) in whole homogenates without the nuclear fraction from Wnk4+/+ and Wnk4D561A/+ mice kidneys (n = 5/group). Semiquantification by densitometry analysis is shown. *, P < 0.05 in Wnk4D561A/+ vs. Wnk4+/+ comparison.
Results

Hypercalciuria in Wnk4<sup>D561A/+</sup> mice

Blood and urine samples were collected from 3-month-old Wnk4<sup>+/+</sup> and Wnk4<sup>D561A/+</sup> littermates. Compared with Wnk4<sup>+/+</sup> control mice, Wnk4<sup>D561A/+</sup> mice demonstrated typical phenotypes of PHAII, including hypertension and hyperkalemia with decreased urine K<sup>+</sup> excretion by fraction extraction rate of K<sup>+</sup>, as per previous report (9). Wnk4<sup>D561A/+</sup> mice also demonstrated increased urine Ca<sup>2+</sup> excretion (urine Ca<sup>2+</sup>/Cr 0.22 ± 0.02 mg/mg in Wnk4<sup>D561A/+</sup> vs. 0.11 ± 0.03 mg/mg in Wnk4<sup>+/+</sup>; P < 0.01, n = 12) despite no significant difference in serum Cr, ionized Ca<sup>2+</sup>, PTH, or 1,25(OH)<sub>2</sub>D<sub>3</sub> (Table 1).

Increased expression of TRPV6 and CBP-D28k in Wnk4<sup>D561A/+</sup> mice

As WNK4 is coexpressed with TRPV5 and TRPV6 in the distal nephron and prior studies have suggested impaired TRPV5 function was the involved in the pathogenesis of hypercalciuria in PHAII (8), we examined TRPV5 and TRPV6 mRNA expression by Q-PCR in this study. Despite no significant difference in TRPV5 mRNA expression between Wnk4<sup>D561A/+</sup> and Wnk4<sup>+/+</sup> controls, Wnk4<sup>D561A/+</sup> mice demonstrated significantly increased TRPV6 mRNA levels (148 ± 10% in Wnk4<sup>D561A/+</sup> vs. 100 ± 12% in Wnk4<sup>+/+</sup>; P < 0.05, n = 6) (Fig. 1A).

We next examined the protein expression of TRPV5, TRPV6, and CBP-D28k in the distal tubules of Wnk4<sup>D561A/+</sup> mice by semiquantitative IB. Cellular localization of the proteins was investigated by IF staining. The antibody for TRPV5 IB (Santa Cruz Biotechnology, Inc.) was verified by comparing the IB pattern using proteins prepared from mouse kidney tissue and Madin-Darby canine kidney (MDCK) cells transfected with p-enhanced green fluorescent protein (EGFP)-C2 expression vector (Invitrogen) with or without insertion of mouse TRPV5 cDNA (Fig. 1B). The antibody for TRPV6 (Alomone Labs Ltd.) has been previously verified (12). TRPV5 protein expression [107 ± 8% in Wnk4<sup>D561A/+</sup> vs. 100 ± 8% in Wnk4<sup>+/+</sup>; not significant (NS), n = 5] (Fig. 1C) and its cellular localization (Fig. 2A) remained unchanged in Wnk4<sup>D561A/+</sup> mice. However, TRPV6 protein expression (171 ± 23% in Wnk4<sup>D561A/+</sup> vs. 100 ± 28% in Wnk4<sup>+/+</sup>; P < 0.01, n = 5) and CBP-D28k (266 ± 65% in Wnk4<sup>D561A/+</sup> vs. 100 ± 60% in Wnk4<sup>+/+</sup>; P < 0.01, n = 5) were significantly increased in the Wnk4<sup>D561A/+</sup> mice (Fig. 1C). IF revealed enhanced TRPV6 staining in the luminal aspect of distal renal tubules in the Wnk4<sup>D561A/+</sup> mice (Fig. 2B).

Expression of NHE3, NKCC2, NCC, and ENaC in Wnk4<sup>D561A/+</sup> mice

It is well established that in the PCT and TAL, Ca<sup>2+</sup> reabsorption runs in parallel to segmental tubular Na<sup>+</sup> reabsorption (5, 6). We next examined protein expression of a major Na<sup>+</sup> transporter, NHE3, located in the PCT by semiquantitative IB and IF. There was no significant difference in NHE3 expression (Fig. 3A) or its cellular localization between Wnk4<sup>/++</sup> and Wnk4<sup>D561A/+</sup> littermates (Fig. 3B). In the TAL, however, a significant decrease in NKCC2 expression (72 ± 5% in Wnk4<sup>D561A/+</sup> vs. 100 ± 15% in Wnk4<sup>+/+</sup>; P < 0.01, n = 5) (Fig. 3A) and IF was observed in Wnk4<sup>D561A/+</sup> mice (Fig. 3B). NCC and ENaC(β) expression were significantly enhanced (Fig. 3), indicative of activated Na<sup>+</sup> reabsorption in the distal nephron of Wnk4<sup>D561A/+</sup> mice.
Urine Ca\(^{2+}\) excretion in response to furosemide in Wnk4\(^{-/-}\)/H11545 mice

To determine whether NKCC2 function was suppressed in Wnk4\(^{-/-}\)/H11545 mice, we treated the Wnk4\(^{-/-}\)/H11545 and Wnk4\(^{-/-}\)/H11545 littermates with furosemide, a NKCC2 inhibitor. The resulting urine calcium excretion rate was used as an index of NKCC2 function. In Wnk4\(^{-/-}\) mice, increased urine Ca\(^{2+}\) excretion was observed after a single dose (acute phase) (urine Ca\(^{2+}\)/Cr 0.25 ± 0.03 vs. 0.13 ± 0.02 mg/mg; \(P < 0.05\), n = 6) or after consecutive doses for 3 d (chronic phase) (urine Ca\(^{2+}\)/Cr 0.23 ± 0.05 vs. 0.13 ± 0.02 mg/mg; \(P < 0.05\), n = 6). However, the urine Ca\(^{2+}\) excretion rate was not significantly increased in either acute (urine Ca\(^{2+}\)/Cr 0.27 ± 0.04 vs. 0.24 ± 0.02
mg/mg; NS, n = 6) and chronic (urine Ca^{2+}/Cr 0.25 ± 0.05 vs. 0.24 ± 0.02 mg/mg; NS, n = 6) furosemide treatment in Wnk4^{D561A/+} mice. These findings support the hypothesis that NKCC2 function is inhibited (Fig. 4).

**Discussion**

In congruence with the human disease, Wnk4^{D561A/+} mice exhibited hypercalciuria in addition to typical PHAII phenotype and entirely confirmed the findings from the previous PHAII transgenic mice model with two additional copies of PHAII (Q562E)-mutant Wnk4 (13). Serum Cr as an index of glomerular filtration and ionized Ca^{2+} in Wnk4^{D561A/+} mice was similar to that in wild-type littermate controls, suggesting that filtered Ca^{2+} load was not a major contributor to hypercalciuria. Thus, increased urine Ca^{2+} excretion in Wnk4^{D561A/+} mice must result from reduced renal tubular Ca^{2+} reabsorption. Serum levels of PTH and 1,25(OH)_{2}D_{3}, two important regulators of renal tubular Ca^{2+} reabsorption (5–7), were also normal and not significantly different between the two groups. These results are consistent with findings in PHAII patients (14), suggesting that these two hormones are unlikely to contribute to hypercalciuria.

TRPV5 and TRPV6, two epithelial Ca^{2+} channels primarily responsible for transcellular Ca^{2+} reabsorption in the distal nephron, are known to colocalize with WNK4. Using the Xenopus oocyte expression system, Jiang et al. (8) showed that PHAII-causing WNK4 mutants significantly enhanced TRPV5-mediated Ca^{2+} transport by increasing TRPV5 membrane abundance to an extent similar to wild-type WNK4. NCC antagonized the activating effects of wild-type WNK4 on TRPV5 in a dose-dependent manner (8). In the presence of NCC, PHAII-causing WNK4 mutants exhibited a more impaired ability to enhance TRPV5-mediated Ca^{2+} uptake compared with wild-type WNK4. Because NCC is overexpressed and activated in PHAII, these in vitro studies suggested that the reduced TRPV5-mediated renal tubular Ca^{2+} reabsorption by the inhibitory effect of NCC may account for hypercalciuria in PHAII patients with WNK4 mutations (8). In addition, reduction in TRPV5 and CBP-D28k mRNA and protein expression caused by the metabolic acidosis of PHAII might also be involved in hypercalciuria (15).

In the case of NCC gain-of-function, PHAII (Q562E) mutant transgenic mice model exhibited the typical phenotype of PHAII and hypercalciuria, which were further enhanced by high NaCl intake and corrected upon crossing with NCC-null mice (13). These findings indicate that activation of NCC is involved in hypercalciuria and increased Na^{+} delivery to distal tubules further augment urine Ca^{2+} excretion. However, TRPV5 and TRPV6 expression in the distal renal tubules and upstream Na^{+} and Ca^{2+} reabsorption were not performed in those animal models. In our Wnk4^{D561A/+} knock-in mice model, we did not find the reduced TRPV5 but rather increased TRPV6 and CBP-D28k expression in the distal tubules of Wnk4^{D561A/+} mice. The discrepancy between our and previously reported results remained unclear. Our results suggested that the diminished mRNA or protein abundance of TRPV5 expression in the distal tubules by metabolic acidosis might be counteracted by the causes that regulated tubular Ca^{2+} reabsorption and delivery to distal tubules. Although TRPV5 expression and cellular localization were not significant difference between wild-type controls and Wnk4^{D561A/+} mice, we could not exclude the possibility that the phosphorylation and activity of TRPV5 could be enhanced by PHAII-causing WNK4 mutants (9) or other causes without affecting its cellular localization in Wnk4^{D561A/+} mice (16). It has recently been shown that patients with PHAII due to WNK4 mutation Q562E had a higher urine Ca^{2+} to Na^{+} ratio despite the reduced endogenous lithium clearance in PHAII patients (17). Reduced lithium clearance can be due to either the increased proximal or distal tubular Na^{+} reabsorption (18). Because increased Na^{+} and Ca^{2+} reabsorption in proximal renal tubules would lead to hypocalciuria but not hypercalciuria, the enhanced distal tubular Na^{+} reabsorption as reflected by an activated NCC and ENaC activity in Wnk4^{D561A/+} mice may account for the decreased lithium clearance in PHAII (9). However, the detailed molecular mechanism of possible aberrant Na^{+} and Ca^{2+} handling in the upstream nephron of PHAII patients remains unclear.

With their hypertension and low renin levels (9), Wnk4^{D561A/+} mice must at minimum live in a state of salt-repletion. Their increased NCC and ENaC function in the distal tubules in the setting of similar urine Na^{+} excretion rate with wild-type littermates suggests reduced...
Na⁺ reabsorption in the upstream tubules might be in compensation for increased distal Na⁺ reabsorption. It is well known that salt-expanded states increase urine Ca²⁺ excretion by reducing both Na⁺ and Ca²⁺ reabsorption in the PCT with increased delivery of Na⁺ and Ca²⁺ to the distal tubules (19, 20). We examined two upstream Na⁺ transporters, NHE3 in PCT and NKCC2 in TAL involved in paracellular Ca²⁺ reabsorption. Although we did not find a significant decrease in gross NHE3 expression, other modifiers of NHE3 function such as increased sub-apical distribution and decreased phosphorylation may have been occurring in Wnk⁴D⁵⁶¹A/+ mice as has been reported in wild-type mice with Na⁺ overload (21). Of interest, we found a significant decrease in NKCC2 expression in Wnk⁴D⁵⁶¹A/+ mice. The blunted urine Ca²⁺ excretion in response to acute and chronic furosemide treatment in Wnk⁴D⁵⁶¹A/+ mice further supported the hypothesis that NKCC2 function was already inhibited. Although WNK4 mRNA was substantially expressed in TAL by in situ hybridization (22), its protein expression could only be detected in distal convoluted tubule and downstream tubules by IF staining (3, 9, 23), suggesting that a direct effect of mutant WNK4 on the expression of NKCC2 in TAL were less likely. The importance of volume expansion on urine Ca²⁺ excretion is also illustrated by the hypercalcuria in mineralocorticoid excess states with ENaC activation and salt-repleted hypertension (24–26). Drawing analogy with the hypercalciuria in furosemide-treated mice (11) or patients with NKCC2 inactivation even when treated with thiazide diuretics (29), in fact, patients with WNK4 mutations. Due to the widespread occurrence in wild-type mice with Na⁺ overload (21). Of interest, we found a significant decrease in NKCC2 expression in Wnk⁴D⁵⁶¹A/+ mice. The blunted urine Ca²⁺ excretion in response to acute and chronic furosemide treatment in Wnk⁴D⁵⁶¹A/+ mice further supported the hypothesis that NKCC2 function was already inhibited. Although WNK4 mRNA was substantially expressed in TAL by in situ hybridization (22), its protein expression could only be detected in distal convoluted tubule and downstream tubules by IF staining (3, 9, 23), suggesting that a direct effect of mutant WNK4 on the expression of NKCC2 in TAL were less likely. The importance of volume expansion on urine Ca²⁺ excretion is also illustrated by the hypercalcuria in mineralocorticoid excess states with ENaC activation and salt-repleted hypertension (24–26). Drawing analogy with the hypercalciuria in furosemide-treated mice (11) or patients with NKCC2 inactivating mutations (Barter’s syndrome) (27), reduced NKCC2 expression in Wnk⁴D⁵⁶¹A/+ mice may diminish lumen-positive potential and in turn decrease Ca²⁺ reabsorption in the TAL. An increase in TRPV6 and CBP-D28k expression was probably a secondary adaptive response to increased Ca²⁺ load from upstream tubules (11).

Given that PHAII patients with WNK1 mutations also have salt-sensitive hypertension and hyperkalemia without hypercalcuria (28), one might argue that salt overload may not play a major role in the hypercalcuria of PHAII patients with WNK4 mutations. Due to the widespread expression of Long-WNK1 in renal tubules, some PHAII patients with WNK1 mutations have persistent hypertension even when treated with thiazide diuretics (29). In fact, NKCC2 and ENaC have been reported to be activated by Long-WNK1 (30, 31). Thus, Na⁺ channels and transporters other than NCC may be involved in the pathogenesis of these patients. Accordingly, Ca²⁺ reabsorption may be governed by a different mechanism in these patients.

In conclusion, the Wnk⁴D⁵⁶¹A/+ knock-in PHAII mouse model exhibits hypercalcuria with normal glomerular filtration rate, serum ionized Ca²⁺, PTH, and 1,25(OH)₂D₃ levels. Inhibition of Ca²⁺ reabsorption in the upstream nephron may be involved in the hypercalcuria as evidenced by the compensatory expression of TRPV6 and CBP-D28k in the distal tubules of Wnk⁴D⁵⁶¹A/+ mice. The detailed mechanisms by which a salt-sensitive hypervolemic state regulates upstream Na⁺ and Ca²⁺ handling in WNK4 mutations causing PHAII merit further investigation.

Acknowledgments

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