The role of calbindin-D28k on renal calcium and magnesium handling during treatment with loop and thiazide diuretics

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Lee C, Ng H, Lee Y, Lai L, Lien YH. The role of calbindin-D28k on renal calcium and magnesium handling during treatment with loop and thiazide diuretics. Am J Physiol Renal Physiol 310: F230–F236, 2016. First published November 18, 2015; doi:10.1152/ajprenal.00057.2015.—Calbindin-D28k (CBD-28k) is a calcium binding protein located in the distal convoluted tubule (DCT) and plays an important role in active calcium transport in the kidney. Loop and thiazide diuretics affect renal Ca and Mg handling: both cause Mg wasting, but have opposite effects on Ca excretion as loop diuretics increase, but thiazides decrease, Ca excretion. To understand the role of CBD-28k in renal Ca and Mg handling in response to diuretics treatment, we investigated renal Ca and Mg excretion and gene expression of DCT Ca and Mg transport molecules in wild-type (WT) and CBD-28k knockout (KO) mice. Mice were treated with chlorothiazide (CTZ; 50 mg·kg⁻¹·day⁻¹) or furosemide (FSM; 30 mg·kg⁻¹·day⁻¹) for 3 days. To avoid volume depletion, salt was supplemented in the drinking water. Urine Ca excretion was reduced in WT, but not in KO mice, by CTZ. FSM induced similar hypercalciuria in both groups. DCT Ca transport molecules, including transient receptor potential vanilloid 5 (TRPV5), TRPV6, and CBD-9k, were upregulated by CTZ and FSM in WT, but not in KO mice. Urine Mg excretion was increased and transient receptor potential subfamily M, member 6 (TRPM6) was upregulated by both CTZ and FSM in WT and KO mice. In conclusion, CBD-28k plays an important role in gene expression of DCT Ca, but not Mg, transport molecules, which may be related to its being a Ca, but not a Mg, intracellular sensor. The lack of upregulation of DCT Ca transport molecules by thiazides in the KO mice indicates that the DCT Ca transport system is critical for Ca conservation by thiazides.

furosemide; thiazides; TRPV5; TRPV6; TRPM6; calbindin-D28k; calbindin-D9k

LOOP AND THIAZIDE DIURETICS are commonly used for treating hypertension, congestive heart failure, and other disorders with fluid overload (21). Loop diuretics are inhibitors of the Na-K-2Cl cotransporter (NKCC) in the thick ascending limb of the loop of Henle (TALH), while thiazides inhibit the Na-Cl cotransporter (NCC) in the distal convoluted tubule (DCT). Both cause renal loss of Na, K, and Mg, but have different effects on renal Ca handling: loop diuretics cause hypercalciuria, while thiazides cause hypocalciuria (6, 19). Those distinct effects on Ca excretion are also observed in patients with mutations in the NKCC (Barter syndrome) and NCC (Gitelman syndrome), respectively (22).

In the kidney, Ca is reabsorbed through passive paracellular transport in the proximal tubule (PT) and TALH and active transcellular transport in the DCT (3). The gatekeeper of this active Ca transport mechanism is TRPV5. Ca enters cells across the apical TRPV5, then binds to calbindin-D28k (CBD-28k), the major intracellular calcium binding proteins in DCT, and diffuses to the basolateral membrane, where Ca is extruded via the Na/Ca exchanger or Ca pumps (5). The underlying mechanism of hypercalciuria induced by loop diuretics is related to the loss of the driving force of paracellular transport of Ca in the TALH. To compensate for the large loss of Ca, active Ca transporters such as TRPV5 and TRPV6 in the downstream DCT are upregulated (15). A similar compensatory mechanism has been reported in the gentamicin- and diabetes-induced hypercalciuria by our group (14, 16). Thiazides induce hypocalciuria via different mechanisms depending on the volume status: with volume depletion, increased passive reabsorption in PT is the key mechanism (18); without volume depletion, the increased Ca reabsorption mainly occurs in the DCT via upregulation of TRPV5/6 and CBD-28k (13).

Both loop and thiazide diuretics cause renal Mg loss. In the kidney, similar to Ca, Mg is also reabsorbed via passive paracellular transport mainly in the TALH and active transcellular transport in the DCT, mainly mediated by the apical active transporter TRPM6 (5). It is likely that the loop diuretics reduce the paracellular transport of Mg by diminishing the driving force via inhibiting NKCC. As for thiazide, it has been assumed that reduction of TRPM6 in the DCT may explain the Mg loss, as Nijenhuis et al. (18) have demonstrated that TRPM6 abundance is reduced in NCC knockout mice.

CBD-28k is well known as an intracellular Ca buffer, not only in DCT cells but also in many other cells, such as neurons (3). Recently, it has been shown that CBD-28k dynamically controls TRPV5-mediated Ca influx by physical interaction with TRPV5 at the plasma membrane and buffering local free Ca to prevent inactivation of the channel (10). In addition, Berggård et al. (1) demonstrated elegantly that CBD-28k can also serve as a Ca sensor. In the present study, we aimed to investigate how CBD-28k ablation affects the renal Ca and Mg handling in response to loop and thiazide diuretics, and the gene regulation of active Ca and Mg transporters, such as TRPV5/6, CBD-9k, and TRPM6, in the DCT.
MATERIALS AND METHODS

Animals

Male 6- to 8-wk-old C57BL/6 mice and CBD-28k knockout mice (purchased from Jackson Laboratories, Bar Harbor, ME) were used for this experiment. The wild-type (WT) and CBD-28k knockout (KO) mice were each divided into three groups (n = 8–10/group): control, chlorothiazide (CTZ), and furosemide (FSM) groups. All animals were maintained under a constant 12-h photoperiod at temperatures between 21 and 23°C. They were allowed free access to selected food and water. The food contained sodium (0.2%), Ca (1.0%), and Mg (0.16%). This study was approved by Institutional Animal Care and Use Committee at Kaohsiung Chang-Gung Memorial Hospital.

Administration of Diuretics

CTZ (50 mg·kg⁻¹·day⁻¹) or FSM (30 mg·kg⁻¹·day⁻¹) was given in two divided doses via intraperitoneal injection for 3 days. To avoid volume depletion induced by diuretics, drinking water with 0.8% NaCl and 0.1% KCl was supplied during the experimental period as in a previous study (15). During the third day of diuretic injection, 24-h urine samples were collected and urine volume was recorded. The next day, blood samples were collected before animals were euthanized for harvesting of their kidneys.

Biochemical Measurement

Urine concentrations of creatinine, sodium (Na), potassium (K), Ca, and Mg and plasma concentration of creatinine, Ca, and Mg were determined using the Synchron CX Delta system (Beckman, Fullerton, CA) according to the manufacturer’s operating protocol. Urine aldosterone level was determined by an Aldosterone ELISA Kit (Abcam, Cambridge, UK). Hematocrit was measured using a standard centrifugation method to assess the volume status.

Molecular Studies

Gene expression analysis. For RNA isolation and complementary DNA synthesis, total renal RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). Reverse transcription for cDNA synthesis was performed using a reverse transcription system (Promega, Madison, WI) as described previously (13).

Gene expression was analyzed by employing real-time PCR as described previously (13). The molecules involved in Ca and Mg transport in the TALH and DCT, including claudin-16, TRPV5, TRPV6, CBD-28k, CBD-9k, and TRPM6 were assessed. The mRNA abundance of β-actin was used as the internal reference for each gene evaluated. The synthesized cDNA was then subjected to real-time PCR using the ABI prism 7900 HT Sequence Detection System (ABI, Foster City, CA). The synthesized cDNA was then subject to real-time PCR using the ABI camera (Evolution VF, Media Cybernetics, Rockville, MD).

PCR of each gene was performed in triplicate to obtain a mean value. The changes in gene expression are presented as percentages (%) of control group animal values.

Immunofluorescence staining. Frozen kidney tissue was used for CBD-28k, TRPV5, and TRPM6 protein assessment. Sections 5 μm in thickness were fixed with 4% paraformaldehyde and incubated with primary antibody (goat anti-mouse CBD-28k monoclonal antibody, 1:500, Sigma; rabbit anti-rat ECaC1, 1:100, Alpha Diagnostic International, San Antonio, TX; and guinea pig polyclonal antibody to TRPM6, 1:1,000, Abcam) for 16 h, and then with FITC-conjugated secondary antibodies for CBD-28k (Jackson ImmunoResearch Laboratories, West Grove, PA): streptavidin/FITC-conjugated secondary antibody for TRPV5 (DakoCytonoma, Dako, Carpinteria, CA), and Cy3-conjugated AffiniPure F(ab')2 and Alexa Fluor 488 goat anti-rabbit IgG (H+L) antibody for TRPM6 (Life Technologies, Carlsbad, CA) for 30 min. The immunofluorescence pictures were then taken using a Zeiss fluorescence microscope connected to a digital photo camera (Evolution VF, Media Cybernetics, Rockville, MD).

Immunoblotting study. Renal sections were frozen at −80°C and then homogenized at 4°C in protein lysis buffer solution containing 20 mM Tris-HCl (pH 7.4), 0.1% SDS, 5 mM EDTA, 1% Triton X-100, and a protease inhibitor cocktail tablet (Roche, Penzberg, Germany). After determination of the concentration, the protein samples were run on 10% SDS-PAGE and then transferred to a nitrocellulose membrane. The membrane was incubated with an anti-TRPV5 antibody (1:1,000, Abcam), anti-CBD-28k antibody (1:20,000, Swant, Marly, Switzerland), anti-TRPV6 antibody (1:1,000, Alomone Laboratory, Jerusalem, Israel), and anti-CLDN16 antibody (1:3,000, Abcam) after blocking with 10% nonfat milk. The membrane was then incubated with an anti-rabbit IgG, horseradish peroxidase-linked antibody (1: 5,000, Cell Signaling Technology) for TRPV5, TRPV6, and claudin-16 blotting. Goat anti-mouse IgG (1:20,000, Jackson ImmunoResearch Laboratories) was utilized for CBD-28k. β-Actin was used as the internal control in this study. The abundance of the above four molecules was then quantified by densitometric analyses. The changes in protein abundance are presented as percentages (%) of control animal values.

Statistical Analyses

Data are presented as means ± SD. Statistical analyses of the data were performed using SPSS-PC software. Unpaired Student’s t-tests were used to compare differences between two groups, and ANOVA followed by post hoc multiple comparisons (least significant difference test) were utilized among four groups. A P value of <0.05 was considered statistically significant for all tests.

RESULTS

Biochemical and Physiological Data

Body weight, urine volume, and serum and urine biochemical data are shown in Table 2. There were no significant changes in the body weight among all animal groups. The hematocrit of different treatment groups was similar to that of control animals. Daily urinary excretion of aldosterone did not differ between treatment and control groups in either WT or KO animals. These results indicate that diuretics treatment did not cause volume depletion in our animals. The serum levels of creatinine, Ca, or Mg did not show any significant difference between WT and KO mice, or after CTZ or FSM treatment in either group. The daily urinary Na, K, and Mg excretion was not different between untreated WT and KO mice, but KO mice excreted more Ca than WT mice (5.0 ± 0.5 vs 3.5 ± 0.3 mmol/day. P < 0.05, Table 2). In both WT and KO groups, treatment with CTZ or FSM increased daily urine volume and

Table 1. Lists of primers used for real-time PCR

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<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>β-Actin</td>
<td>ttccaccaaacacagtagggg</td>
<td>gaacgccctgtagtcceaatag</td>
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<tr>
<td>CBD-28k</td>
<td>cagaaacctccatctgaatca</td>
<td>tccgctggatagctccaatcc</td>
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<tr>
<td>CBD-9k</td>
<td>aggccccagaaaaattgtctc</td>
<td>taatttcgtagtcceaatcc</td>
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<td>gacggcctgccaataacaga</td>
<td>tggggctgatgttgcacaa</td>
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<td>TRPV6</td>
<td>atccgctgccaataacaga</td>
<td>tctctggatttgcacaaag</td>
</tr>
<tr>
<td>TRPM6</td>
<td>ctgcattttctgacacagca</td>
<td>ctgcagttcagaacagttg</td>
</tr>
<tr>
<td>Claudin-16</td>
<td>ttttcctacccctgctttgg</td>
<td>gcccctgtagtcceaatag</td>
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CBD-28k, calbindin-D28k; CBD-9k, calbindin-D9k; TRPV5, TRPV6, and TRPM6, transient receptor potential V5, V6, and M6.
Fig. 1. Gene expression of transient receptor potential vanilloid 5 (TRPV5; A), TRPV6 (B), calbindin D-28k (CBD-28k; C), CBD-9k (D), transient receptor potential subfamily M, member 6 (TRPM6; E), and claudin-16 (F) in wild-type and CBD-28k knockout (KO) mice following chlorothiazide (CTZ) and furosemide (FSM) treatment. *P < 0.05 vs. wild-type control (Ctrl). #P < 0.05 vs. CBD-28k KO control.

Table 2. Body weight, urine volume, and biochemical data

<table>
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<tr>
<th></th>
<th>Wild-Type Control</th>
<th>CTZ</th>
<th>FSM</th>
<th>Calbindin-D28k Knockout</th>
<th>Control</th>
<th>CTZ</th>
<th>FSM</th>
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<tr>
<td>Body weight, g</td>
<td>21.1 ± 0.5</td>
<td>21.0 ± 0.2</td>
<td>20.7 ± 0.7</td>
<td>20.2 ± 0.3</td>
<td>20.5 ± 0.4</td>
<td>20.2 ± 0.5</td>
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<tr>
<td>Urine volume, ml/day</td>
<td>2.31 ± 0.17</td>
<td>2.92 ± 0.18*</td>
<td>3.45 ± 0.27*</td>
<td>1.87 ± 0.17</td>
<td>2.64 ± 0.22*</td>
<td>4.52 ± 0.39*</td>
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<tr>
<td>Hematocrit, %</td>
<td>43.6 ± 1.5</td>
<td>42.7 ± 1.2</td>
<td>44.1 ± 1.6</td>
<td>44.1 ± 1.2</td>
<td>45.0 ± 1.1</td>
<td>45.3 ± 1.7</td>
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<td>Urine aldosterone, pg/day</td>
<td>71.2 ± 7.7</td>
<td>65.3 ± 5.9</td>
<td>73.3 ± 4.6</td>
<td>59.1 ± 2.7</td>
<td>62.3 ± 6.2</td>
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<td>Serum Cr, mg/dl</td>
<td>0.28 ± 0.02</td>
<td>0.24 ± 0.02</td>
<td>0.28 ± 0.04</td>
<td>0.25 ± 0.03</td>
<td>0.29 ± 0.01</td>
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<td>Serum Mg, mg/dl</td>
<td>9.5 ± 0.2</td>
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<td>10.0 ± 0.2</td>
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<td>Serum Na, mmol/day</td>
<td>1.8 ± 0.1</td>
<td>2.0 ± 0.4</td>
<td>1.8 ± 0.1</td>
<td>1.9 ± 0.3</td>
<td>1.7 ± 0.3</td>
<td>1.9 ± 0.3</td>
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<tr>
<td>Urine K, mmol/day</td>
<td>252 ± 15</td>
<td>395 ± 25*</td>
<td>379 ± 50*</td>
<td>248 ± 46</td>
<td>352 ± 31*</td>
<td>386 ± 41*</td>
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<tr>
<td>Urine Mg, mmol/day</td>
<td>417 ± 64</td>
<td>567 ± 19*</td>
<td>574 ± 26*</td>
<td>382 ± 41</td>
<td>521 ± 61*</td>
<td>507 ± 16*</td>
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<td>Urine Ca, mmol/day</td>
<td>17.1 ± 2.5</td>
<td>28.8 ± 2.1*</td>
<td>32.5 ± 2.9*</td>
<td>19.6 ± 2.5</td>
<td>30.8 ± 2.9*</td>
<td>27.9 ± 3.4*</td>
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</tr>
</tbody>
</table>

Values are means ± SD. CTZ, chlorothiazide; FSM, furosemide; Cr, creatinine; Na, sodium; K, potassium; Ca, calcium; Mg, magnesium. *P < 0.05 vs. wild-type control. #P < 0.05 vs. calbindin-D28k knockout control.

urinary excretion of Na and K significantly (all P < 0.05, Table 2). In WT group, CTZ reduced, but FSM increased Ca excretion, compared with control animals (both P < 0.05, Table 2). In KO animals, CTZ treatment did not cause significant change in Ca excretion, while FSM induced a 45% increase in Ca excretion (P < 0.05, Table 2). As for Mg excretion, in WT and KO animals both CTZ and FSM injections were associated with a significant increase in daily Mg excretion (both P < 0.05, Table 2). There were no significant differences between CTZ and FSM treatment.

Gene Expression Analysis

As shown in Fig. 1, the gene expression of TRPV5, TRPV6, and CBD-9K were not different between WT and KO mice. Similar findings were observed in the expression of TRPM6 and claudin-16.

CTZ administration with a salt supplement in WT mice was associated with a significant increase in mRNA abundance of TRPV5 (193 ± 4%), TRPV6 (183 ± 4%), CBD-28k (216 ± 4%), and CBD-9k (211 ± 3%). The expression of TRPM6 was
also increased (193 ± 4%, \(P < 0.05\)), but claudin-16 was not affected (91 ± 4%). In KO mice, gene expression of TRPV5, TRPV6, and CBD-9k were not changed significantly after CTZ treatment (108 ± 3, 124 ± 5, and 99 ± 4%, all \(P > 0.05\)). There was a significant increase in TRPM6 (201 ± 3%). No significant change was observed in claudin-16 (82 ± 4%) (Fig. 1).

FSM injection with a salt supplement produced a significant increase in mRNA abundance of TRPV5 (227 ± 5%), TRPV6 (198 ± 4%), CBD-28k (192 ± 4%), and CBD-9k (231 ± 3%) in WT mice (all \(P < 0.05\)). The expression of TRPM6 was also increased (227 ± 5%, \(P < 0.05\)). There was no significant change in claudin-16 (106 ± 5%, \(P > 0.05\)). TRPV5, TRPV6, and CBD-9k were not affected by FSM in KO mice (126 ± 4, 107 ± 3, and 118 ± 2%, all \(P > 0.05\)). There was a significant increase in TRPM6 (183 ± 5%, \(P < 0.05\)) but not in claudin-16 (106 ± 5%, \(P > 0.05\)).

**Immunofluorescence Staining and Immunoblotting**

The immunofluorescence staining studies revealed an increase in staining for CBD-28k (Fig. 2A), TRPV5 (Fig. 2B), and TRPM6 (Fig. 2C) after CTZ treatment with a salt supplement in WT mice. In CBD-28k knockout mice, the TRPV5-specific staining was not affected by CTZ, but staining for TRPM6 was increased. Administration of FSM with a salt supplement resulted in an increase in staining CBD-28k (Fig. 2A), TRPV5 (Fig. 2B), and TRPM6 (Fig. 2C) in WT mice. For KO mice, FSM treatment increased staining for TRPM6, but not for TRPV5.

For quantitative studies of protein abundance in the kidney after diuretics treatment, immunoblotting for CBD-28k, TRPV5, TRPM6, and claudin-16 were performed with specific antibodies. There was a two- to threefold increase in CBD-28k abundance in the WT group when treated with either CTZ or FSM (235 ± 4 and 274 ± 7%, both \(P < 0.05\), Fig. 3A). No CKD-28k bands were seen in renal tissue of KO mice as expected. TRPV5 was increased significantly after CTZ and FSM injection in WT animals (204 ± 5 and 186 ± 5%, both \(P < 0.05\), Fig. 3B), but not in KO mice (127 ± 6 and 113 ± 4%, both \(P > 0.05\), Fig. 3B). Treatment with CTZ and FSM both induced a significant increase in TRPM6 in the WT group (231 ± 5 and 229 ± 4%, both \(P < 0.05\)) and the KO group (226 ± 6 and 218 ± 5%, both \(P < 0.05\), Fig. 3C). The abundance of claudin-16 was not affected by CTZ or FSM in both WT and KO mice (Fig. 3D).

**DISCUSSION**

Our study demonstrates that CBD-28k has a significant impact on renal Ca handling during diuretic treatment via its role in TRPV5/6 gene regulation, but has no impact on renal Mg handling as its ablation does not affect TRPM6 gene expression. In WT mice, when hypovolemia is prevented by a salt supplement, CTZ treatment causes hypocalciuria, which is associated with upregulation of DCT Ca transport molecules (13). The hypocalciuric effect of CTZ and CTZ-induced upregulation of TRPV5/6 and CBD-9k is completely abolished by the ablation of CBD-28k.

As for Mg, both diuretics cause similar Mg wasting and upregulation of TRPM6, and there are no differences between WT and KO mice.

CBD-28k acts as a dynamic Ca buffer, which regulates Ca transport in the DCT. Lambers et al. (10) reported that CBD-28k translocates to the plasma membrane, where it directly associates with TRPV5 when intracellular Ca\(^{2+}\) concentration is low. With Ca influx into the cell, CBD-28k buffers Ca, thereby countering local accumulation of cytosolic free Ca, which inactivates TRPV5. Upon Ca binding, CBD-28k diffuses from TRPV5 and facilitates transport of Ca to the basolateral membrane. CBD-28k and TRPV5 apparently form a functional protein couple essential for renal Ca handling (10). Another role of CBD-28k is to serve as an intracellular Ca sensor. Berggård et al. (1) have demonstrated elegantly that Ca and Mg share the same binding sites in CBD-28k and that CBD-28k has a 1,000-fold selectivity for Ca over Mg. Furthermore, upon Ca binding, CBD-28k undergoes conformational change with exposure of a hydrophobic surface, allowing interaction with other proteins (1). It is reasonable to assume that CBD-28k senses the increased distal delivery of Ca in the DCT and sends out secondary messengers to enhance the expression of Ca-transporting molecules. It may explain why TRPV5/6 and CBD-9k are not upregulated by diuretics in the CBD-28k KO mice.

We have previously shown that dietary salt intake can modulate the expression of TRPV5/6, CBD-28k, and TRPM6: high salt intake upregulates these genes, while low salt intake downregulates them (17). It is most likely that salt will increase distal delivery of Ca and Mg, which triggers a compensatory mechanism to increase active Ca and Mg transport in the DCT via upregulation of transport molecules. When the distal delivery is enhanced by furosemide, gentamicin, or streptozocin-induced diabetes, similar upregulation occurs (14–16). Other investigators have reported similar results. Rizzo et al. (20) found that bumetanide, a loop diuretic, upregulates CBD-28k. Yatabe et al. (25) reported that a high-sodium diet upregulates TRPV5, CBD-28k, and the Na/Ca exchanger (25). It has been suggested that the concerted upregulation of more distal Ca\(^{2+}\)- and Mg\(^{2+}\)-transporting molecules may be a physiological response to reduce the loss of Ca and Mg. It is possible that Ca and Mg may serve as the stimulators for respective Ca- and Mg-transporting molecules. CBD-28k serves as a sensor only for Ca, not for Mg; therefore, TRPM6 upregulation by FSM is not affected in the CBD-28k KO mice. It should be mentioned that the distribution of TRPM6 and CBD-28k in the DCT is not identical: TRPM6 is expressed in both DCT1 and DCT2, while CBD-28k is expressed predominantly in DCT2 (23). Therefore, the CBD-28k effect on TRPM6 regulation would be somewhat limited even if it is a magnesium sensor.

CTZ inhibits the NCC in the DCT, where both TRPV5/6 and TRPM6 locate. Unlike loop diuretics, CTZ reduces urinary Ca excretion. The mechanism of a CTZ-induced hypocalciuric effect is volume dependent (19). When volume depletion occurs due to diuresis, Ca reabsorption in the proximal tubule is markedly increased, resulting in hypocalciuria. There are no changes in gene expression of calcium transport molecules in DCT. When volume depletion is prevented by a salt supplement, chronic thiazide treatment induces hypocalciuria by increasing Ca uptake in DCT through activation of the transcellular Ca transport system and enhancement of gene expression in TRPV5, CBD-28k, and CBD-9k (13). It is difficult to determine whether upregulation of Ca-transporting molecules
is due to the salt supplement alone or if there is any contribution of a direct effect from thiazides. Recently, Yang et al. (24) created a mouse model of Gitelman syndrome by knocking in a gene with a known point mutation of NCC (24). The phenotype of this mouse model is identical to human Gitelman syndrome: relatively hypotensive, hypokalemic, hypomagnesemic, and hypocalciuric. They found that TRPV5/6 and CBD-28k are all upregulated in this Gitelman mouse model, indicating that inactivating NCC in fact upregulates Ca transport molecules in the DCT. Their results support the theory that thiazides may upregulate Ca-transporting molecules in the DCT, resulting in Ca conservation. Our studies indicate that the lack of upregulation of TRPV5/6 in CBD-28k KO mice does have functional consequences. In KO mice, the hypocalciuric effect of CTZ is completely abolished. Our findings may have clinical impacts since thiazides have been used for reducing Ca excretion in renal stone-forming patients. For those patients who do not respond to thiazides, we may need to explore whether those patients have a deficiency in CBD-28k.

Our CBD-28k KO mice manifested moderate, but significant hypercalciuria when fed a high-Ca diet (1%). Using the same CBD-28k KO mice, Gkika et al. (7) found no hypercalciuria.
after feeding mice a 2% Ca diet for 5 wk. The differences between the two studies may be related to the duration of the 2% Ca diet. In the study by Gkika et al., the prolonged treatment with a high-Ca diet in KO mice had markedly reduced the serum 1,25(OH)2D3 level and downregulated both CBD-9k and TRPV6 in the duodenum. It is possible that the CBD-28k KO mice adapted to a long-term 2% Ca diet by diminishing intestinal absorption of Ca, and therefore their hypercalciuric phenotype was masked.

Mg depletion is a common side effect of thiazides and a severe problem in patients with Gitelman syndrome (4). It has been assumed that decreased TRPM6 in the DCT is the mechanism of hypomagnesemia in the state of NCC inactivation based on the report by Nijenhuis et al. (18), in which TRPM6 abundance is markedly reduced in NCC KO mice. Our study showed that with a salt supplement, CTZ in fact upregulated TRPM6. One possibility is that inactivation of NCC downregulates TRPM6, which is counteracted by the effect of a salt supplement via increasing the distal delivery of Mg to the DCT, thus upregulating TRPM6 (17). Alternatively, the effect of thiazides on Mg wasting may be independent of TRPM6. The Mg extrusion from the basolateral membrane of DCT cells is dependent on the Na gradient set by the Na-K-ATPase. The inhibition of NCC by thiazides blocks Na entry and subsequently diminishes the Na-K-ATPase activity and hinders the basolateral Mg extrusion (2). Further studies are needed to elucidate the mechanism of Mg renal loss induced by thiazides.

Fig. 3. Immunoblotting study of CBD-28k (A), TRPV5 (B), TRPM6 (C), and claudin-16 (D) in renal tissue of wild-type and CBD-28k KO mice following CTZ and FSM treatment. The whole blots are shown with molecular size markers labeled on the left. The specific bands are labeled with arrows on the right. The specificity of the 234-kDa band as TRPM6 is confirmed as it disappeared completely when the anti-TRPM6 antibody was preincubated with the control peptide antigen provided by the manufacturer (data not shown). *P < 0.05 vs. control (Ctrl). #P < 0.05 vs. CBD-28k KO control.
Our study demonstrated that the expression of claudin-16 was not affected by CTZ or FSM treatment in either WT or CBD-28k KO mice. Claudin-16 is one of major components forming tight junctions in the TALH (8). A genetic defect in claudin-16 causes hypomagnesemia with hypercalciuria and nephrocalcinosis (9). The expression of claudin-16 is not affected by salt or fluid intake (17), or Mg-wasting drugs such as gentamicin (16) and cisplatin (11). Since CTZ acts on the DCT, it is unlikely to affect the expression of claudin-16, which locates to the TALH. Similarly, the expression of claudin-16 is not affected in CBD-28k KO mice as CBD-28k is only located in the DCT. The fact that FSM does not affect claudin-16 gene expression indicates that loop diuretic-induced Ca and Mg wasting is probably due to blocking the driving force of paracellular transport of Ca and Mg through abolishing luminal positivity.

In conclusion, our studies indicate that CBD-28k plays an important role in gene expression of DCT Ca transport molecules, but has no impact on the DCT Mg transport molecule TRPM6. This is probably due to the fact that CBD-28k is an intracellular Ca sensor, but not a sensor for Mg (1). The lack of upregulation of DCT Ca transport molecules by thiazides in the KO mice is associated with abolishment of Ca conservation mediated by thiazides. These results suggest that the active Ca transport system in the DCT is critical for thiazide-induced hypocalciuria.

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

AUTHOR CONTRIBUTIONS

REFERENCES
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