RESEARCH ARTICLE

Effects of a high-sodium/low-potassium diet on renal calcium, magnesium, and phosphate handling

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Effects of a high-sodium/low-potassium diet on renal calcium, magnesium, and phosphate handling. Am J Physiol Renal Physiol 315: F110–F122, 2018. First published January 10, 2018; doi:10.1152/ajprenal.00379.2017.—The distal convoluted tubule (DCT) of the kidney plays an important role in blood pressure regulation by modulating Na⁺ reabsorption via the Na⁺/Cl⁻ cotransporter (NCC). A diet containing high salt (NaCl) and low K⁺ activates NCC, thereby causing Na⁺ retention and a rise in blood pressure. Since high blood pressure, hypertension, is associated with changes in serum calcium (Ca²⁺) and magnesium (Mg²⁺) levels, we hypothesized that dietary Na⁺ and K⁺ intake affects Ca²⁺ and Mg²⁺ transport in the DCT. Therefore, the present study aimed to investigate the effect of a high-Na⁺/low-K⁺ diet on renal Ca²⁺ and Mg²⁺ handling. Mice were divided in four groups and fed a normal-Na⁺/HCl, high-Na⁺/low-K⁺, normal-Na⁺/low-K⁺, or high-Na⁺/low-K⁺ diet for 4 days. Serum and urine were collected for electrolyte and hormone analysis. Gene and protein expression of electrolyte transporters were assessed in kidney and intestine by qPCR and immunoblotting. Whereas Mg²⁺ homeostasis was not affected, the mice had elevated urinary Ca²⁺ and phosphate (Pi) excretion upon high Na⁺ intake, as well as significantly lower serum Ca²⁺ levels in the high-Na⁺/low-K⁺ group. Alterations in the gene and protein expression of players involved in Ca²⁺ and Pi transport indicate that reabsorption in the proximal tubular and TAL is affected, while inducing a compensatory response in the DCT. These effects may contribute to the negative health impact of a high-salt diet, including kidney stone formation, chronic kidney disease, and loss of bone mineral density.

INTRODUCTION

The majority of people worldwide consume a diet containing high salt (NaCl) and low potassium (K⁺) levels, which is associated with increased blood pressure (hypertension) (44). Hypertension is one of the most important public health issues due to its increased risk of cardiovascular-renal disease, resulting in an overall elevated mortality (24). Although mechanisms for salt-dependent hypertension have been mainly related to the consumption of Na⁺, it has recently been shown that K⁺ intake plays an essential role in blood pressure regulation. Dietary K⁺ intake is inversely correlated to the risk of hypertension and cardiovascular-related mortality (31, 34). A K⁺-rich diet is expected to have a protective effect and can reduce blood pressure in hypertensive patients (1).

The kidney is the central organ for maintaining the body’s Na⁺ balance by controlling Na⁺ reabsorption along different nephron segments. Specifically, the aldosterone-sensitive distal part of the nephron, consisting of the distal convoluted tubule (DCT), connecting tubule (CNT), and collecting duct (CD), is essential for Na⁺ reabsorption (50). The DCT can be further subdivided into the early and late DCT, DCT1 and DCT2, respectively. Na⁺ reabsorption is mediated by the Na⁺/Cl⁻ cotransporter (NCC) in DCT1 and DCT2, as well as by the epithelial Na⁺ channel (ENaC) in DCT2, CNT, and CD. Here, Na⁺ reabsorption through ENaC is coupled to K⁺ secretion via the renal outer medullary K⁺ channel (ROMK). In short, Na⁺ reabsorption in the DCT1 determines the amount of Na⁺ delivery to the downstream segments and thereby has an indirect role in K⁺ secretion. This is highlighted by the molecular mechanisms underlying Gitelman syndrome and pseudohypoaldosteronism type 2 (familial hyperkalemic hypertension, FHH). Gitelman syndrome results from inactivating mutations in the NCC gene (48), which leads to reduced NCC function. Patients suffer from hypokalemia that can partly be explained by enhanced Na⁺ delivery to ENaC and subsequent K⁺ secretion by ROMK due to disturbed NCC-mediated Na⁺ reabsorption in the DCT. On the contrary, FHH patients suffer from hyperkalemia as a result of mutations in the genes encoding for proteins regulating NCC, including Cullin3, KLHL3, and the with-no-lysine kinases WNK1 and WNK4 (7, 60). Activation of the WNK kinases leads to phosphorylation and thereby activation of two related proteins, SPAK (STE20/SPS1-related proline/alanine-rich kinase) and OSR1 (oxidative stress-responsive kinase 1) (58). Activated SPAK and OSR1 kinases then phosphorylate key residues in the amino-terminal region of NCC, resulting in enhanced Na⁺ reabsorption by either increased transport activity or enriched NCC abundance at the apical membrane (35). Hence, Na⁺ reabsorption and subsequent K⁺ secretion are diminished in downstream segments, which can explain the hyperkalemia in...
FHHt. In addition, WNK kinases have also been shown to directly alter ENaC and ROMK function (23).

Interestingly, a recent study by Terker et al. (53) postulated an additional relationship between K⁺ and Na⁺ in which dietary K⁺ can regulate renal Na⁺ handling through plasma K⁺ levels. They suggested that lower plasma K⁺ induces a Cl⁻ efflux from the DCT cell, thereby releasing the Cl⁻-dependent inhibition of WNK1. Subsequently, this results in WNK1 autoprophosphorylation and activation (38), which in turn phosphorylates and activates NCC through the SPAK/OSR1 kinases (53). Conversely, increasing dietary K⁺ intake was shown to reduce NCC phosphorylation (and activity) in a linear range (40, 49, 56).

In addition to regulating renal Na⁺ and K⁺ handling, the distal part of the nephron also determines the final urinary Ca²⁺ and Mg²⁺ excretion since no Ca²⁺ or Mg²⁺ reabsorption takes place beyond the DCT/CNT. Interestingly, hypertension is associated with altered serum Ca²⁺ and Mg²⁺ levels (41, 42). However, the underlying molecular mechanisms are not well understood. Since renal Na⁺ and K⁺ handling plays a key role in the pathogenesis of hypertension, we hypothesized that the disturbances in the Ca²⁺ and Mg²⁺ balance can be explained by Na⁺ and/or K⁺-induced changes in Ca²⁺ and Mg²⁺ reabsorption in the distal part of the nephron. The present study aimed to identify the molecular players involved in the effect of dietary Na⁺ and K⁺ intake on renal Ca²⁺ and Mg²⁺ handling in wild-type mice fed a high-Na⁺ diet combined with normal or low K⁺. Furthermore, we examined renal phosphate (P³) handling.

MATERIALS AND METHODS

Antibodies. The following primary antibodies were used: Rabbit anti-NCC [Millipore, Billerica, MA; no. AB3553; immunoblotting (IB) 1:2,000; immunohistochemistry (IHC) 1:200], Rabbit anti-pNCC-T58 [kindly provided by Dr. Robert Fenton (36); IB 1:2,000], pNCC-T58 [kindly provided by Dr. Robert Fenton (36); IB 1:2,000], anti-NCC [Millipore, Billerica, MA; no. AB3553; immunoblotting (IB) 1:2,000]; Rabbit anti-rabbit Alexa Fluor 594-conjugated secondary antibody (Invitrogen, West Grove, PA; no. 515–035–003; IB 1:10,000); goat anti-rabbit (Sigma-Aldrich; no. A4914; IB 1:10,000); and Guinea pig anti-TRPV5 [(25), IHC 1:1000].

Buffers. Lysis buffer was composed of 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (vol/vol) Triton X-100, 1 mM sodium-orthovanadate, 10 mM sodium-glycerophosphate, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.27 mM sucrose, containing freshly added tablet of complete protease inhibitor cocktail (Roche, Basel, Switzerland), and 0.1% (vol/vol) β-mercaptoethanol. TBS-T (Tris-buffered saline) was composed of Tris-HCl (200 mM, pH 7.5), 0.15 M NaCl, and 0.2% (vol/vol) Tween-20. SDS-PAGE sample buffer was composed of 5x 10% (wt/vol) SDS, 10 mM β-mercaptoethanol, 50% (vol/vol) glycerol, 0.3 M Tris-HCl (pH 6.8), and 0.05% (wt/vol) bromophenol blue.

Animals. Studies were approved by the Central Animal Laboratory Nijmegen and the animal ethics board of the Radboud University Nijmegen (CCD no. AVD10300216382). All mice were 10- to 12-wk-old males, 20–25 g, and had a C57BL/J6 background. Prior to dietary intervention, they were housed in standard cages in a temperature- and light-controlled room, with control synthetic pellet chow and drinking water available ad libitum for 4 days. Afterward, the mice were randomly divided in four groups of 10 mice and fed their respective diet for 4 days. At the last day, they were housed in metabolic cages for 24 h to collect urine and feces. After being anesthetized with 4% (vol/vol) isoflurane, the mice were euthanized, and blood, kidneys, and intestines were collected and snap-frozen in liquid nitrogen.

Animal diets. All diets were prepared by SNNiff Spezialdiäten, Soest, Germany. High Na⁺ was obtained by adding NaCl to make a 6% (wt/wt) NaCl diet. Low-K⁺ diets [≤0.05% (wt/wt) K⁺] had no additional K⁺ added, and normal K⁺ was obtained by adding KCl to make a 1% (wt/wt) K⁺ diet. This resulted in the following diets: normal Na⁺/normal K⁺ (NN/LK), normal Na⁺/low K⁺ (NN/LK), high Na⁺/normal K⁺ (HN/LK), and high Na⁺/low K⁺ (HN/LK).

Electrolyte and renin measurements. Serum and urinary total Mg²⁺ concentrations were determined using a colorimetric xylidiI-II blue assay kit according to the manufacturer’s protocol (Roche/Hitachi), on a Bio-Rad plate reader at 600 nm. Serum and urinary total Ca²⁺ were colorimetrically determined with a chromogenic/buffer dual-component kit (Sigma-Aldrich), on a Bio-Rad plate reader at 570 nm. The obtained values were calibrated using a Precinorm standard solution (Precinorm U, Roche). Serum and urinary Na⁺, K⁺, and Cl⁻ determinations were performed at the university hospital central clinical laboratory on an automated system according to the manufacturer’s protocol (Abbott Diagnostics, Hoofddorp, The Netherlands). Total urine volume was used to calculate 24 h excretion. Active plasma renin concentration (APRC) was measured with a radioimmunoassay that detects the amount of angiotensin I (ANG I) produced per hour in the presence of excess angiotensinogen (ng of ANG I produced per ml of plasma per h) (9).

Immunohistochemistry. Immunohistochemistry was performed as previously described (25). In brief, costaining of TRPV5 with NCC was performed on 5-µm sections of poly-l-lysine paraformaldehyde (PLP) fixed frozen mouse kidney samples. Sections were incubated for 16 h at 4°C with guinea pig anti-TRPV5 primary antibody. TRPV5 staining was enhanced using TSA fluorescence System according to the manufacturer’s protocol (Perkin Elmer, Groningen, The Netherlands). Subsequently, sections were incubated for 2 h at room temperature with rabbit anti-NCC primary antibody. The NCC proteins were visualized with anti-rabbit Alexa Fluor 594-conjugated secondary antibody. Fluorescence microscopy was performed using 10x and 40x objectives on a Leica DMi6000 confocal microscope, and images were processed using LAS AF software.

Quantitative real-time PCR. Total kidney RNA was isolated using TRIzol agent (Invitrogen, Breda, The Netherlands) according to the manufacturer’s protocol, and subsequently treated with DNase (Promega, Fitchburg, MA) to remove genomic DNA. Reverse transcription of the mRNA was performed by M-MLV reverse transcriptase (Invitrogen) for 1 h at 37°C. Gene expression levels were determined by SYBR Green (Bio-Rad, Venendaal, The Netherlands) real-time PCR on a Bio-Rad (Hercules) analyzer and normalized for glyceraldehyde 3-phosphate dehydrogenase (Gapdh) expression levels. Relative expression was calculated according to the Livak method [2⁻ΔΔCt; ΔCt (cycle threshold) = Ct gene of interest – Ct housekeeping gene] and presented as fold change of expression compared with control diet (NN/LK). Primer sequences are indicated in Table 1.

Protein isolation. Cortex and medulla sections were macroscopically isolated from kidney tissue and homogenized in ice-cold lysis buffer. The kidney lysates were centrifuged at 4°C for 15 min at 16,000 g, and supernatants were snap-frozen in aliquots and stored at −80°C. Western blot analysis was performed on a gel and transferred to nitrocellulose membranes. Membranes were blocked with a 5% (wt/vol) milk solution and then incubated with primary antibodies overnight at 4°C. Membranes were subsequently incubated with secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence (ECL) reagents (Pierce), and quantification was achieved using ImageJ software.
stored at −80°C. Protein concentrations were determined using the Bradford method according to the manufacturer’s protocol (Bio-Rad).

**Immunoblotting.** Lysates (50 μg) in SDS sample buffer were subjected to electrophoresis on Criterion TGX precast gels (Bio-Rad) and then transferred to PVDF membranes. The membranes were blocked in TBS-T containing 5% (wt/vol) nonfat dry milk (NFDM) for 30 min at room temperature. Subsequently, they were immunoblotted overnight at 4°C with primary antibody [in TBS-T containing 5% (wt/vol) NFDM]. Next day, the blots were washed with TBS-T to remove unbound primary antibody and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. After subsequent washes, the protein was visualized with chemiluminescent reagent (SuperSignal West Femto/Pico; Thermo Scientific, Waltham, MA) and processed with the Bio-Rad ChemiDoc XRS. The NCC and NKCC2 bands on the immunoblots were quantified with ImageJ software (NIH).

**Statistical analysis.** All data are shown as means ± SE. Statistical significance (P < 0.05) was determined by analysis of variance and a Bonferroni post hoc test. All data were analyzed using GraphPad Prism 7.0 (GraphPad Software).

## RESULTS

**Effect of dietary intervention on electrolyte handling.** To investigate the role of dietary Na⁺ and K⁺ intake on renal electrolyte handling, the mice were fed four different diets [NN/NK (control), NN/LK, HN/NK, and HN/LK], and blood and 24-h urine were collected using metabolic cages. No differences in body weight or food intake were observed between the groups (Table 2). Importantly, water intake and urine production were significantly elevated in the high Na⁺ groups (HN/NK and HN/LK) compared with all other groups. Second, serum K⁺ levels were significantly lower in the groups fed a low-K⁺ diet compared with the normal K⁺ groups. Of note, high Na⁺ intake did not affect serum K⁺. Third, serum Cl⁻ levels decreased significantly in the NN/LK group, while the other groups showed no differences. Next, urinary excretion of Na⁺, K⁺, and Cl⁻ was determined in 24-h urine. Urinary K⁺ excretion corresponded to the serum K⁺ levels and was for instance drastically reduced in the low-K⁺ groups. The urinary Na⁺ and Cl⁻ excretion was significantly enhanced in groups fed a high-Na⁺ diet. To confirm the dietary effect on NCC phosphorylation as previously described (53), kidney cortex samples were subjected to immunoblotting demonstrating enhanced phosphorylation of NCC in both low-K⁺ groups (Fig. 1A). Interestingly, the low-K⁺ diet resulted in a reduced renal expression of Na⁺-K⁺-Cl⁻ cotransporter (NKCC2), the family member of NCC that transports Na⁺, K⁺, and Cl⁻ in the thick ascending limb of Henle’s loop (TAL) (Fig. 1B). Furthermore, we analyzed the expression of the proximal tubular Na⁺/H⁺ exchanger NHE3 by qPCR. The HN/LK group showed a significant downregulation of Slc9a3, the gene encoding NHE3 (Fig. 1C), compared with groups fed normal K⁺ (NN/NK and HN/NK).

After establishing that the dietary intervention resulted in electrolyte changes, serum levels and 24-h urine excretion of Ca²⁺ and Mg²⁺ were analyzed. There were no differences in serum Mg²⁺ levels or urinary Mg²⁺ excretion between the groups (Fig. 2, A and B). Interestingly, Ca²⁺ excretion was elevated upon high Na⁺ intake and an additional rise was demonstrated in the HN/LK group compared with HN/NK (Fig. 2C). Serum Ca²⁺ levels were also significantly decreased in the HN/LK group, while no changes were observed for the mice fed a HN/NK diet (Fig. 2D). In addition, phosphate (P) levels in urine and serum were examined. Urinary Pexcretion was significantly higher in mice fed a high-Na⁺ diet, independent of dietary K⁺. Serum P, was not different between the groups (Fig. 2, E and F).

**Expression of renal electrolyte transporters.** The expression of genes encoding for proteins involved in Mg²⁺ (re)absorp-

### Table 1. Sequences of the primers used for real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer 5’→3’</th>
<th>Reverse Primer 5’→3’</th>
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<tbody>
<tr>
<td>Gapdh</td>
<td>TAAACTTCACTATGAGG</td>
<td>GGTTGACACCACTACAAAC</td>
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<tr>
<td>Tprn6</td>
<td>AAAGCCATGGGATTTGCCCA</td>
<td>CTGCTGATGGAAAGCAAAC</td>
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<tr>
<td>Tprn7</td>
<td>TCTGCCTGCTGTTTGCTTT</td>
<td>CGGCATGTCCTGTCAGTT</td>
</tr>
<tr>
<td>Tprn5</td>
<td>CTGAGCTGCTGCTGCTGCT</td>
<td>TGGGACAGGTGAAAGACGATT</td>
</tr>
<tr>
<td>Tprn6</td>
<td>GGCTGACATCTCCATCTGAC</td>
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</tr>
<tr>
<td>Cyp2a4</td>
<td>GGAGTCTGAGGGTTCCTTTGG</td>
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</tr>
<tr>
<td>Cyp2b1</td>
<td>GTTGGAGATTGGTATTGTGG</td>
<td>ATGGAGGAGTTGGTACAGTT</td>
</tr>
<tr>
<td>Calb1</td>
<td>GAGGAGAAGTGGTTACCTGGA</td>
<td>ATGGAGGAGTTGGTACAGTT</td>
</tr>
<tr>
<td>Renin</td>
<td>GCAGGACATGGGATTTGCCCA</td>
<td>GTGAGTATGAGAAGGAAAC</td>
</tr>
<tr>
<td>Nap2a</td>
<td>TACGAGGACATGGGATTTGCCCA</td>
<td>GTGAGTATGAGAAGGAAAC</td>
</tr>
<tr>
<td>Nap2c</td>
<td>GTGAGGACATGGGATTTGCCCA</td>
<td>GTGAGTATGAGAAGGAAAC</td>
</tr>
<tr>
<td>Cldns1</td>
<td>GGTGCTTCTTGTGCTGAC</td>
<td>GCTGACATGGGATTTGCCCA</td>
</tr>
<tr>
<td>Cldns4</td>
<td>GTAGGAGTGTGAATGGACGAT</td>
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<td>Cldns6</td>
<td>GACAGGACATGGGATTTGCCCA</td>
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</tr>
<tr>
<td>Cldns2</td>
<td>GCGGAGAAGTGGTTACCTGGA</td>
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<td>Slc9a1</td>
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<tr>
<td>Slc9a1</td>
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### Table 2. Metabolic parameters

<table>
<thead>
<tr>
<th></th>
<th>NN/NK</th>
<th>NN/LK</th>
<th>HN/NK</th>
<th>HN/LK</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>24.0 ± 0.3</td>
<td>23.7 ± 0.3</td>
<td>23.4 ± 0.3</td>
<td>23.1 ± 0.3</td>
</tr>
<tr>
<td>Food intake, g</td>
<td>3.4 ± 0.2</td>
<td>3.8 ± 0.5</td>
<td>3.0 ± 0.2</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>Water intake, ml</td>
<td>3.2 ± 0.6</td>
<td>3.9 ± 0.5</td>
<td>6.6 ± 0.6*</td>
<td>9.4 ± 1.4*</td>
</tr>
<tr>
<td>Urine volume, ml</td>
<td>1.4 ± 0.1</td>
<td>1.7 ± 0.3</td>
<td>4.4 ± 0.4b</td>
<td>5.9 ± 0.7b</td>
</tr>
</tbody>
</table>

Mice were fed the indicated diets for 4 days and were kept in individual metabolic cages for the last 24 h. Values are expressed as means ± SE (n = 10). Significant differences are indicated as *P < 0.05 compared with NN/NK and **P < 0.05 compared with NN/LK.

### Table 3. Urinary electrolyte excretion and serum electrolyte levels

<table>
<thead>
<tr>
<th></th>
<th>NN/NK</th>
<th>NN/LK</th>
<th>HN/NK</th>
<th>HN/LK</th>
</tr>
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<tbody>
<tr>
<td>Serum electrolytes, mM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺</td>
<td>157 ± 1</td>
<td>157 ± 1</td>
<td>159 ± 1</td>
<td>163 ± 1*</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>113 ± 1</td>
<td>107 ± 1b</td>
<td>112 ± 1b</td>
<td>115 ± 1b</td>
</tr>
<tr>
<td>K⁺</td>
<td>5.2 ± 0.1</td>
<td>4.3 ± 0.2</td>
<td>5.3 ± 0.1b</td>
<td>3.9 ± 0.1b</td>
</tr>
<tr>
<td>Ureine electrolytes, μmol/24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na⁺</td>
<td>230 ± 19</td>
<td>131 ± 10</td>
<td>1,939 ± 146b</td>
<td>1,847 ± 155b</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>530 ± 44</td>
<td>155 ± 14</td>
<td>2,444 ± 195b</td>
<td>2,023 ± 155b</td>
</tr>
<tr>
<td>K⁺</td>
<td>502 ± 40</td>
<td>123 ± 1.7b</td>
<td>549 ± 32b</td>
<td>26.8 ± 2.5b</td>
</tr>
</tbody>
</table>

Data were collected after housing the mice in metabolic cages for the last 24 h. Urinary excretion (24 h) and serum levels of Na⁺, K⁺, and Cl⁻ from mice kept on the indicated diets are shown as means ± SE (n = 10), and significance is indicated as *P < 0.05 compared with NN/NK, **P < 0.05 compared with NN/LK, and ***P < 0.05 compared with HN/NK.
tion in kidney and intestine was analyzed in the different groups using RT-qPCR (Fig. 3). Renal transcript levels of Trpm6, encoding the apical Mg²⁺ channel TRPM6, were significantly higher in the high-Na⁺ groups, while no significant differences were observed for Trpm6 expression levels in colon (Fig. 3, A and C). Of note, the renal expression of Trpm7, encoding the ubiquitous Mg²⁺ channel, was significantly lower in the HN/LK group (Fig. 3B).

Next, the expression of several calciotropic genes in kidney and intestine was examined. There was no difference between the groups in the expression levels of renal Trpv5 or intestinal Trpv6, coding for luminal epithelial Ca²⁺ channels in the kidney and duodenum, respectively (Fig. 4, A and B). Immunohistochemical analysis of mouse kidney sections also showed that there are no differences in TRPV5 protein abundance between the groups (Fig. 5, A and B). The sections were costained with NCC to confirm localization of TRPV5 in the DCT2, and demonstrate that NCC protein expression was not altered (Fig. 5, A and B). Interestingly, the expression levels of the genes coding for the basolateral Ca²⁺ transporter NCX1
(Slc8a1) and for Ca\(^{2+}\)-binding protein calbindin-D\(_{28k}\) (CaBP\(_{28k}\)) were significantly downregulated upon a low dietary K\(^{+}\) intake (Fig. 4, C and D). This was restored to control levels by addition of high Na\(^{+}\), as the HN/LK group had significantly higher expression levels than NN/LK and did not significantly differ from NN/NK (Fig. 4, C and D). In addition, the high-Na\(^{+}\) diet (both HN/NK and HN/LK) was shown to increase CaBP\(_{28k}\) protein levels in the kidney cortex (Fig. 4E).

The expression of several members of the paracellular pathway was also investigated. Claudin-16/19 play an important role in transport of Ca\(^{2+}\) and Mg\(^{2+}\) in the TAL (26). Whereas renal expression of Cldn19 was not different, the Cldn16 expression significantly decreased in mice fed a low K\(^{+}\) diet (Fig. 3, D and E). In addition, claudin-14 expression was examined as it is thought to be involved in claudin-16/19 function (22). There were no differences in Cldn14 expression (Fig. 3F). Furthermore, expression of claudin-2, which participates in paracellular Ca\(^{2+}\) transport in the proximal tubule (33), is unaltered by the diet (Fig. 4F).

To assess the differences in urinary Pi excretion, expression of phosphate transporters was examined. The major part of...
filtered Pi is reabsorbed in the proximal tubule by the Na\(^+\)-dependent Pi cotransporters NaPi-2a and NaPi-2c (45). Genes encoding these transporters, \(Napi2a\) and \(Napi2c\), respectively, were significantly downregulated in mice fed a HN/LK diet compared with control (NN/NK) (Fig. 6, A and B). Of note, \(Napi2c\) was also expressed significantly lower in the NN/LK group compared with NN/NK and HN/NK groups. Immunoblotting confirmed the mRNA expression data of \(Napi2a\) demonstrating a lower renal NaPi-2a protein expression in the HN/LK group compared with control (Fig. 6 C). In addition, NaPi-2c protein levels were significantly lower in the mice fed the HN/LK diet compared with other groups (Fig. 6 C).

**Hormonal regulation upon dietary intervention.** To assess the effect of the dietary regimes on hormonal regulation, the expression of renin in the kidney as well as APRC was examined. Both renin mRNA expression and APRC were significantly lower in the high Na\(^+\) conditions (HN/NK and HN/LK), but there was no difference upon K\(^+\) restriction in a high-Na\(^+\) diet (Fig. 7, A and B). Of note, K\(^+\) restriction alone (NN/LK) led to higher APRC compared with all other groups (Fig. 7 B).

Next, the expression of \(Cyp27b1\) and \(Cyp24a1\), encoding for proteins that are responsible for the production and degradation of active vitamin D, respectively, was examined. While renal \(Cyp24a1\) expression levels did not differ between the groups,
the expression of Cyp27b1 was lower in the groups fed either a low-K⁺ (NN/LK), high-Na⁺ (HN/NK), or a combined (HN/LK) diet compared with control (Fig. 7, C and D). Moreover, the expression was lowest in the HN/LK group, which was significant compared with HN/NK (Fig. 7C).

To understand whether hormones involved in the maintenance of the Ca²⁺ balance were altered, plasma PTH (PTH 1–84) and FGF23 (FGF23 COOH-terminal) levels were analyzed. There were no differences in these hormone levels between the groups (Fig. 7, E and F).

**DISCUSSION**

The present study demonstrates that a diet containing high Na⁺ with low K⁺ can have significant effects on renal Ca²⁺ handling, while not affecting the Mg²⁺ balance. Serum and urine Mg²⁺ levels were not changed upon 4 days of diet. In contrast, the urinary Ca²⁺ excretion was significantly enhanced by high Na⁺ intake, which was aggravated upon lowering dietary K⁺ in combination with high Na⁺. The latter also resulted in decreased serum Ca²⁺ levels. In addition, there was
a significant increase in urinary Pi excretion upon high Na\(^+\) intake. This study provides more insight into the effects of an altered Na\(^+\) and K\(^+\) intake on renal Ca\(^{2+}\), Mg\(^{2+}\), and Pi handling and offers further insight into the observed molecular changes in the kidney.

There is a growing body of evidence demonstrating that dietary K\(^+\) intake associates inversely with blood pressure, often as strong as the positive correlation with dietary salt (1, 2, 17, 21, 31, 34). Importantly, it has recently been postulated that low blood K\(^+\) levels are a key regulator of renal Na\(^+\) reabsorption through stimulation of NCC-mediated Na\(^+\) transport in the DCT (53). Our study demonstrated that low dietary K\(^+\) intake enhances pNCC abundance in both NN/LK and HN/LK conditions, in line with a previous study by Terker et al. (53). To preserve extracellular fluid (ECF) volume, and hence blood pressure, NCC is phosphorylated and activated via the renin-angiotensin-aldosterone system (RAAS) during dietary salt restriction (53). We showed an enhanced NCC phosphorylation despite low RAAS activation at HN/LK condition. Suppressed activation of RAAS was validated by decreased APRC in both HN/NK and HN/LK conditions. Together, this indicates that the effect of K\(^+\) deficiency is more powerful than ECF volume by controlling Na\(^+\) reabsorption in the DCT. High dietary K\(^+\) was shown to suppress NCC phosphorylation during low-Na\(^+\) conditions likely to preserve kaliuresis (57).

Next to regulating Na\(^+\) reabsorption, the DCT is also the main segment of active Mg\(^{2+}\) transport. The Mg\(^{2+}\)-permeable channel TRPM6 is considered the gatekeeper of the Mg\(^{2+}\) balance as it determines the final urinary Mg\(^{2+}\) excretion (59). Importantly, our study showed that high Na\(^+\) and/or K\(^+\) deficiency does not affect Mg\(^{2+}\) transport in the DCT in a similar way as Na\(^+\) transport, suggesting that the signaling pathway affecting NCC function is not linked to TRPM6-

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Fig. 5. Protein expression of TRPV5 in kidney. A: representative microphotographs are shown for TRPV5 (green) and NCC (red) protein expression in mouse kidney tissue, with a 10× (top) and 40× (bottom) magnification. Groups are indicated above the images. B: protein expression of TRPV5 and NCC was quantified by fluorescence microscopy (Leica DMI6000B). Bar graph of the analysis shows arbitrary units for TRPV5 and NCC protein expression as means ± SE (n = 10).
mediated transport. In contrast, a previous study demonstrated increased urinary Mg²⁺ excretion in rats fed a high-Na⁺ diet (29). This discrepancy could be due to the period of the diets (7 days vs. 4 days), suggesting a time-dependent effect. A high-Na⁺ diet likely reduces paracellular Mg²⁺ transport in the proximal tubule and the TAL, resulting in an increased distal delivery of Mg²⁺ that is subsequently responsible for the observed upregulation of TRPM6. This could be the first defense mechanism to maintain blood Mg²⁺ levels constant.

Although the various diets had no major effect on renal Mg²⁺ handling, there were prominent outcomes on urinary Ca²⁺ excretion and serum Ca²⁺ levels. Ca²⁺ homeostasis is maintained through the interplay between the intestines, bone, and kidney. Dietary Ca²⁺ is absorbed via the intestine into the blood, subsequently filtered by the glomerulus, and reabsorbed along the nephron or stored in bone. The amount of filtered Ca²⁺ that is not reabsorbed along the nephrons will be excreted in the urine, demonstrating the important role of the kidney in maintaining the Ca²⁺ balance. An inappropriately high amount of urinary Ca²⁺ excretion, also known as hypercalciuria, contributes to the development of osteoporosis and kidney stone formation (4, 13). It has been established that a high-salt diet induces hypercalciuria (12, 16), and that restricted salt intake is often used for prevention of kidney stones recurrence, particularly in cases of Ca²⁺-containing stones (14, 30, 43, 47). Together, there is a clinical association between salt intake,
hypertension, hypercalciuria, and increased risk of kidney stones (10, 55). Interestingly, our study reveals a rapid renal response to dietary changes as inappropriate loss of urinary Ca²⁺ was observed in the HN/LK diet group after 4 days. Furthermore, urinary Pi excretion follows the same trend and is also significantly elevated in the HN/LK-fed mice. This is particularly interesting given the fact that ~10% of all kidney stones are calcium-phosphate stones (61). Our findings demonstrated that short-term dietary changes can already result in hypercalciuria and phosphaturia, which are important risk factors for stone formation. There is an increasing interest in dietary intervention for kidney stone prevention and a diet high in K⁺ was recently associated with a lower risk of kidney stones (18). Future studies should aim at understanding the underlying molecular effects of low-salt and high-K⁺ diet on progression of age-related bone loss and development of kidney stones.

The effect on urinary Ca²⁺ and Pi excretion suggests that the high-Na⁺/low-K⁺ diet initially affects proximal tubular reabsorption. It is widely assumed that the degree of hypercalciuria increases proportionately with the amount of Na⁺ excreted in the urine and that dietary Na⁺ restriction can decrease urinary Ca²⁺ excretion in both stone formers and healthy individuals (28, 37, 47, 52, 54). Earlier micropuncture studies have also demonstrated that Ca²⁺ reabsorption correlates with Na⁺ reabsorption along the proximal tubule (39). Our study now demonstrated a reduced NHE3 expression, which indicates that Na⁺ reabsorption in the proximal tubule could be reduced.
However, there was no increased urinary Na\(^+\) excretion in the HN/LK group compared with high-Na\(^+\) diet alone, while urinary Ca\(^{2+}\) was significantly enhanced upon dietary K\(^+\) depletion in combination with high Na\(^+\) (HN/LK). This suggests an additional effect of low plasma K\(^+\) levels on renal Ca\(^{2+}\) handling, next to the proposed Na\(^+\)-dependent Ca\(^{2+}\) reabsorption in the proximal tubule (51). We propose that the TAL-mediated reabsorption could be altered as a result of low-K\(^+\) diet. Both groups of mice, either NN/LK or HN/LK, have lower NKCC2 and claudin-16 expression. Therefore, we propose that the high-Na\(^+\)-diet alone mainly affects proximal tubular reabsorption via extracellular fluid expansion, resulting in increased urinary Ca\(^{2+}\) and P\(_i\) excretion. However, the aggravated hypercalciuria in HN/LK animals might be due to a combined effect on the proximal tubule and TAL, which could not be completely compensated by the DCT. Furthermore, Terker et al. showed that NCC activation plays an essential role in the Ca\(^{2+}\) excretion under low-K\(^+\) condition. NCC knockout mice exhibited a diminished urinary Ca\(^{2+}\) excretion upon receiving a HN/LK diet (53). Our study demonstrated that CasBP28\(_k\) protein levels were significantly enhanced in the high-Na\(^+\) groups, suggesting a compensatory mechanism for increased Ca\(^{2+}\) transport in the DCT. However, there was no change in expression of the apical Ca\(^{2+}\) channel TRPV5. It should be noted that expression changes do not necessarily reflect alterations in protein function and transport capacity.

The body usually compensates for urinary Ca\(^{2+}\) loss by increased intestinal absorption of Ca\(^{2+}\). Importantly, TRPV6 expression was not changed in duodenum, the main site for active (regulated) Ca\(^{2+}\) absorption. However, we demonstrated downregulation of renal Cyp27bl, encoding for 25-hydroxyvitamin D\(_3\) 1-alpha-hydroxylase (1\(\alpha\)-hydroxylase), in the groups receiving either a low-K\(^+\) diet (NN/LK) or high-Na\(^+\)-Na\(^+\) diet (HN/NK), or a combination diet (HN/LK). The 1\(\alpha\)-hydroxylase is responsible for the generation of active vitamin D, which in turn acts toward increasing serum Ca\(^{2+}\) levels. Vitamin D can subsequently increase serum Ca\(^{2+}\) levels by stimulating intestinal Ca\(^{2+}\) absorption, renal Ca\(^{2+}\) reabsorption, and Ca\(^{2+}\) release from bone (19). Lower levels of this enzyme could partly explain the unchanged TRPV5 and TRPV6 mRNA expression despite extreme hypercalciuria. The Cyp27bl expression in the HN/LK group was even significantly lower than high Na\(^+\) alone (HN/NK), in line with an aggravated hypercalciuria.

Interestingly, the trend of Cyp27bl expression coincided with that of Napi2c in the different dietary groups, as well as Napi2a downregulation in the HN/LK group. NaPi-2a and NaPi-2c protein levels were also significantly reduced in this group, which is in line with the phosphaturic response upon a HN/LK diet. Of note, it is proposed that the abundance of NaPi-2a protein reflects the capacity of the kidney to reabsorb P\(_i\) (5, 32). In contrast to our results, previous studies reported a significant decrease in NaPi-2c protein abundance upon longer (14 days) dietary K\(^+\)-deficiency in rats and mice, while the abundance of NaPi-2a increased (8, 62). This discrepancy could be due to the different dietary time frames (4 vs. 14 days) as we did not observe hyperphosphaturia or hypophosphatemia upon diet-induced hypokalemia by the low-K\(^+\) diet alone. The lack of changes in urinary or plasma P\(_i\) levels could also be explained by the significantly elevated APRC in the NN/LK group, which induces high ANG II levels that in turn stimulate proximal tubular reabsorption of Na\(^+\) and other electrolytes.

In addition, there is a clear interrelationship between hormonal control of Ca\(^{2+}\) and P\(_i\) handling, which involves the PTH-FGF23-vitamin D axis (6). Although vitamin D is known to regulate intestinal P\(_i\) absorption, it has not been established whether vitamin D is directly involved in the regulation of renal P\(_i\) reabsorption. There are vitamin D-responsive elements in human genes encoding NaPi-2a and NaPi-2c, but data regarding its effect on expression have remained controversial so far (11, 20, 27). Vitamin D is known to act in concert with FGF23 and PTH to control renal P\(_i\) and Ca\(^{2+}\) reabsorption. For example, FGF23 can have instant effects on P\(_i\), homeostasis through reduction of renal NaPi-2a and Cyp27bl levels (46). Interestingly, it was recently shown in mice deficient in Fgf23 that FGF23 can regulate renal Na\(^+\) reabsorption via altering NCC expression and function (3). We have examined the impact of the diets on serum FGF23 and PTH levels, but did not find changes in response to the diets. Future studies should increase our understanding of the role of the PTH-FGF23-vitamin D axis in high-Na\(^+\) and/or low-K\(^+\) diet-induced changes in electrolyte handling.

In conclusion, during short-term modification of Na\(^+\) and K\(^+\) intake, the kidney rapidly develops adaptation with corresponding changes in urinary electrolyte excretion. Our results suggest that instant dietary modifications have primary effects on proximal tubular reabsorption with a compensatory response in the DCT. As the majority of Ca\(^{2+}\) and P\(_i\) is reabsorbed in the proximal tubule, the distal part of the nephron is unable to recover for the excessive loss, while the mechanism is sufficient to maintain Mg\(^{2+}\) homeostasis.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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