Thiazide-induced hypocalciuria is accompanied by a decreased expression of Ca\(^{2+}\) transport proteins in kidney

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Thiazide-induced hypocalciuria is accompanied by a decreased expression of Ca\(^{2+}\) transport proteins in kidney.

Introduction. Thiazide diuretics have the unique characteristic of increasing renal Na\(^{+}\) excretion, while decreasing Ca\(^{2+}\) excretion. However, the molecular mechanism responsible for this thiazide-induced hypocalciuria remains unclear. The present study investigates the effect of thiazides on the expression of the proteins involved in active Ca\(^{2+}\) transport as well as the role of extracellular volume (ECV) status.

Methods. Hydrochlorothiazide (HCTZ), 12 mg/24 hours, was administered during 7 days to Wistar rats by osmotic minipumps. In addition, ECV contraction was either prevented by Na\(^{+}\) repletion or induced by a low-salt diet. Expression levels of the proteins involved in active Ca\(^{2+}\) transport (i.e., epithelial Ca\(^{2+}\) channel (TRPV5/ECaCl), calbindin-D\(_{28K}\), Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX1)), as well as the thiazide-sensitive Na\(^{+}\) Cl\(^{-}\) co-transporter (NCC) were determined by real-time quantitative polymerase chain reaction (PCR) and semiquantitative immunohistochemistry.

Results. HCTZ significantly reduced urinary Ca\(^{2+}\) excretion (22% ± 5% relative to controls). Hematocrit was significantly increased, confirming ECV contraction. In addition, Na\(^{+}\) depletion virtually abolished Ca\(^{2+}\) excretion (8% ± 1%), while Na\(^{+}\) repletion during HCTZ treatment prevented both ECV contraction and hypocalciuria. HCTZ significantly decreased mRNA expression of TRPV5 (71% ± 6%), calbindin-D\(_{28K}\) (53% ± 6%), NCX1 (51% ± 8%) and NCC (50% ± 11%), regardless of ECV status or calciuresis. Immunohistochemistry revealed reduced TRPV5 (43% ± 2%), calbindin-D\(_{28K}\) (59% ± 1%) and NCC (56% ± 4%) abundance. Furthermore, during HCTZ treatment, the subset of tubules coexpressing NCC and calbindin-D\(_{28K}\) was significantly reduced (43% ± 5%) and a disturbed cellular localization of NCC was observed.

Conclusion. These data suggest that ECV contraction is a critical determinant of the thiazide-induced hypocalciuria, which is accompanied by a decreased expression of Ca\(^{2+}\) transport proteins.

Key words: ECaC, TRPV5, calbindin, extracellular volume.

Thiazides are among the most widely prescribed drugs today, particularly by being the mainstay of first-line therapy in hypertension. In addition, these diuretics have, in contrast to loop diuretics, the unique characteristic of increasing Ca\(^{2+}\) reabsorption, while decreasing Na\(^{+}\) reabsorption [1]. This hypocalciuric effect provides therapeutic opportunities in for instance idiopathic hypercalciuria and nephrolithiasis. Furthermore, thiazides have been shown to increase bone mineral density and decrease fracture risk, spiking interest in the favorable long-term effects of these diuretics in countering osteoporosis [2–4]. Thiazides increase renal Na\(^{+}\) excretion by inhibiting the Na\(^{+}\) Cl\(^{-}\) cotransporter (NCC or thiazide receptor) present in the apical membrane of distal convoluted tubule (DCT) cells [5]. Surprisingly, the exact molecular mechanism responsible for the thiazide-induced hypocalciuria remains unclear. Increased passive proximal Ca\(^{2+}\) reabsorption as well as direct stimulation of active Ca\(^{2+}\) transport in the DCT have been suggested to explain the hypocalciuric effects of chronic thiazide administration [6–8].

The inhibition of Na\(^{+}\) reabsorption in DCT by thiazides results in increased renal salt and water loss and thereby decreases extracellular volume (ECV) [5]. This ECV contraction has been shown to result in a compensatory enhancement of proximal Na\(^{+}\) reabsorption, thereby increasing the electrochemical gradient driving passive Ca\(^{2+}\) transport in proximal tubule segments [9]. Therefore, the hypocalciuric effect of chronic thiazide administration was postulated to be the result of an enhancement of passive paracellular Ca\(^{2+}\) reabsorption secondary to ECV contraction [10, 11].

Previously, acute administration of thiazides into the tubular lumen was shown to stimulate transcellular Ca\(^{2+}\) transport in DCT [1]. Subsequently, several mechanisms have been postulated to explain the hypocalciuria of chronic thiazide treatment by a similar stimulatory effect [7, 12]. Stimulation of apical Ca\(^{2+}\) entry has been suggested, mediated by hyperpolarization of the plasma.

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membrane secondary to NCC inhibition. After the recent identification and characterization of the epithelial Ca\(^{2+}\) channel TRPV5 (previously named ECaCl) as the rate-limiting apical entry step in transepithelial Ca\(^{2+}\) transport, several authors postulated that the molecular and electrophysiologic properties of TRPV5 were in line with the aforementioned hypothesis [10, 13, 14]. Alternatively, enhanced basolateral Na\(^{+}/Ca\(^{2+}\) exchange by up-regulation of the Na\(^{+}/Ca\(^{2+}\) exchanger (NCX1), secondary to decreased intracellular Na\(^{+}\) concentration, has also been suggested as the principal responsible mechanism [7, 10]. Both hypotheses rely on substantial colocalization in DCT of NCC and the proteins involved in active Ca\(^{2+}\) transport. Extensive immunohistochemical studies demonstrated that NCC and TRPV5 only overlap in part, whereas the Ca\(^{2+}\) transporters (i.e., TRPV5, calbindin-D\(_{28K}\), NCX1, and PMCA1b) completely colocalize [6, 15].

The aim of the present study was to determine the effect of chronic thiazide treatment and the ECV status on the expression levels of the major Ca\(^{2+}\) transporters in the kidney. To this end, rats were treated for 7 days with hydrochlorothiazide (HCTZ) and housed in metabolic cages. Subsequently, the expression of the Ca\(^{2+}\) transporters was determined by real-time polymerase chain reaction (PCR) and immunohistochemical analysis.

**METHODS**

**Animal studies**

Young adult male Wistar rats, initial weight 200 to 225 g, were housed individually in metabolic cages enabling 24-hour urine collection. The animals were kept in a light- and temperature-controlled room with ad libitum access to standard pelleted chow and deionized water, and were maintained under these same conditions during a 4-day run-in period. Thereafter, rats were randomized into five groups: (1) a control group (controls; \(N = 6\)), (2) a group to receive only thiazide treatment (HCTZ; \(N = 5\)), (3) a group to receive thiazide treatment as well as oral NaCl/KCl repletion via the drinking water (HCTZ/Na\(^{+}\)-repleted; \(N = 5\)), (4) a Na\(^{+}\)-depleted group fed a synthetic low-sodium diet (Na\(^{+}\)-depleted; \(N = 5\)), and (5) a Na\(^{+}\)-depleted group receiving thiazide treatment (HCTZ/Na\(^{+}\)-depleted; \(N = 5\)). Starting from the fifth day, all animals were ration-fed (20 g/day) either the standard rat chow [0.25% (wt/vol) NaCl; 1% (wt/vol) Ca\(^{2+}\)] or a Na\(^{+}\)-depleted diet [0.02% (wt/vol) NaCl; 1% (wt/vol) Ca\(^{2+}\)], rendering dietary intake equal in all animals with the exception of NaCl. On the ninth day, designated animals were anaesthetized and either had osmotic minipumps (model 2ML1; Alzet, Palo Alto, CA, USA) containing HCTZ implanted subcutaneously in the neck region, or were sham-operated, receiving minipumps containing vehicle only. Osmotic minipumps contained HCTZ in 50% (vol/vol) dimethyl sulfoxide (DMSO), resulting in continuous rate-controlled delivery of HCTZ at a dose of 12 mg/24 hours. While this dosage is, relative to body weight, higher than the dosage used in humans in clinical practice, pilot experiments indicated this to be the lowest dosage resulting in a significant diuresis in these rats. HCTZ was preferred over other thiazides because of its limited carbonic anhydrase activity. The animals in the HCTZ/Na\(^{+}\)-repleted group were switched to drinking water containing 0.9% (wt/vol) NaCl and 0.1% (wt/vol) KCl in deionized water the next day. Six days postimplantation, body weight was determined, blood samples were taken, and the animals were sacrificed. Kidney cortex was sampled and immediately frozen in liquid nitrogen. In addition, kidney cortex was fixated for immunohistochemistry by immersion in 1% (wt/vol) periodate-lysine-paraformaldehyde (PLP) for 2 hours and 15% (wt/vol) sucrose in phosphate-buffered-saline (PBS) overnight. Subsequently, samples were stored at –80°C until further processing. The Animal Ethics Board of the University Medical Center Nijmegen approved all experimental procedures.

**Analytic procedures**

Serum and urine Ca\(^{2+}\) concentrations were determined using a colorimetric assay as described previously [16]. Serum and urine [Na\(^{+}\)] and [K\(^{+}\)] were measured flame-spectrophotometrically (Eppendorf FCM 6343, Hamburg, Germany). Hematocrit was determined using a standard centrifugation protocol [17].

**Real-time quantitative PCR**

Total RNA was extracted from kidney cortex using Trizol Total RNA Isolation Reagent (Gibco BRL, Breda, The Netherlands). The obtained RNA was subjected to DNase treatment to prevent genomic DNA contamination. Thereafter, 2 \(\mu\)g RNA was reverse transcribed by Moloney-Murine Leukemia Virus-Reverse Transcriptase (M-MLV-RT; Gibco BRL) as described previously [18]. The obtained cDNA was used to determine renal TRPV5, calbindin-D\(_{28K}\), NCC, and NCX1 mRNA levels, as well as mRNA levels of the housekeeping gene hypoxanthine-guanine phosphoribosyl transferase (HPRT) as an endogenous control. Expression levels were quantified by real-time quantitative PCR on an ABI Prism 7700 Sequence Detection System (PE Biosystems, Rotkreuz, Switzerland). Primer and probe sequences are listed in Table 1 (Biolegio, Malden, The Netherlands).

**Immunohistochemistry**

Immunohistochemical staining was performed as described previously [19]. In short, 7 \(\mu\)m sections of PLP-fixed frozen kidney cortex samples were stained for TRPV5, calbindin-D\(_{28K}\), and NCC. TRPV5 staining involved immersion of the kidney sections in boiled citrate
Table 1. Primers and probes used in real-time quantitative polymerase chain reaction (qPCR)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPRT</td>
<td>5'-GCTAATGTCTCTCCGCTAGG-3'</td>
<td>5'-AACCCTTGGCCGTCATTCG-3'</td>
<td>5'-GTGGTCGGTGGAGTGGATG-3'</td>
</tr>
<tr>
<td>TRPV5</td>
<td>5'-GTTCACTGGGGGTGGTGGATG-3'</td>
<td>5'-GCTGAGGGAACAGGTGGATG-3'</td>
<td>5'-TGATTCTCGTTCTCTCTCGCG-3'</td>
</tr>
<tr>
<td>CaBP-D28K</td>
<td>5'-GGAGCTGGAGCTGACAGAGAT-3'</td>
<td>5'-TCTCAAGTCTTTCAGCCATGT-3'</td>
<td>5'-ACCAGTGCAGGAAAATTTCCTTCTTAAATTCCA-3'</td>
</tr>
<tr>
<td>NCX1</td>
<td>5'-TTGATCCCAATCTGTCAGG-3'</td>
<td>5'-GCTGAGGGAACAGGTGGATG-3'</td>
<td>5'-ACCTTGACTGATATTGTTTTGACTATTTCATCATTCTGGA-3'</td>
</tr>
<tr>
<td>NCC</td>
<td>5'-CGCTCCGAGGCTGACAGAGAT-3'</td>
<td>5'-GCTGAGGGAACAGGTGGATG-3'</td>
<td>5'-CGGCAATCACCTGCTGACTATGCTACC-3'</td>
</tr>
</tbody>
</table>

Abbreviations are: HPRT, hypoxanthine-guanine phosphoribosyl transferase; TRPV, epithelial Ca$_2^+$ channel (previously known as ECaC1); CaBP-D$_{28K}$, calbindin-D$_{28K}$; NCX1, Na$^+$/Ca$^2^+$ exchanger; NCC, Na$^+$/Cl$^{-}$ cotransporter. PCR primers and fluorescent probes (5'-FAM–3' TAMRA) were designed using the computer program Primer Express (Applied Biosystems) and purchased from Biolegio (Malden, The Netherlands).

Immunoblotting

Kidney cortex sections were homogenized in homogenization buffer A (HbA) [20 mmol/L Tris/HCl, pH = 7.4), 5 mmol/L MgCl$_2$, 5 mmol/L Na$_2$PO$_4$, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 80 mmol/L sucrose, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 5 μg/mL leupeptin and pepstatin]. Protein concentration of the homogenates was determined (Bio-Rad Protein Assay; Bio-Rad Munchen, Germany) and samples were normalized accordingly. Subsequently, 50 μg protein samples were separated on 12% (wt/vol) SDS-PAGE gels and blotted to polyvinylidene difluoride (PVDF)-nitrocellulose membranes (Immobilon-P; Millipore Corporation, Bedford, MA, USA). Blots were incubated for 1 hour with mouse anti-calbindin-D$_{28K}$ (Swant) (1:10,000). Thereafter, blots were incubated with a goat anti-mouse peroxidase coupled secondary antibody and immunoreactive protein was detected using the chemiluminescence method. Immunopositive bands were scanned using an imaging densitometer (Bio-Rad Gs-690) to de...
termine pixel density (Molecular Analyze Software; Bio-
Rad Laboratories, Hercules, CA, USA).

**Statistical analysis**

Data are expressed as means ± SEM. Statistical compara-
tions were tested by one-way analysis of variance
(ANOVA) and Fisher’s multiple comparison. P values
less than 5% were considered statistically significant. All
analyses were performed using the Statview Statistical
Package software (Power PC, version 4.51, Berkley, CA,
USA) on a Macintosh computer.

**RESULTS**

**Urine and serum analysis**

Electrolyte values of the serum and 24-hour urine sam-
cles, creatinine clearance, hematocrit, and body weight
data of the five experimental groups are shown in Table
2. In the HCTZ group, a significant hypocalciuria was
induced. Urine volume was significantly increased,
whereas Na<sup>+</sup> and K<sup>+</sup> excretion did not significantly differ
from controls. Hematocrit was significantly increased,
while body weight in these animals was significantly
reduced. In the HCTZ/Na<sup>+</sup>-repleted animals, oral supple-
mentation of NaCl/KCl completely prevented the thia-
side-induced hypocalciuria. Likewise, hematocrit and
body weight in these animals did not significantly differ
from controls, whereas urine volume, Na<sup>+</sup> and K<sup>+</sup> excre-
tion were significantly enhanced. Interestingly, Ca<sup>2+</sup>
excretion was virtually abolished by Na<sup>+</sup> depletion, mim-
icking the action of thiazides. Similarly, in the HCTZ/
Na<sup>+</sup>-depleted group, Ca<sup>2+</sup> excretion was significantly
decreased relative to controls. In addition, hematocrit was
dramatically increased in both the Na<sup>+</sup>-depleted and
HCTZ/Na<sup>+</sup>-depleted animals, whereas body weight was
significantly reduced in the HCTZ/Na<sup>+</sup>-depleted ani-
mals, but unaffected in the Na<sup>+</sup>-depleted group. Of note,
the inhibition of the calciuresis was comparable in the
HCTZ, Na<sup>+</sup>-depleted, and HCTZ/Na<sup>+</sup>-depleted groups.
Serum levels of Ca<sup>2+</sup>, Na<sup>+</sup>, and K<sup>+</sup> were not significantly
affected in the experimental groups, except in HCTZ/
Na<sup>+</sup>-depleted animals, a small, albeit significant, de-
crease of serum K<sup>+</sup> concentration was observed. 
Serum creatinine levels and creatinine clearance were not sig-
nificantly different between the various groups.

**Real-time quantitative PCR**

Kidney cortex mRNA levels of the Ca<sup>2+</sup> transport
proteins and NCC were determined by real-time quanti-
tative PCR (Fig. 1). In all HCTZ-treated groups, chronic
HCTZ administration consistently and significantly de-
creased mRNA expression of TRPV5, calbindin-D<sub>28K</sub>,
NCX1, and NCC. There were no significant differences in
expression levels between the three HCTZ-treated
groups. On the contrary, expression of TRPV5, calbin-
din-D<sub>28K</sub>, and NCC in the Na<sup>+</sup>-depleted group did not
significantly differ from controls, whereas NCX1 mRNA
expression was reduced.

**Immunohistochemistry**

To assess whether the decreased mRNA levels resulted in
reduced protein expression, protein abundance was
semiquantified by immunohistochemistry. Figure 2 shows
that the above-mentioned results are indeed accompa-
nied by similar effects on the protein level. A reduced
protein expression of TRPV5, calbindin-D<sub>28K</sub>, and NCC
was consistently observed in all HCTZ-treated groups.
In contrast, in the Na<sup>+</sup>-depleted group, protein expression
levels did not significantly differ from controls.

Interestingly, in addition to the characteristic apical
NCC staining, several tubules in HCTZ-treated animals
displayed a disturbed immunopositive staining [21, 22].
In these tubules NCC was not, as in controls (Fig. 3A),
localized to the apical domain, but a diffuse intracy-
Fig. 1. Effect of hydrochlorothiazide (HCTZ) and extracellular volume (ECV) status on mRNA expression levels of distal convoluted tubule (DCT) transport proteins. mRNA expression levels of TRPV5 (A), calbindin-D<sub>28K</sub> (B), Na<sup>+</sup>/Cl<sup>-</sup> cotransporter (NCC) (C), and Na<sup>+</sup>/Ca<sup>2+</sup>/H<sup>+</sup> exchanger (NCX1) (D) in kidney cortex were determined by real-time quantitative polymerase chain reaction (PCR). Results are presented relative to controls. Controls, sham-operated animals; HCTZ, 12 mg/24 hours HCTZ; HCTZ/Na<sup>+</sup>-repleted, 12 mg/24 hours HCTZ supplemented with NaCl/KCl via the drinking water; Na<sup>+</sup>-depleted, animals fed the low-sodium diet [0.02% (wt/vol) NaCl]; and HCTZ/Na<sup>+</sup>-depleted, Na<sup>+</sup>-depleted animals receiving 12 mg/24 hours HCTZ. Data are presented as means ± SEM. *P < 0.05 vs. controls.

To confirm the semiquantitative determination of protein abundance by immunohistochemistry, immunoblotting was performed for calbindin-D<sub>28K</sub> as shown in Figure 5. A significant reduction in calbindin-D<sub>28K</sub> protein abundance was observed in all thiazide-treated animals. Denitometric analysis of the individual immunoblots revealed significantly reduced calbindin-D<sub>28K</sub> protein expression levels in the HCTZ (61% ± 7%), HCTZ/Na<sup>+</sup>-repleted (52% ± 5%), and HCTZ/Na<sup>+</sup>-depleted (60% ± 5%) groups, whereas the protein expression in the Na<sup>+</sup>-depleted group (94% ± 3%) did not significantly differ from controls (100% ± 4%). These data demonstrated that protein abundance as determined by immunoblotting is consistent with the immunohistochemical results and confirmed the significant down-regulation of calbindin-D<sub>28K</sub> in the thiazide-treated animals.

**DISCUSSION**

In the present study, chronic HCTZ treatment induced a significant hypocaliuria that was accompanied by ECV contraction. Interestingly, a similar reduction in urinary Ca<sup>2+</sup> excretion was observed during Na<sup>+</sup> depletion, while prevention of ECV contraction during HCTZ treatment prohibited the development of hypocaliuria. Furthermore, HCTZ consistently decreased the mRNA expression and protein abundance of several transporters responsible for active Ca<sup>2+</sup> reabsorption, regardless of volume status or calciuresis. Taken together, this suggests that ECV contraction is a critical determinant of the thiazide-induced hypocaliuria, which is accompanied by decreased expression of proteins involved in the active Ca<sup>2+</sup> transport process.

In this study, the HCTZ-induced hypocaliuria was accompanied by a significant increase in hematocrit and concomitant decrease in body weight, confirming that ECV contraction occurred. Since Na<sup>+</sup> depletion resulted in a similar hypocalciuria and hypovolemia, it is tempting to speculate that ECV contraction by itself is responsible for the thiazide-induced hypocaliuria. This is further supported by our finding that Na<sup>+</sup> repletion during HCTZ treatment, thereby preventing the ECV contraction, normalized the Ca<sup>2+</sup> excretion. In the literature, volume contraction has been suggested as a causative factor in thiazide-induced hypocaliuria [7, 10]. The underlying mechanism is a compensatory enhancement of proximal Na<sup>+</sup> reabsorption, observed both in volume contraction by diuretics and Na<sup>+</sup> restriction [9, 23–25], which leads to an increase in the electrochemical driving force for passive Ca<sup>2+</sup> reabsorption [26]. Indeed, Walter and Shirley [9] showed an increased Na<sup>+</sup> reabsorption by the proximal tubule secondary to HCTZ-induced Na<sup>+</sup> depletion. Furthermore, it is a general finding that during chronic thiazide treatment natriuresis normalizes and volume balance is eventually restored, which possibly results from a persistently increased proximal reabsorption of Na<sup>+</sup> [9]. Therefore, hypocaliuria can continue...
Fig. 3. Disturbed distribution of the Na\(^+\)/Cl\(^-\) cotransporter (NCC) in hydrochlorothiazide (HCTZ)-treated animals. Detail of distal convoluted tubule (DCT) showing NCC immunopositive staining of control (A) and HCTZ-treated (B) rats demonstrating that the characteristic apical NCC signal as shown in the controls is lost in a portion of tubules present in the HCTZ-treated rats. Instead, a diffuse intracellular appearance is visible (arrows).

Fig. 4. Hydrochlorothiazide (HCTZ) treatment reduced the amount of Na\(^+\)/Cl\(^-\) and calbindin-D\(_{28k}\) coexpressing tubules. Number of tubules coexpressing NCC and calbindin-D\(_{28k}\) was determined by immunohistochemistry and presented relative to controls. Controls, sham-operated animals; HCTZ, 12 mg/24 hours HCTZ; HCTZ/Na\(^{+}\)-repleted, 12 mg/24 hours HCTZ supplemented with NaCl/KCl via the drinking water; Na\(^{+}\)-depleted, animals fed the low-sodium diet [0.02% (wt/vol) NaCl]; and HCTZ/Na\(^{+}\)-depleted, Na\(^{+}\)-depleted animals receiving 12 mg/24 hours HCTZ. Data are presented as means ± SEM. *P < 0.05 vs. controls.

Fig. 5. Hydrochlorothiazide (HCTZ) treatment reduced calbindin-D\(_{28k}\) protein abundance as determined by immunoblotting. Immunoblots for calbindin-D\(_{28k}\) of individual kidney samples. Controls, sham-operated animals; HCTZ, 12 mg/24 hours HCTZ; HCTZ/Na\(^{+}\)-repleted, 12 mg/24 hours HCTZ supplemented with NaCl/KCl via the drinking water; Na\(^{+}\)-depleted, animals fed the low-sodium diet [0.02% (wt/vol) NaCl]; and HCTZ/Na\(^{+}\)-depleted, Na\(^{+}\)-depleted animals receiving 12 mg/24 hours HCTZ.

Despite a restored volume balance during chronic thiazide treatment, ECV contraction prior to thiazide administration was shown to result in an accelerated and more pronounced hypocalciuric response [26]. The reverse also holds true, since ECV expansion has been shown to induce hypercalciuria by a mechanism dependent on inhibition of proximal Na\(^+\) reabsorption [27–29]. Furthermore, in hypertension a high prevalence of hypercalciuria is reported [30]. In these hypercalciuric situations, thiazide diuretics successfully normalized the Ca\(^{2+}\) excretion. Moreover, the mild diuretic amiloride, which inhibits Na\(^+\) reabsorption in the collecting system, is known to enhance Ca\(^{2+}\) reabsorption and to potentiate the Ca\(^{2+}\)-sparing effects of thiazides [31]. This favors a general mechanism, not restricted to DCT, underlying diuretic-induced hypocalciuria. Together with our findings this suggests that ECV contraction is an important determinant of the chronic thiazide-induced hypocalciuria.

Costanzo and Windhager [1] showed in pioneering
micropuncture and microperfusion experiments that chlorothiazide could stimulate transcellular Ca\(^{2+}\) transport in DCT when directly instilled in the tubular lumen. It is, however, important to distinguish acute from chronic effects of thiazide diuretics. Acute administration results in a marked natriuresis and a decrease of the urinary Ca\(^{2+}\)/Na\(^{+}\) ratio, while the net Ca\(^{2+}\) excretion has been shown to either decrease, remain at control levels, or increase [7, 32]. Chronic administration, however, produces the overt thiazide-induced hypocalciuria. The aforementioned microperfusion studies were performed during acute thiazide administration and these data are, therefore, restricted to the acute diuretic effects [1]. Whether this process contributes to the chronic hypocalciuric effect of thiazides, studied in this paper, is not clear. However, several theories, emphasizing an activation of transcellular Ca\(^{2+}\) transport processes in DCT, were advanced to explain the hypocalciuria during chronic thiazide treatment. NCC inhibition leading to decreased Cl\(^{-}\) entry could reduce the electrogenic exit of Cl\(^{-}\) across the basolateral membrane. This has been suggested to result in hyperpolarization of the apical membrane and thereby enhance Ca\(^{2+}\) entry from the tubular lumen [7, 10, 33]. The observation that Ca\(^{2+}\) currents through the apical Ca\(^{2+}\) channel TRPV5 are maximal at hyperpolarizing membrane potentials apparently supports this hypothesis [14]. However, the decreased TRPV5 expression consistently observed during HCTZ treatment does not support stimulation of apical Ca\(^{2+}\) entry by thiazides. Furthermore, thiazide-induced hyperpolarization has been demonstrated in the basolateral membrane only and it is unclear how this hyperpolarization translates to the proposed apical effect [13]. Alternatively, it has been suggested that stimulation of active Ca\(^{2+}\) reabsorption by thiazides is mediated by a primary activation of the basolateral NCX1 [10, 34]. Inhibition of apical Na\(^{+}\) entry by thiazides would reduce the intracellular Na\(^{+}\) concentration, resulting in stimulation of NCX1 and in enhancement of basolateral Ca\(^{2+}\) efflux. However, the significant down-regulation of NCX1 transcripts during thiazide treatment shown in this study does not seem consistent with the proposed stimulation of Na\(^{+}\)/Ca\(^{2+}\) exchange activity. Furthermore, calbindin-D\(_{28K}\) mRNA and protein abundance were also decreased during HCTZ treatment. Calbindin-D\(_{28K}\) facilitates diffusion of Ca\(^{2+}\) through the cytosol and simultaneously serves as an intracellular Ca\(^{2+}\) buffer to protect the cell from toxic Ca\(^{2+}\) levels [35]. This substantial decrease of the Ca\(^{2+}\) diffusion and buffering capacity would be detrimental in the presence of increased apical Ca\(^{2+}\) entry and increased transcellular Ca\(^{2+}\) transport. Furthermore, both hypotheses imply a significant colocalization of NCC and the Ca\(^{2+}\) transport proteins in the DCT. The present study shows that the population of tubules held responsible for the thiazide-related enhancement of Ca\(^{2+}\) reabsorption is dramatically reduced during HCTZ treatment. Thus, the consistently decreased expression of the Ca\(^{2+}\) transporters seems at variance with a direct stimulation of active Ca\(^{2+}\) transport processes during chronic thiazide treatment.

An additional question emerging from our data is how this decreased expression of the Ca\(^{2+}\) transporters in all HCTZ-treated groups can be explained. This reduction in the expression was accompanied by a comparable reduction in NCC abundance, which was confirmed by loss of tubules coexpressing Ca\(^{2+}\) transporters and NCC. These findings suggest a thiazide-specific deleterious effect on DCT. This would explain the decreased expression levels consistently obtained in all HCTZ-treated groups, regardless of ECV status and calciuresis. Important in this respect is the finding by Loffing et al [22] that thiazide treatment induces structural degeneration of DCT cells and apoptotic cell death, apparent on light and electron microscopy, which is accompanied by a decreased amount of NCC mRNA transcripts. Furthermore, they showed that in degenerating cells NCC immunostaining was virtually absent in the apical membrane, but accumulated in the cytoplasm [22]. In line with their data is the present, albeit less frequent, observation of a similar disturbed cellular localization of NCC in all HCTZ-treated rats, which was never encountered in controls. This atypical, intracellular distribution might be the result of structural damage, suggesting advanced cellular degeneration. In contrast, a recently published study showed that thiazides can increase NCC expression levels [36]. These authors could not explain this discrepancy. Nevertheless, the present data suggest that thiazides induce structural damage to and loss of DCT cells, explaining the reduced expression of transporters present in this nephron segment (i.e., TRPV5, calbindin-D\(_{28K}\), NCX1, and NCC).

Alternatively, the decreased expression levels could result from a compensatory down-regulation to counteract a positive Ca\(^{2+}\) balance. Indeed, the reduced Ca\(^{2+}\) excretion in thiazide treatment is reported to be accompanied by a small, transient rise in plasma Ca\(^{2+}\) concentration [37]. This would result in a compensatory decrease of plasma PTH and calcitriol levels in an effort to normalize plasma Ca\(^{2+}\). Reduced calcitriol concentrations have been reported during thiazide treatment [38–40] and expression of the proteins involved in active Ca\(^{2+}\) transport is positively regulated by calcitriol [16, 41]. Therefore, compensatory down-regulation of the Ca\(^{2+}\) transport proteins might ensue. This suggestion is, however, at variance with our findings that reversal of hypocalciuria by Na\(^{+}\) repletion did not normalize the expression levels of the transporters and that their expression was not affected in the Na\(^{+}\)-depleted animals, which exhibit overt hypocalciuria.

In Gitelman syndrome, a recessive disorder caused by...
mutations in the gene encoding NCC, hypocalciuria is invariably present [20, 42]. Current hypotheses concerning the mechanism responsible for this hypocalciuria also center on stimulation of active Ca\(^{2+}\) transport [11]. Therefore, results from the present study could be applicable to explain the hypocalciuria in Gitelman syndrome. For instance, Gitelman patients are hyperreninemic, having significantly lower blood pressures compared to their unaffected relatives, and in mice harboring a NCC null mutation, kidney renin mRNA levels are elevated [11, 43]. This indicates that compensatory mechanisms to correct hypovolemia are activated. In addition, in NCC null mice, a more than threefold decrease of readily identifiable DCT cells was observed, showing that the absence of functional NCC is not well tolerated by a major population of DCT cells, leading to significant structural changes [20]. This implies that, like during chronic thiazide treatment in our study, the population of cells supposedly harboring the hypocalciuric mechanism is decreased. Therefore, similar experiments in NCC knockout mice might add important new data in unraveling the mechanism responsible for the hypocalciuria in Gitelman syndrome.

**CONCLUSION**

The consistent down-regulation of proteins involved in active Ca\(^{2+}\) transport as well as the observed deleterious effects of HCTZ on DCT cells do not support a stimulation of active Ca\(^{2+}\) transport during chronic thiazide treatment. Our data suggest that ECV contraction is a critical determinant of the hypocalciuric effect of chronic thiazide treatment. Detailed and elaborate micropuncture experiments are needed to measure the rate of Ca\(^{2+}\) reabsorption in the distal convoluted and connecting tubules during chronic thiazide-induced hypocalciuria.

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