Effect of thiazide on renal gene expression of apical calcium channels and calbindins

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THIAZIDE DIURETICS ARE SOME OF THE MOST COMMONLY USED AGENTS FOR TREATING HYPERTENSION AND EDEMA (8). IN ADDITION TO THEIR Natriuretic EFFECT, THIAZIDES ARE ALSO KNOWN TO REDUCE URINE CALCIUM EXCRETION (4). BECAUSE OF THEIR HYPERCALCIURIC EFFECT, THIAZIDES HAVE BEEN USED TO PREVENT NEPHROLITHIASIS IN PATIENTS WITH HYPERCALCERIA (18, 24). THIAZIDES AFFECT THE HORMONAL REGULATION AND DRUG TARGETING (26). RECENT IDENTIFICATION OF APICAL CALCIUM CHANNELS, TRANSIENT RECEPTOR POTENTIAL VANILLOID 5 (TRPV5) AND TRPV6, AS WELL AS CALBINDIN-D28K AND CALBINDIN-D9K, BOTH OF WHICH ARE CALCIUM TRANSPORT FACILITATORS LOCATED IN THE DCT, UPREGULATION OF RETINAL TRPV5 WAS FOUND 4 H AFTER INTRAPERITONEAL INJECTION OF CHLOROTHIAZIDE (CTZ) AT 25 AND 50 MG/KG, BUT NOT AT 100 MG/KG. CHRONIC TREATMENT WITH CTZ AT 25 MG/KG TWICE DAILY FOR 3 DAYS, WITH OR WITHOUT SALT SUPPLEMENTATION OF 0.8% NaCl AND 0.1% KCl IN THE DRINKING WATER, CAUSED HYPERCALCIURIA, BUT THE GENE EXPRESSION PATTERNS WERE DIFFERENT. WITHOUT SALT SUPPLEMENTATION, MICE DEVELOPED VOLUME CONTRACTION AND THERE WERE NO CHANGES IN GENE EXPRESSION. WHEN VOLUME CONTRACTION WAS PREVENTED BY SALT SUPPLEMENTATION, THERE WAS A SIGNIFICANT INCREASE IN GENE EXPRESSION OF TRPV5, CALBINDIN-D28K, AND CALBINDIN-D9K. SALT SUPPLEMENTATION ALONE ALSO INDUCED SIGNIFICANT UPREGULATION OF TRPV5, TRPV6, AND BOTH CALBINDINS. THE UPREGULATION OF TRPV5 BY CTZ AND SALT SUPPLEMENTATION AND SALT ALONE WAS FURTHER CONFIRMED WITH IMMUNOFRESCENT STAINING STUDIES. OUR STUDIES SUGGEST THAT THIAZIDES INDUCE HYPERCALCIURIA THROUGH DIFFERENT MECHANISMS DEPENDING ON VOLUME STATUS. WITH VOLUME CONTRACTION, INCREASED CALCIUM REABSORPTION IN THE PROXIMAL TUBULE PLAYS THE MAJOR ROLE. WITHOUT VOLUME CONTRACTION, HYPERCALCIURIA IS PROBABLY ACHIEVED THROUGH INCREASED CALCIUM REABSORPTION IN THE DCT BY THE ACTIVATION OF A TRANSCELLULAR CALCIUM TRANSPORT SYSTEM AND UPREGULATION OF APICAL CALCIUM CHANNEL TRPV5, CALBINDIN-D28K, AND CALBINDIN-D9K. CHLOROTHIAZIDE; TRANSIENT RECEPTOR POTENTIAL VANILLOID 5; TRANSIENT RECEPTOR POTENTIAL VANILLOID 6; CALBINDIN-D28K; CALBINDIN-D9K

MATERIALS AND METHODS

Animals

Eight-week-old male C57BL/6NHsd mice were purchased from Harlan (Madison, WI) and housed at the University of Arizona animal care facility. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
facilities. All animals were allowed free access to both food and drinking water. Mice were fed a diet containing 0.3% Na and 1.2% Ca (Harlan Teklad LM-485 diet). All animal procedures were performed according to University of Arizona Institutional Animal Care and Use Committee policy.

Study Design

**Acute effect of chlorothiazide.** Mice were given a single dose of chlorothiazide (CTZ; Sigma, St. Louis, MO) intraperitoneally and killed 4 h later. Three different dosages of CTZ, i.e., 25, 50, and 100 mg/kg body wt, were tested. Urine samples were collected in individualized metabolic cages before and after CTZ injection. Blood was collected for biochemical measurements. Total RNA was isolated from the whole kidney for gene expression studies.

**Chronic effect of thiazides.** To assess the chronic effect of CTZ and the role of extracellular volume status on renal calcium excretion, four groups of mice were used: 1) control group; 2) CTZ only group, in which mice were treated with CTZ (25 mg/kg) twice daily for 3 days; 3) CTZ and salt supplementation group, in which in addition to CTZ treatment, mice were given 0.8% NaCl and 0.1% KCl in the drinking water; and 4) salt supplementation group, in which mice were given 0.8% NaCl and 0.1% KCl in the drinking water without CTZ treatment. It has been shown that this preparation is effective in restoring normal volume status in animal treated with diuretics (19, 20). Hematocrit (Hct) was measured using a standard centrifugation method to assess volume status. Blood and urine samples were collected after 3 days of treatment for biochemical measurements. All mice were killed on the fourth day. The left kidneys were quickly collected for biochemical measurements. Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA), and first-strand cDNA was synthesized using the Superscript system (Invitrogen) according to the manufacturer’s protocols.

**Biochemical Measurements**

Urine and serum creatinine and calcium levels were determined by colorimetric assay kits (Sigma) as described previously (15). Renal calcium excretion was expressed as the urine calcium-to-creatinine (Ca/Cr) ratio.

**RNA Isolation and cDNA Synthesis**

Total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA), and first-strand cDNA was synthesized using the Superscript system (Invitrogen) according to the manufacturer’s protocols.

**Real-Time RT-PCR**

The gene expression of apical calcium channels (TPRV5 and TPRV6) and calbindins (calbindin-D28k and calbindin-D9k) was assessed by quantitative real-time RT-PCR. The first-strand cDNA was subjected to real-time PCR using the Cepheid Smart Cycle II System (Cepheid, Sunnyvale, CA) and the fluorescent dye SYBR Green (Molecular Probes, Eugene, OR) as a tracer. The β-actin gene was used as an internal control. For a given target gene, the relative gene expression was determined as 2^(-ΔΔCt) where Gt is the first cycle at which the reporter signal significantly exceeds the baseline signal. Each PCR reaction was performed in triplicate and averaged to obtain a mean Ct value. Melting curve analysis was carried out at the end of a 40-cycle amplification to validate the size of the PCR products. The primer sequences used for the studied genes are listed in Table 1.

**Immunofluorescent Staining**

Frozen kidney samples embedded in OCT compound were cut into 5-μm sections in a cryostat, fixed with 4% paraformaldehyde for 15 min, and incubated with primary antibodies (rabbit anti-rat ECaC1 1:100, Alpha Diagnostic International, San Antonio, TX) for 1 h, then with Alexa 488-conjugated secondary antibodies (goat anti-rabbit Alexa Flour, 1:500, Molecular Probes) for 30 min, all at room temperature. The sections were counterstained with DAPI and mounted with Vectashield Hard Set mounting medium (Vector Laboratories, Burlingame, CA). Images were captured with a CoolSnap digital camera (RS Photometrics, Tucson, AZ) attached to a Nikon E600 fluorescence microscope. To semiquantify TPRV5 expression levels, immunopositive tubules in 10 microscopic fields/sample were counted blindly without prior knowledge of sample identification.

**Statistical Analysis**

All values are expressed as means ± SE. Statistical comparisons were performed by one-way ANOVA and Scheffé’s method for multiple comparisons. For comparisons of data between pretreated and posttreated animals, paired Student’s t-tests were used. P values <0.05 were considered statistically significant.

**RESULTS**

**Acute Effect of CTZ**

Administration of a single dose of CTZ at 25 or 50 mg/kg induced a significant reduction in urinary calcium excretion in 4 h (urine Ca/Cr: 0.03 ± 0.01 for 25 mg/kg group, 0.03 ± 0.01 for 50 mg/kg group, and 0.13 ± 0.04 for control group, both P < 0.05 vs. control, Fig. 1). At 100 mg/kg, the change in urine Ca/Cr ratio did not reach statistical significance (Fig. 1). There were no changes in serum creatinine or calcium levels in mice receiving CTZ treatment at any of the tested doses.

Quantitative real-time RT-PCR studies revealed that there was an 82% increase in TRPV5 gene expression in the kidney

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tbody>
<tr>
<td>β-Actin</td>
<td>ACCACACCTTCTCATCAATGAGC</td>
<td>GGC ACA GTGTTGGTGACCC</td>
</tr>
<tr>
<td>TPRV5</td>
<td>AAACTTCTGCAATGTGTTGGTACG</td>
<td>TTTGGCGAAGTCACAGT</td>
</tr>
<tr>
<td>TPRV6</td>
<td>GGAACTGCCTCCAGCAAGCA</td>
<td>GTACTCCGACCCTCCTCAGT</td>
</tr>
<tr>
<td>CBD28k</td>
<td>GAAGATGGCAGACTGAGTCG</td>
<td>GTA GTA AGCTGGCAGCTCTG</td>
</tr>
<tr>
<td>CBD9k</td>
<td>GTGCGTCGAGAAGCTCGCC</td>
<td>CATGTCGAA AGCTTTTGT</td>
</tr>
</tbody>
</table>

TRPV5 and TRPV6, transient receptor potential vanilloid 5 and 6, respectively; CBD28k and CBD9k, calbindin-D28k and calbindin-D9k, respectively.
after a single dose of CTZ at 25 mg/kg and a 39% increase after CTZ injection at 50 mg/kg (both *P < 0.05, Fig. 2A). However, no significant change was found in animals treated with 100 mg/kg of CTZ. In contrast, acute CTZ treatment did not cause a significant change in gene expression of TRPV6 (140, 111, and 103% of control at 25, 50, and 100 mg/kg, respectively, all *P < 0.05 vs. control, Fig. 2B), nor did it alter calbindin-D_{28k} (Fig. 2C) or calbindin-D_{9k} (Fig. 2D) gene expression.

**Chronic Effect of Low Dose of CTZ**

Table 2 shows the data for Hct and serum calcium and creatinine levels in the chronic treatment study. CTZ treatment alone caused a significant increase in Hct compared with mice in the control, salt supplementation alone, and CTZ plus salt supplementation groups. These findings indicate that CTZ treatment alone causes significant volume depletion, which can be prevented by salt supplementation. There were no significant alternations in serum creatinine or calcium levels in any groups.

The urine Ca/Cr ratio results are shown in Fig. 3. CTZ administration at 25 mg/kg twice daily for 3 days caused a significant decrease in the urine Ca/Cr ratio (0.06 ± 0.03 vs. control group: 0.15 ± 0.03, *P < 0.05). When volume depletion was prevented by providing 0.8% NaCl and 0.1% KCl in the drinking water, there was a significant decrease in the urine Ca/Cr ratio (0.06 ± 0.03, *P < 0.05 vs. control). Salt supplementation without CTZ treatment did not affect the urine Ca/Cr ratio. These results indicate that a 3-day CTZ treatment increases renal calcium reabsorption and that this hypocalciuric effect is not abolished by volume repletion.

As for gene expression, chronic CTZ treatment without salt repletion did not induce any change in gene expression of calcium channels (TRPV5 and TRPV6, Fig. 4, A and B) or calcium-binding proteins (calbindin-D_{28k} and calbindin-D_{9k}, Fig. 4, C and D). When volume depletion was prevented by salt supplementation in the drinking water, there was a 100% increase in TRPV5 mRNA abundance (*P < 0.01) and a 56% increase in TRPV6 (*P > 0.05), but the latter did not reach statistical difference. This treatment also increased gene expression of calbindin-D_{28k} (142% increase, *P < 0.05) and calbindin-D_{9k} (122% increase, *P < 0.05). Salt load alone induced upregulation of TRPV5, TRPV6, calbindin-D_{28k}, and calbindin-D_{9k} (69, 89, 190, and 142% increase, respectively, all *P < 0.05 vs. control). There were no differences in the gene expression profile of the above genes between the groups with CTZ plus salt supplementation and salt supplementation alone.

To verify whether upregulation of TRPV5 mRNA by CTZ leads to increased protein expression, we performed immunofluorescent staining studies using antibodies against TRPV5. Figure 5 shows that there are more TRPV5-positive tubules in
the kidney from mice treated with CTZ plus salt supplementation and salt alone compared with control. Semiquantitative studies show that TRPV5 protein expression increased by 95 and 66% (both $P < 0.05$) in the kidneys from mice treated with CTZ plus salt supplementation and salt alone, respectively (Fig. 6). There was a small but insignificant increase in mice treated with CTZ alone. These results are in agreement with the TRPV5 mRNA data.

**Chronic Effect of High Dose of HCTZ**

Mice treated with 60 mg·kg$^{-1}$·day$^{-1}$ of HCTZ for 7 days had a lower urine Ca/Cr ratio, whereas those treated with HCTZ and salt supplementation showed no changes in urine Ca/Cr ratio (Table 3). As for gene expression, HCTZ alone or HCTZ with salt supplementation for 7 days had no significant effects on renal mRNA abundance of TRPV5/6, calbindin-D9k, or calbindin-D28k (Table 3). Immunofluorescent staining studies using anti-TRPV5 antibodies did not show any changes after HCTZ treatment either (data not shown). There were no apparent histopathological changes in renal tubules induced by the high dose of HCTZ (hematoxylin and eosin staining, data not shown).

**DISCUSSION**

Our study revealed distinct patterns of gene expression in response to acute and chronic treatment with thiazides. While acute CTZ treatment induced hypocalciuria, which was accompanied by upregulation of TRPV5, chronic CTZ treatment without salt supplementation did not change gene expression of calcium channels or calcium-binding proteins. With salt supplementation, CTZ treatment was associated with upregulation of TRPV5 and calbindins. These results indicate that different mechanisms are involved in thiazide-induced hypocalciuria, depending on volume status.

**Table 2. Effects of chronic chlorothiazide administration on hematocrit and biochemical data**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CTZ (50 mg·kg$^{-1}$·day$^{-1}$)</th>
<th>CTZ+Salt</th>
<th>Salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematocrit, %</td>
<td>47.8±0.7</td>
<td>50.7±0.9*</td>
<td>45.3±1.0</td>
<td>46.6±1.3</td>
</tr>
<tr>
<td>Serum Ca, mg/dl</td>
<td>9.4±0.2</td>
<td>8.9±0.2</td>
<td>9.3±0.1</td>
<td>9.3±0.2</td>
</tr>
<tr>
<td>Serum Cr, mg/dl</td>
<td>0.25±0.03</td>
<td>0.25±0.03</td>
<td>0.31±0.04</td>
<td>0.29±0.06</td>
</tr>
</tbody>
</table>

Values are means ± SE; $n = 4.6$/group. CTZ, chlorothiazide; salt, salt supplementation; Ca, calcium; Cr, creatinine. *$P < 0.05$ vs. the other 3 groups.

It has been well established that acute treatment with thiazides inhibits sodium reabsorption while enhancing calcium reabsorption in the DCT. It has been proposed that as thiazides inhibit activity of NCC, decreased sodium influx on the apical site may enhance basolateral sodium influx, resulting in a decrease in intracellular calcium level. The reduced intracellular calcium concentration can drive more apical calcium entry.
This mechanism, however, has not been proven (9). More recently, Gesek and Friedman (10) demonstrated that thiazides induced hyperpolarization of cell membrane in DCT cells, which in turn triggers apical calcium uptake. This mechanism is supported by the observation that TRPV5 is activated by hyperpolarization in an oocyte expression system (14). Our results showed that after acute treatment, animals developed hypocalciuria, which was accompanied by upregulation of the TRPV5 gene in the kidney. It is possible that thiazide-induced hyperpolarization not only activates TRPV5 but also serves as a driving force for its gene expression. Interestingly, at a higher dose (100 mg/kg), not only the hypocalciuric effect but also the upregulation of TRPV5 diminished, which we suspect may be caused by thiazide-induced toxicity. Acute CTZ treatment also induced a trend toward an increase in TRPV6 gene expression. TRPV5 and TRPV6 are similar in structure and function; both genes are located in the same chromosome and probably are duplicated evolutionally (32). It is likely that they are regulated similarly; for example, both are upregulated by vitamin D (11, 30). The different responses to acute CTZ treatment between TRPV5 and TRPV6 reported in our studies may be due to their different distribution in the kidney, whereas TRPV5 is restricted to the DCT, TRPV6 has a wider distribution, i.e., from the DCT to the inner medullary collecting duct (23). It is possible that CTZ also upregulates TRPV6 in the DCT, but the effect is masked by its expression in non-DCT segments, which may not be affected by CTZ.

Our results of chronic treatment with CTZ demonstrated that although CTZ still induced hypocalciuria, the upregulation of TRPV5 seen after acute treatment was completely reversed. Because this change was associated with volume depletion, we speculate that volume depletion increases sodium reabsorption in the proximal tubules, thus decreasing distal delivery of sodium to the DCT. As a result, hyperpolarization of cell membrane does not occur even in the presence of CTZ; consequently, the driving force for TRPV5 expression is lost. This theory is supported by our finding that upregulation of TRPV5 was maintained when volume depletion was prevented by salt supplementation. Based on our and others’ studies (1, 21), it is clear that in the presence of volume depletion, enhanced proximal tubule reabsorption of calcium is the major physiological response that contributes to hypocalciuria. With less distal delivery of sodium and calcium, the role of the DCT in calcium preservation may be significantly diminished.

Table 3. Effects of chronic HCTZ administration on calcium excretion and mRNA abundance of apical calcium channels and calcium-binding proteins

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HCTZ (60 mg·kg⁻¹·day⁻¹)</th>
<th>HCTZ + Salt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine Cu/Cr</td>
<td>0.17±0.04</td>
<td>0.07±0.01*</td>
<td>0.11±0.03</td>
</tr>
<tr>
<td>TRPV5, %</td>
<td>100±10</td>
<td>110±2</td>
<td>116±13</td>
</tr>
<tr>
<td>TRPV6, %</td>
<td>100±17</td>
<td>92±11</td>
<td>86±10</td>
</tr>
<tr>
<td>Calbindin-D₉k, %</td>
<td>100±6</td>
<td>102±25</td>
<td>117±17</td>
</tr>
<tr>
<td>Calbindin-D₂₈k, %</td>
<td>100±10</td>
<td>83±24</td>
<td>87±14</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4/group. HCTZ, hydrochlorothiazide. *P < 0.05 vs. control.
When mice were given a salt supplement in the drinking water to expand extracellular volume, we found that TRPV5, TRPV6, calbindin-D<sub>28k</sub>, and calbindin-D<sub>9k</sub> were all upregulated. It is well known that salt load inhibits proximal tubule absorption of sodium and calcium and increases distal delivery of both ions (17). We speculate that upregulation of calcium channels and calbindins may be a physiological response to the increased calcium load in the DCT as well as in more distal nephron segments. By doing so, excessive calcium loss can be prevented. Upregulation of calbindin-D<sub>28k</sub> in the rat distal tubules by increased calcium load has been observed in chronic metabolic acidosis. After rats were given NH₄Cl in the drinking water for 7 days, renal calbindin-D<sub>28k</sub> mRNA abundance increased almost threefold (27). It is possible that increased distal calcium load may stimulate synthesis of calcium channels and calbindins to compensate for renal calcium loss. Further studies are needed to test this hypothesis.

In mice treated with both CTZ and salt supplementation, calcium reabsorption was increased. This finding is consistent with the notion that thiazides may decrease calcium excretion without extracellular volume contraction (3). Interestingly, both TRPV5 mRNA and protein were markedly upregulated under this treatment, and there was a trend toward an increase in TRPV6 expression. It is possible that both thiazide-induced hyperpolarization and calcium load are the driving force for upregulation of TRPV5 and, possibly TRPV6, in the DCT. Furthermore, both calbindin-D<sub>28k</sub> and calbindin-D<sub>9k</sub> are upregulated after the combination of CTZ and salt supplementation, presumably due to an increased calcium load. It is likely that the increased gene expression of calcium channels and calbindins plays an important role in thiazide-induced calcium preservation in the absence of volume contraction. The role of CTZ in gene regulation of TRPV5 in this situation is not clear because TRPV5 is upregulated by salt supplementation alone. It is possible that CTZ may have an independent effect because acute treatment with CTZ alone upregulates TRPV5. Further investigations are needed to delineate mechanisms of TRPV5 upregulation observed in this study.

Our results are markedly different from those reported by Nijenhuis et al. (21). In their study, 12 mg of HCTZ were given daily to Wistar rats with a body weight of 200–225 g (~60 mg·kg<sup>−1</sup>·day<sup>−1</sup>). Because the potency of HCTZ is ~10-fold that of CTZ (31), their animals received an equivalent of 600 mg/kg CTZ. They found that there was a decrease in the expression of NCC, the Na/Ca exchanger, calbindin-D<sub>28k</sub>, and TRPV5 in HCTZ-treated rats, regardless of their volume status. This decrease is probably due to the apoptosis of DCT cells because similar doses of thiazides have been previously shown to induce apoptosis in rats (16). When we used a similar dose of HCTZ (60 mg·kg<sup>−1</sup>·day<sup>−1</sup>) in mice, we found no effects on gene expression of calcium channels and binding proteins (Table 3) or on renal tubular morphology. It is possible that these differences between rats and mice are due to the fact that rats are more sensitive to thiazides than mice, as previously shown by Bucher et al. (2). In their studies, rats developed nephropathy when fed a diet containing 250 parts/million HCTZ, whereas mice did not, even after being fed a diet containing 50,000 parts/million HCTZ for 2 yr.

In conclusion, we demonstrated that acute treatment with thiazides upregulates TRPV5, whereas chronic treatment using thiazides is associated with upregulation of TRPV5, calbindin-D<sub>28k</sub> and calbindin-D<sub>9k</sub> in the absence of volume contraction. In addition, salt load alone upregulates both calcium channels and calbindins. When thiazides are administered chronically, there are at least two mechanisms involved in its hypocalciuric effect. If there is volume contraction, hypocalciuria is mainly due to an increase in proximal calcium reabsorption. Consequently, no changes in gene expression of calcium transport molecules in the DCT occur because of decreased distal delivery of both sodium and calcium. In the absence of volume contraction, chronic thiazide treatment induces hypocalciuria by increasing calcium uptake in the DCT through activation of transcellular calcium transport system and enhancement of gene expression in the key component, the apical calcium channel TRPV5, as well as through cytoplasmic facilitators calbindin-D<sub>28k</sub> and calbindin-D<sub>9k</sub>.