Downregulation of Ca\(^{2+}\) and Mg\(^{2+}\) Transport Proteins in the Kidney Explains Tacrolimus (FK506)-Induced Hypercalciuria and Hypomagnesemia

TOM NIJENHUIS, JOOST G.J. HOENDEROP, and RENÉ J.M. BINDELS

Department of Physiology, Nijmegen Center for Molecular Life Sciences, University Medical Center Nijmegen, Nijmegen, the Netherlands

Abstract. FK506 (tacrolimus) and dexamethasone are potent immunosuppressants known to induce significant side effects on mineral homeostasis, including hypercalciuria and hypomagnesemia. However, the underlying molecular mechanisms remain unknown. The present study investigated the effects of FK506 and dexamethasone on the expression of proteins involved in active Ca\(^{2+}\) reabsorption: the epithelial Ca\(^{2+}\) channel TRPV5 and the cytosolic Ca\(^{2+}\)-binding protein calbindin-D\(_{28K}\). In addition, the renal expression of the putative Mg\(^{2+}\) channel TRPM6, suggested to be involved in transcellular Mg\(^{2+}\) reabsorption, was determined. Administration of FK506 to rats by daily oral gavage during 7 d significantly enhanced the urinary excretion of Ca\(^{2+}\) and Mg\(^{2+}\) and induced a significant hypomagnesemia. FK506 significantly decreased the renal mRNA expression of TRPV5 (62 ± 7% relative to controls), calbindin-D\(_{28K}\) (9 ± 1%), and TRPM6 (52 ± 8%), as determined by real-time quantitative PCR analysis. Furthermore, semiquantitative immunohistochemistry showed reduced renal protein abundance of TRPV5 (24 ± 5%) and calbindin-D\(_{28K}\) (29 ± 4%), altogether suggesting that down-regulation of these transport proteins is responsible for the FK506-induced Ca\(^{2+}\) and Mg\(^{2+}\) wasting. In contrast, dexamethasone significantly enhanced renal TRPV5 (150 ± 15%), calbindin-D\(_{28K}\) (177 ± 23%), and TRPM6 (156 ± 20%) mRNA levels along with TRPV5 (211 ± 8%) and calbindin-D\(_{28K}\) (176 ± 5%) protein abundance in the presence of significantly increased Ca\(^{2+}\) and Mg\(^{2+}\) excretion. This indicated that these proteins are directly or indirectly regulated by dexamethasone. In conclusion, FK506 and dexamethasone induce renal Ca\(^{2+}\) and Mg\(^{2+}\) wasting, albeit by different mechanisms. Downregulation of specific Ca\(^{2+}\) and Mg\(^{2+}\) transport proteins provides a molecular mechanism for FK506-induced hypercalciuria and hypomagnesemia, whereas dexamethasone positively regulates these proteins.

Immunosuppressants such as the calcineurin inhibitors cyclosporin A and FK506 (tacrolimus) along with glucocorticoids such as dexamethasone are widely prescribed in numerous disorders and to organ transplant recipients. Although their immunosuppressive actions are achieved by distinct mechanisms, FK506 and dexamethasone both are known to induce significant side effects on mineral homeostasis. These drugs are associated with an increased bone turnover, a negative Ca\(^{2+}\) balance and hypercalciuria, perturbations that can ultimately result in osteoporosis (1–3). Furthermore, hypomagnesemia is a widely known additional side effect of FK506 treatment (4, 5). The kidney is crucial to both Ca\(^{2+}\) and Mg\(^{2+}\) homeostasis by providing the main excretory route for these divalent ions. However, the exact mechanisms by which these immunosuppressants provoke renal cation wasting are unknown.

The hypercalciuria during treatment with these drugs has been attributed to increased bone resorption as well as decreased renal Ca\(^{2+}\) reabsorption (2, 6, 7). In the kidney, the bulk of Ca\(^{2+}\) reabsorption is accomplished by Na\(^{+}\)-driven paracellular Ca\(^{2+}\) transport in the proximal tubule and thick ascending limb of Henle (TAL) (8). Fine-tuning of Ca\(^{2+}\) excretion is achieved by active transcellular reabsorption of Ca\(^{2+}\) in the distal convoluted tubule (DCT) and connecting tubule (CNT), which involves apical Ca\(^{2+}\) entry through the epithelial Ca\(^{2+}\) channel TRPV5 (previously ECalC1), intracellular buffering and facilitated diffusion by the Ca\(^{2+}\)-binding protein calbindin-D\(_{28K}\), and basolateral extrusion by the Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX1) and the plasma membrane Ca\(^{2+}\)-ATPase (PMCA1b) (9, 10). Theoretically, inhibition of active Ca\(^{2+}\) reabsorption could be involved in the immunosuppressant-induced hypercalciuria.

FK506 treatment has been associated with an inappropriately high fractional excretion of Mg\(^{2+}\), suggesting that inhibition of passive or active Mg\(^{2+}\) reabsorption could contribute to the hypomagnesemia (11). Active reabsorption of Mg\(^{2+}\) has been localized to the DCT, but in contrast to Ca\(^{2+}\) reabsorption, little is known about the specific proteins that mediate...
transcellular Mg\(^{2+}\) transport (12, 13). Recently, it was shown that autosomal recessive hypomagnesemia, characterized by disturbed intestinal Mg\(^{2+}\) absorption and inappropriately high fractional Mg\(^{2+}\) excretion rates, is caused by mutations in the gene encoding TRPM6 (14, 15). Like other ion channels in the transient receptor potential (TRP) family, this protein contains six transmembrane domains and is highly homologous to the Mg\(^{2+}\)-permeable ion channel TRPM7, and TRPM6 mRNA expression was detected in kidney and intestine (15). Recently, we demonstrated that TRPM6 confines a Mg\(^{2+}\) permeable channel that is exclusively expressed in the apical membrane of mouse DCT and small intestine, suggesting that TRPM6 constitutes the apical Mg\(^{2+}\) entry channel in transcellular Mg\(^{2+}\) (re)absorption (16). The identification of TRPM6 provides an important tool to study renal active Mg\(^{2+}\) transport during FK506 treatment on a molecular level.

Taken together, the cascade of cellular and molecular events that lead to impaired renal Ca\(^{2+}\) and Mg\(^{2+}\) handling during FK506 and glucocorticoid treatment is largely unknown. Hypothetically, downregulation of Ca\(^{2+}\) and Mg\(^{2+}\) transport proteins in the distal part of the nephron may be involved in the pathogenesis of hypercalcuria and hypermagnesuria during drug treatment. The aim of the present study, therefore, was to determine the effects of FK506 and dexamethasone on the expression of the Ca\(^{2+}\) transport proteins TRPV5 and calbindin-D\(_{28K}\) and the putative Mg\(^{2+}\) channel TRPM6 in the kidney. To this end, rats were treated for 7 d with these immunosuppressants and housed in metabolic cages to collect samples for urine analysis. Subsequently, expression levels of TRPV5, calbindin-D\(_{28K}\), and TRPM6 were determined by real-time quantitative PCR and immunohistochemical analysis.

Materials and Methods

Animal Studies

Young adult male Wistar rats with an initial weight of 225 to 250 g were housed individually in metabolic cages to collect 24-h urine samples. The animals were kept in a light- and temperature-controlled room with ad libitum access to standard pelleted diet and water. The animals were randomly assigned to either the control group (\(n = 10\)) or the two treatment groups, receiving FK506 (\(n = 6\)) or dexamethasone (\(n = 6\)). These immunosuppressants were dissolved in peanut oil and administered daily by oral gavage. Control animals received vehicle only, whereas the two treatment groups received either FK506 (Fujisawa Pharmaceutical Co., Osaka, Japan) at a single dose of 1 mg/d or dexamethasone (Sigma-Aldrich, Zwijndrecht, the Netherlands) at 300 \(\mu\)g/d. Animals were treated for 7 d, after which blood samples were taken and the animals were killed. Kidney cortex and duodenum were sampled and immediately frozen in liquid nitrogen. In addition, kidney cortex was fixed for immunohistochemistry by immersion in 1\% (wt/vol) periodate-lysine-paraformaldehyde for 2 h and 15\% (wt/vol) sucrose in PBS overnight (17). Subsequently, samples were stored at \(-80^\circ\)C until further processing. The animal ethics board of the University Medical Center Nijmegen approved all experimental procedures.

Analytical Procedures

Serum and urine Ca\(^{2+}\) concentrations were determined using a colorimetric assay as described previously (18). Serum and urine [Mg\(^{2+}\)], [Na\(^{+}\)], [creatinine] [glucose], alkaline phosphatase, and \(\gamma\)-glutamyl transpeptidase were measured on a Hitachi autoanalyzer (Hitachi Corp., Tokyo, Japan).

Real-Time Quantitative PCR

Total RNA was extracted from kidney cortex and duodenum using Trizol Total RNA Isolation Reagent (Life Technologies BRL, Breda, the Netherlands). The obtained RNA was subjected to DNase treatment to prevent genomic DNA contamination. Thereafter, 2 \(\mu\)g of RNA was reverse transcribed by Moloney-murine leukemia virus-reverse transcriptase (Life Technologies BRL) as described previously (19). The obtained cDNA was used to determine TRPV5, calbindin-D\(_{28K}\), and TRPM6 mRNA levels in kidney cortex as well as TRPV6, calbindin-D\(_{28K}\), and TRPM6 mRNA expression in duodenum. Expression levels were quantified by real-time quantitative PCR on an ABI Prism 7700 Sequence Detection System (PE Biosystems, Rotkreuz, Switzerland). PCR primers and fluorescence probes were designed using the computer program Primer Express (Applied Biosystems, Foster City, CA) and are listed in Table 1 (Biologio, Malden, the Netherlands).

Immunohistochemistry

Immunohistochemical staining was performed as described previously (17). In short, either single or double staining of sections for TRPV5, calbindin-D\(_{28K}\), kallikrein, and the Na\(^{+}\)-Cl\(^{-}\) co-transporter (NCC) was performed on 7-\(\mu\)m sections of fixed frozen kidney samples. TRPV5 staining involved immersion of the kidney sections in boiled citrate target retrieval buffer (0.01 M sodium citrate and 0.01 M citric acid [pH 6.0]), which was then left to cool for 30 min and subsequent incubation in 0.3\% (vol/vol) \(\mathrm{H}_2\)O\(_2\) in buffer (0.15 M NaCl, 0.1 M Tris-HCl [pH 7.5]) for 30 min. Sections were incubated for 16 h at 4\(^\circ\)C with primary antibody, affinity-purified guinea pig TRPV5 antibody (1:1000) (17), mouse anti–calbindin-D\(_{28K}\) (Swant, Bellinzona, Switzerland; 1:500), rabbit anti-NCC (1:300) (20), and rabbit anti-kallikrein (1:5000; Calbiochem, San Diego, CA). After incubation with biotin-coated goat anti–guinea pig secondary antibody, TRPV5 was visualized using a tyramide signal amplification kit (NEN Life Science Products, Zaventem, Belgium). For detection of calbindin-D\(_{28K}\), NCC, and kallikrein, sections were incubated with an Alexa 488–conjugated goat anti-rabbit or an Alexa 594–conjugated goat anti-mouse secondary antibody. Images were made using a Zeiss fluorescence microscope equipped with a digital photo camera (Nikon DXM1200). For semiquantitative determination of protein levels, images were analyzed with the Image Pro Plus 4.1 image analysis software (Media Cybernetics, Silver Spring, MD), resulting in quantification of the protein levels as the mean of integrated optical density.

Statistical Analyses

Data are expressed as means ± SEM. Statistical comparisons were tested by one-way ANOVA and Fisher multiple comparison. \(P < 0.05\) was considered statistically significant. Statistical analysis was performed using the Statview Statistical Package software (Power PC version 4.51; Berkeley, CA) on a Macintosh computer.

Results

Body Weight, Urine, and Serum Analysis

Net urinary excretion of Ca\(^{2+}\) and Mg\(^{2+}\) is depicted in Figure 1. Additional biochemical analysis of the serum and 24-h urine samples of the experimental groups is shown in
Table 2. In the FK506 group, net and fractional excretion of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) was significantly enhanced. \( \text{Na}^{+} \) excretion was reduced in this treatment group, whereas urine volume did not significantly differ from controls. Although serum \( \text{Ca}^{2+} \) and \( \text{Na}^{+} \) levels were unaffected, FK506 treatment induced a striking hypomagnesemia. Dexamethasone administration enhanced urinary \( \text{Ca}^{2+} \) as well as \( \text{Mg}^{2+} \) excretion. Likewise, \( \text{Na}^{+} \) excretion and urine volume were significantly increased. In the dexamethasone-treated animals, serum \( \text{Ca}^{2+} \) levels did not significantly differ from controls, whereas serum \( \text{Na}^{+} \) levels were decreased. Serum \( \text{Mg}^{2+} \) levels were significantly reduced relative to controls but remained within the normal range. Both serum creatinine and GFR, as determined by creatinine clearance, did not significantly differ from controls in both the FK506 and dexamethasone groups. Serum glucose levels were significantly increased in both the FK506- and dexamethasone-treated groups, resulting in increased glucose excretion in the dexamethasone group only. In addition, urinary excretion of markers of tubular damage (alkaline phosphatase and \( \gamma \)-glutamyl transpeptidase) was detectable, albeit not significantly increased in the FK506 group, whereas the dexamethasone-treated group showed significant enzymuria (21). Although baseline body weight did not differ significantly between the groups (data not shown), at the end of the experiment, dexamethasone-treated animals had a significantly lower mean body weight compared with controls, whereas FK506 treatment did not affect body weight.

Renal mRNA Expression Levels of TRPV5, Calbindin-D\(_{28K}\), and TRPM6

For evaluating the possible association between increased urinary excretion of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) and the expression levels of the respective transport proteins, renal mRNA levels of the epithelial \( \text{Ca}^{2+} \) channel TRPV5, the cytosolic \( \text{Ca}^{2+} \)-binding protein calbindin-D\(_{28K}\), and the putative epithelial \( \text{Mg}^{2+} \) channel TRPM6 were determined by real-time quantitative PCR analysis. FK506 significantly decreased TRPV5, calbindin-

Table 1. Sequences of primers and Taqman probes for real-time quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRPV5</td>
<td>5'-CTGTTGTGTTGAGGCTGACAG-3'</td>
<td>5'-AGGAACTTTTCTTTCAGCTG-3'</td>
<td>5'-FAM-TAATGGCCCTTGAGAGGTGGAAC-3'</td>
</tr>
<tr>
<td>CaBP28</td>
<td>5'-GAGGCCCAGGCTGACAG-3'</td>
<td>5'-TGGCTTCTTCTTGTACTTCCTCCTTGT-3'</td>
<td>5'-FAM-AGCGCGGTAATGATGCTCTCTTATTCG-3'</td>
</tr>
<tr>
<td>TRPM6</td>
<td>5'-CCTGGTCTGAGGATGATGTTCTCAAGCC-3'</td>
<td>5'-ATCCGCCGCTATGCACA-3'</td>
<td>5'-FAM-TAGTTTTTCTCCTGAGTCTTTTTCCA-3'</td>
</tr>
<tr>
<td>CaBP9</td>
<td>5'-CCCGAAGAAATGAAGAGCATTTT-3'</td>
<td>5'-TTCTCCATCACCGTTCTTATCCA-3'</td>
<td>5'-FAM-CAAAAAATATGCAGCCAAGGAAGGCGA-3'</td>
</tr>
</tbody>
</table>

Primer pairs and fluorescent probes (5'-FAM-3'-TAMRA) were designed using the computer program Primer Express (Applied Biosystems) and purchased from Biolegio. TRPV5, epithelial \( \text{Ca}^{2+} \) channel 1 (previously known as ECaC1); CaBP28, calbindin-D\(_{28K}\), channel 1.2 (previously known as ECaC2/CaT1); TRPM6, putative \( \text{Mg}^{2+} \) channel; TRPV6, epithelial \( \text{Ca}^{2+} \) channel 2 (previously known as ECaC2).

Figure 1. Effect of FK506 and dexamethasone treatment on net urinary \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) excretion in the rat. Net excretion of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) was determined by analysis of 24-h urine samples of rats individually housed in metabolic cages. Ctr, controls; FK506, FK506 1 mg/d by oral gavage; Dex, dexamethasone 300 \( \mu \)g/d by oral gavage. Data are presented as means ± SEM. ** \( P < 0.05 \) versus controls.
Conversely, dexamethasone treatment significantly enhanced the renal mRNA expression of both Ca$^{2+}$/H$^{+}$ transport proteins and TRPM6.

Renal Protein Abundance of TRPV5 and Calbindin-D$_{28K}$

For assessing whether the changes in renal mRNA levels resulted in altered protein expression, TRPV5 and calbindin-D$_{28K}$ protein abundance was semiquantified by immunohistochemistry (Figure 3A). These results illustrated that the above-mentioned mRNA results were indeed accompanied by similar effects on the protein level. Computerized analysis of the immunohistochemical staining showed significantly reduced TRPV5 and calbindin-D$_{28K}$ protein expression during FK506 treatment, whereas dexamethasone significantly enhanced the abundance of these Ca$^{2+}$ transport proteins in the kidney (Figure 3B). Of note, the effects on TRPV5 protein expression were more pronounced compared with the mRNA level, suggesting that in addition to affecting gene transcription, additional translational changes might have occurred.

For confirming the specificity of the observed effects on the Ca$^{2+}$/H$^{+}$ and Mg$^{2+}$/H$^{+}$ transport proteins, protein abundance of kallikrein and NCC were determined by immunohistochemistry (Figure 3A). The renal abundance of tissue kallikrein, a specific marker for the DCT and CNT (22), did not significantly differ from controls in both the FK506 and dexamethasone groups (Figure 3B). In addition, the protein expression of NCC, exclusively expressed in the DCT, was not significantly affected by FK506 treatment but showed a

Table 2. Urine and serum analyses and body weight$^a$

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>FK506</th>
<th>Dexamethasone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Urine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FE Ca$^{2+}$ (%)</td>
<td>0.62 ± 0.07</td>
<td>1.08 ± 0.17$^b$</td>
<td>1.02 ± 0.11$^b$</td>
</tr>
<tr>
<td>FE Mg$^{2+}$ (%)</td>
<td>4.7 ± 1.1</td>
<td>35.1 ± 3.4$^c$</td>
<td>20.4 ± 2.2$^b$</td>
</tr>
<tr>
<td>Na$^+$ excretion (mmol/24 h)</td>
<td>1.6 ± 0.1</td>
<td>1.0 ± 0.2$^b$</td>
<td>2.4 ± 0.2$^b$</td>
</tr>
<tr>
<td>FE Na$^+$ (%)</td>
<td>0.64 ± 0.06</td>
<td>0.46 ± 0.04$^b$</td>
<td>1.15 ± 0.05$^b$</td>
</tr>
<tr>
<td>glucose excretion (mmol/24 h)</td>
<td>0.1 ± 0.1</td>
<td>2.7 ± 1.3</td>
<td>20.8 ± 4.8$^b$</td>
</tr>
<tr>
<td>diuresis (ml/24 h)</td>
<td>8.8 ± 1.0</td>
<td>18.6 ± 4.8</td>
<td>42.5 ± 6.2$^b$</td>
</tr>
<tr>
<td>C cr (ml/min)</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1</td>
<td>1.1 ± 0.1$^b$</td>
</tr>
<tr>
<td>ALP excretion (U/24 h)</td>
<td>0.1 ± 0.1</td>
<td>0.4 ± 0.2</td>
<td>1.1 ± 0.4$^b$</td>
</tr>
<tr>
<td>γ-GT excretion (U/24 h)</td>
<td>0.1 ± 0.1</td>
<td>1.3 ± 0.7</td>
<td>4.9 ± 2.5$^b$</td>
</tr>
<tr>
<td><strong>Serum</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca$^{2+}$ (mmol/L)</td>
<td>2.53 ± 0.03</td>
<td>2.51 ± 0.03</td>
<td>2.49 ± 0.02$^b$</td>
</tr>
<tr>
<td>Mg$^{2+}$ (mmol/L)</td>
<td>1.01 ± 0.02</td>
<td>0.65 ± 0.02$^b$</td>
<td>0.92 ± 0.01$^b$</td>
</tr>
<tr>
<td>Na$^+$ (mmol/L)</td>
<td>142.1 ± 0.5</td>
<td>141.0 ± 0.4</td>
<td>134.0 ± 0.5$^b$</td>
</tr>
<tr>
<td>glucose (mmol/L)</td>
<td>6.9 ± 0.2</td>
<td>11.1 ± 0.9$^b$</td>
<td>18.7 ± 2.4$^b$</td>
</tr>
<tr>
<td>creatinine (μmol/L)</td>
<td>37 ± 1</td>
<td>38 ± 1</td>
<td>42 ± 2</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>310 ± 9.3</td>
<td>296 ± 6.1</td>
<td>277 ± 9.7$^b$</td>
</tr>
</tbody>
</table>

$^a$ Controls, animals receiving vehicle only; FK506, FK506 1 mg/d; Dexamethasone, dexamethasone 300 μg/d; FE, fractional excretion; C cr, creatinine clearance; ALP, alkaline phosphatase; γ-GT, gamma-glutamyl transpeptidase. Data are presented as means ± SEM. $^b p < 0.05$ versus controls.
significant, albeit modest, increase in the dexamethasone-treated animals (Figure 3B). Furthermore, no signs of a general deleterious effect of FK506 (inclusion bodies, tubular vacuolization, and atrophy) were detected on light microscopic analysis (4). GFR was unaffected and enzynuria was not significantly increased, although markers of tubular damage were detectable. Importantly, the unaltered expression of kallikrein and NCC suggested that no overt FK506 nephrotoxicity was present in DCT.

In addition, co-staining of kidney sections for the presence of TRPV5 and kallikrein was performed, which showed a full co-localization of these proteins in DCT and CNT in the control animals (Figure 4A). Importantly, Figure 4B clearly illustrates that TRPV5 protein abundance in DCT and CNT is significantly reduced in the FK506-treated animals, whereas kallikrein expression levels are not affected. Dexamethasone treatment did not influence the co-localization of TRPV5 and kallikrein, substantiating that the dexamethasone-induced increase of TRPV5 abundance is confined to these nephron segments (Figure 4C). Co-staining of kidney sections for kallikrein and calbindin-D_28K showed similar results, with the exception that calbindin-D_28K expression extended further into the cortical collecting duct (data not shown).

Intestinal mRNA Expression Levels of TRPV6, Calbindin-D_{28K}, and TRPM6

Theoretically, upregulation of Ca^{2+} transport proteins in the intestine could contribute to the increased Ca^{2+} wasting, and decreased intestinal TRPM6 expression could contribute to the hypomagnesemia. Therefore, the duodenal mRNA expression of the epithelial Ca^{2+} channel TRPV6, the cytosolic Ca^{2+}-binding protein calbindin-D_{28K}, and TRPM6 were determined (Figure 5). mRNA expression of all three transporters was not significantly altered by FK506 treatment. Dexamethasone significantly increased TRPV6 and calbindin-D_{28K} mRNA expression levels, whereas TRPM6 mRNA expression was not significantly affected.

Discussion

The present study demonstrated that FK506 and dexamethasone significantly enhance renal Ca^{2+} and Mg^{2+} excretion, albeit by different molecular mechanisms. The decreased expression of the Ca^{2+} transport proteins TRPV5 and calbindin-D_{28K} and the putative Mg^{2+} channel TRPM6 during FK506 treatment suggested that downregulation of these transport proteins contributes to the pathogenesis of FK506-induced hypercalciuria and hypomagnesemia. In contrast, dexametha-
FK506 treatment significantly increased urinary Ca\(^{2+}\) excretion, accompanied by a downregulation of the renal mRNA expression of TRPV5 and calbindin-D\(_{28K}\) and a specific reduction of the protein abundance of these Ca\(^{2+}\) transport proteins in DCT and CNT, recognized as the main sites of transcellular Ca\(^{2+}\) reabsorption. Previous reports showed reduced calbindin-D\(_{28K}\) levels during FK506 treatment, suggesting that FK506 could affect active Ca\(^{2+}\) transport (23). That serum Ca\(^{2+}\) concentrations and GFR did not differ from controls confirmed that impaired Ca\(^{2+}\) reabsorption rather than an increased filtered load caused the hypercalciuria. Morphologic features of tubular toxicity were not detected. Furthermore, the excretion of urinary markers of tubular damage was not significantly increased and the expression of specific markers for DCT and CNT was unaltered, excluding that a general deleterious effect of FK506 on DCT is responsible for the observed downregulation. In addition, the expression of the major intestinal Ca\(^{2+}\) transport proteins was not increased by FK506 treatment, excluding that hypercalciuria is secondary to up-regulation of active Ca\(^{2+}\) absorption. The present data strongly supported our hypothesis that FK506 induces a primary defect of renal active Ca\(^{2+}\) reabsorption by specifically downregulating the proteins involved in active Ca\(^{2+}\) transport.

The molecular mechanism underlying the downregulation of the Ca\(^{2+}\) transport proteins by FK506 remains elusive. In previous studies, plasma calcitriol (1,25(OH)\(_2\)D\(_3\)) was either unaltered or moderately increased, whereas plasma parathyroid levels were not affected by similar doses of FK506, which excludes that the reduced Ca\(^{2+}\) transport protein expression levels are secondary to decreased circulating levels of these calcitropic hormones (11, 23, 24). The immunosuppressive action of FK506 depends on the inhibition of the Ca\(^{2+}\)-dependent phosphatase calcineurin in T lymphocytes (25–27). Calcineurin is not known to be involved in renal Ca\(^{2+}\) reabsorption, but another calcineurin inhibitor, cyclosporine A, increased urinary Ca\(^{2+}\) excretion and decreased calbindin-D\(_{28K}\) protein levels, suggesting that calcineurin inhibition may play a role in the impairment of Ca\(^{2+}\) reabsorption by these drugs (23, 28). In addition, FK506 binds to intracellular immunophilins called FK506-binding proteins (FKBP), which have been implicated as ion channel regulators (29–31). In particular, FKBP4 was shown to bind and regulate the Ca\(^{2+}\)-permeable Drosophila TRPL channel, and this binding was disrupted by the addition of FK506 (32). Furthermore, several intracellular Ca\(^{2+}\)-release channels were shown to be modulated by binding and dissociation of FKBP (33–35). Therefore, it is tempting to speculate that FKBP are potential associated proteins regulating TRPV5 expression or activity.

Hypomagnesemia is a widely known adverse effect of FK506 treatment (4, 5). The increased fractional excretion of Mg\(^{2+}\) in the presence of a profound hypomagnesemia indicated that renal Mg\(^{2+}\) reabsorption is seriously impaired by FK506. This renal Mg\(^{2+}\) wasting was accompanied by reduced renal expression of the putative Mg\(^{2+}\) channel TRPM6. Mutations in the gene encoding TRPM6 were recently shown to cause hereditary hypomagnesemia, which was also accompanied by an inappropriately increased fractional excretion of Mg\(^{2+}\) (14, 15). Importantly, RT-PCR analysis of microdissected rat nephrons showed strong TRPM6 expression in the DCT, the main site of active Mg\(^{2+}\) reabsorption (15). Together with the high structural homology to TRPM7, which has been previously identified to constitute a Mg\(^{2+}\) permeable cation channel, these data indicated that this protein constitutes the

![Figure 4. Co-immunohistochemical staining of rat kidney for the epithelial Ca\(^{2+}\) channel TRPV5 and kallikrein. Co-staining of kidney sections for TRPV5 and kallikrein, a marker for the distal convoluted tubule (DCT) and connecting tubule (CNT), in controls (A), FK506-treated animals (B), and dexamethasone-treated animals (C). Controls, rats receiving vehicle only; FK506, rats receiving FK506 1 mg/d by oral gavage; Dexamethasone, rats receiving dexamethasone 300 μg/d by oral gavage.](image-url)
apical Mg$^{2+}$ entry channel in renal transcellular Mg$^{2+}$ transport in DCT (36, 37). Of note, the DCT has been previously indicated as a possible site of the tubular Mg$^{2+}$ leak during FK506 treatment (13). The duodenal TRPM6 mRNA levels were not significantly altered by FK506 treatment, which is at variance with primary role of duodenal TRPM6 in the pathogenesis of hypomagnesemia. Altogether, our data showed that in addition to mutations in TRPM6, drug-induced downregulation of this ion channel in kidney is associated with increased urinary Mg$^{2+}$ excretion and hypomagnesemia, further substantiating the importance of TRPM6 in renal Mg$^{2+}$ reabsorption. Dexamethasone-treated animals displayed a significant hypercalciuria, but, in contrast to FK506 treatment, dexamethasone increased the expression levels of TRPV6 and calbindin-D$_{28K}$. However, in line with previous data that showed decreased tubular reabsorption of Ca$^{2+}$ during glucocorticoid treatment, the unaltered serum Ca$^{2+}$ levels and GFR suggested that a primary impairment of Ca$^{2+}$ reabsorption is responsible for the hypercalciuria observed in our study (2, 6). In the proximal tubule and TAL, passive reabsorption of Ca$^{2+}$ takes place, which is altogether responsible for reabsorbing approximately 70 to 90% of the filtered Ca$^{2+}$ (8). In this paracellular pathway, Ca$^{2+}$ is transported down an electrochemical gradient that is dependent on Na$^{+}$ reabsorption, and impairment of the latter is known to result in increased Ca$^{2+}$ excretion. In this study, net Na$^{+}$ excretion was indeed considerably increased and serum Na$^{+}$ levels were reduced, whereas NCC expression was enhanced. Previously, glucocorticoid excess was demonstrated to enhance the expression of the distally located NCC and epithelial Na$^{+}$ channel, whereas dexamethasone was shown to inhibit the function and expression of the proximal tubular Na$^{+}$/phosphate co-transporter (38–41). Therefore, reduced reabsorption of Na$^{+}$ in the proximal tubule or TAL could be responsible for the increased Na$^{+}$ excretion and, by decreasing passive Ca$^{2+}$ reabsorption, the dexamethasone-induced hypercalciuria. In addition, enzymuria was significantly increased by dexamethasone, suggesting that structural damage to the proximal tubule or TAL could contribute to the increased Na$^{+}$ and Ca$^{2+}$ excretion. The increased expression of the proteins involved in active Ca$^{2+}$ transport in DCT indicated that these proteins are directly or indirectly regulated by dexamethasone. The latter might reflect a compensatory effect secondary to the increased distal Ca$^{2+}$ load, similar to the increased expression of distally located Na$^{+}$ transporters, which has been shown after inhibition of Na$^{+}$ reabsorption in the loop of Henle (42–44). The concomitant increase in expression of the major intestinal Ca$^{2+}$ transport proteins suggests a direct stimulatory effect of dexamethasone on active Ca$^{2+}$ (re)absorption in kidney and intestine.

It is interesting that dexamethasone treatment resulted in an increased urinary excretion of Mg$^{2+}$ with a concomitant decrease of serum Mg$^{2+}$ levels. However, the latter effect was much smaller than that observed in the FK506-treated animals and remained within the normal range, possibly because of the milder renal Mg$^{2+}$ leak. Alternatively, increased intestinal Mg$^{2+}$ absorption mediated by upregulated intestinal TRPM6 expression levels might counterbalance renal Mg$^{2+}$ losses, but this is not supported by the present study. In analogy to Ca$^{2+}$ reabsorption, a substantial part of Mg$^{2+}$ filtered by the glomerulus is reabsorbed via the paracellular Na$^{+}$-driven pathway (12). Therefore, impairment of the passive reabsorption of divalent ions in proximal tubule and TAL could explain both the hypercalciuria and hypermagnesuria. Subsequently, the enhanced TRPM6 expression levels could result from a direct stimulatory effect of dexamethasone or a compensatory response, serving to limit the renal Mg$^{2+}$ wasting.

Indeed, serum glucose levels were significantly increased in both the FK506- and dexamethasone-treated animals. In the FK506-treated group, urinary glucose excretion did not significantly differ from controls, but the trend toward increased glycosuria could very well explain the concomitant trend toward increased urine volume. Furthermore, dexamethasone significantly increased glycosuria, which in conjunction with the increased Na$^{+}$ excretion might cause the observed polyuria.

In conclusion, FK506-induced downregulation of Ca$^{2+}$ and Mg$^{2+}$ transport proteins could be a critical factor in the pathogenesis of hypercalciuria and hypomagnesemia. Elucidation of the molecular mechanism responsible for these inhibitory effects will extend our knowledge of the in vivo regulation of these transporters and will identify novel targets for pharmacologic therapy.
Acknowledgments
This study was supported by the Dutch Kidney Foundation (C10.1881) and the Dutch Organization of Scientific Research (Zon-Mw 016.006.001).

We thank the Fujisawa Pharmaceutical Co. for generously supplying tacrolimus (FK506).

References