Expression of renal distal tubule transporters TRPM6 and NCC in a rat model of cyclosporine nephrotoxicity and effect of EGF treatment

Kristien J. Ledeganck,1 Gaëlle A. Boulet,2 Caroline A. Horvath,2 Marleen Vinckx,1 Johannes J. Bogers,2 Rita Van Den Bossche,3 Gert A. Verpooten,1,4 and Benedicte Y. De Winter1

1Laboratory of Experimental Medicine and Pediatrics, 2Applied Molecular Biology Research Group, and 3Laboratory of Pharmacology, University of Antwerp, and 4Department of Nephrology-Hypertension, Antwerp University Hospital, Edegem, Antwerp, Belgium

Submitted 24 February 2011; accepted in final form 6 June 2011

Cyclosporine (CsA) is a calcineurin inhibitor which inhibits T cell activation by blocking the transcription of cytokine genes (29). The most serious and dose-limiting side effect of this immunosuppressive drug is nephrotoxicity. Acute nephrotoxicity is characterized by renal vasoconstriction, which is dose related and reversible after dose reduction or discontinuation of CsA. Chronic CsA-induced nephrotoxicity can be progressive and irreversible. The histological changes include tubular atrophy, medial arteriolar hyalinosis, and striped fibrosis with infiltration of mononuclear cells. Tubulointerstitial fibrosis is the most important and determining factor, which leads to irreversible loss of kidney function in chronic kidney diseases (1, 13, 28). CsA also induces functional alterations and ion homeostasis disturbances like hypomagnesemia and magnesium (Mg2+) wasting, hyponatremia, hyperkalemia, hyperchloremic metabolic acidosis, and hyperuricemia (5, 18).

Hypomagnesemia after treatment with calcineurin inhibitors is due to renal Mg2+ loss (3, 33). The major site of passive Mg2+ reabsorption is the thick ascending limb (TAL), where 70% of the Mg2+ is reabsorbed paracellularly. The tight junction proteins claudin-16 (also known as paracellin-1) and claudin-19 are key players in this paracellular Mg2+ transport (20, 22). It was shown by Chang et al. (7) that CsA reduces claudin-16 expression in vitro in TAL cells.

Recently, two other ion channels which play an important role in Mg2+ homeostasis were identified, TRPM6 and TRPM7. They belong to the transient receptor potential potential subfamily melanopsin (TRP). TRPM6 has an expression pattern predominantly present in absorbing epithelia. In the kidney, TRPM6 is expressed in the distal convoluted tubule (DCT), known as the major site of active transcellular Mg2+ reabsorption along the nephron. TRPM7 is ubiquitously expressed and implicated in cellular Mg2+ homeostasis, cell death, and cell cycle regulation (19, 42).

Ikari et al. (21) found that CsA decreased TRPM6 expression in vitro in cell culture of rat kidney epithelial cells (NRK-52E). Furthermore, it is known that CsA administration results in a decreased renal expression of epidermal growth factor (EGF) in the rat (9). Recently, it was shown that EGF stimulates Mg2+ reabsorption in the DCT via its receptor on the basolateral membrane and via activation of TRPM6 in the apical membrane. EGF-mediated stimulation of TRPM6 occurs via signaling through SRC kinases and Rac1 in vitro, thereby redistributing endomembrane TRPM6 to the plasma membrane (16, 41).

Calcineurin inhibitors also affect sodium (Na+) reabsorption, since hyponatremia due to renal sodium loss has been described in renal transplant patients treated with tacrolimus or CsA (18, 51). The epithelial sodium channel and mainly the thiazide-sensitive Na+/Cl− cotransporter (NCC) in the DCT reabsorbs sodium to make the final adjustment of renal sodium excretion (15, 27). The renin-angiotensin-aldosterone system (RAAS) stimulates NCC activity. On the one hand, ANG II induces rapid trafficking of the NCC from stores in subapical vesicles to the plasma membrane, and on the other hand both ANG II and aldosterone independently phosphorylate and thereby activate the NCC (37, 44).

The aim of this study was to gain more insight into the molecular mechanisms of CsA-induced hypomagnesemia and...
hyponatremia. We investigated whether we could confirm the in vitro finding that claudin-16 and TRPM6 are downregulated after CsA administration in an in vivo rat model of CsA nephrotoxicity. The animal model has been validated with regard to histological characteristics, the inflammatory component, and the expression of profibrotic factors transforming growth factor (TGF)-β and plasminogen activator inhibitor (PAI)-1. We further hypothesized that EGF administration leads to an increase in TRPM6 expression in the DCT in control rats and to a reduction of TRPM6 downregulation in CsA-treated rats. Furthermore, we investigated the effect of CsA on the expression of the NCC in the DCT. To determine the influence of the RAAS on NCC expression in the rat model, serum aldosterone levels and renal preprorenin mRNA levels were measured.

MATERIALS AND METHODS

To validate the animal model of CsA nephrotoxicity, the following analyses were performed: determination of serum creatinine, visualization of CsA-induced morphological lesions, phenotyping of inflammatory cells, and assessment of renal mRNA levels of the proinflammatory factors TGF-β and PAI-1.

The study’s aims were addressed by determination of the following parameters: the fractional excretion of Mg2+ (FE Mg2+) and Na+ (FE Na+), the expression of TRPM6, TRPM7, claudin-16, and claudin-19, EGF, the EGF receptor (EGFR), the NCC, and preprorenin and serum aldosterone levels. Subsequently, the effect of human (h) EGF administration on these parameters was evaluated.

Ethical approval for animal studies was achieved from the Medical Ethical Committee on Animal Experimentation at the University of Antwerp, Belgium.

Animal model. Forty-eight male Wistar rats (Charles River, Brussels, Belgium), weighing at the start of the experiments between 180 and 200 g, were housed four per cage, at 22 ± 2°C with a 12:12-h light-dark cycle. They had free access to water and food with a normal-Mg2+-, low-sodium diet (0.05% sodium, Harlan Laboratories, Horst, The Netherlands), a necessary constituent of the CsA nephrotoxicity rat model: salt depletion accelerates CsA-induced morphological lesions.

Harlan Laboratories, Horst, The Netherlands), a necessary constituent of the rat model: salt depletion accelerates CsA-induced morphological lesions.

Forty-eight male Wistar rats (Charles River, Brussels, Belgium), weighing at the start of the experiments between 180 and 200 g, were housed four per cage, at 22 ± 2°C with a 12:12-h light-dark cycle. They had free access to water and food with a normal-Mg2+-, low-sodium diet (0.05% sodium, Harlan Laboratories, Horst, The Netherlands), a necessary constituent of the CsA nephrotoxicity rat model: salt depletion accelerates CsA-induced morphological lesions.

Animal model. Forty-eight male Wistar rats (Charles River, Brussels, Belgium), weighing at the start of the experiments between 180 and 200 g, were housed four per cage, at 22 ± 2°C with a 12:12-h light-dark cycle. They had free access to water and food with a normal-Mg2+-, low-sodium diet (0.05% sodium, Harlan Laboratories, Horst, The Netherlands), a necessary constituent of the CsA nephrotoxicity rat model: salt depletion accelerates CsA-induced morphological lesions.

Animal model. Forty-eight male Wistar rats (Charles River, Brussels, Belgium), weighing at the start of the experiments between 180 and 200 g, were housed four per cage, at 22 ± 2°C with a 12:12-h light-dark cycle. They had free access to water and food with a normal-Mg2+-, low-sodium diet (0.05% sodium, Harlan Laboratories, Horst, The Netherlands), a necessary constituent of the CsA nephrotoxicity rat model: salt depletion accelerates CsA-induced morphological lesions.

Harlan Laboratories, Horst, The Netherlands), a necessary constituent of the CsA nephrotoxicity rat model: salt depletion accelerates CsA-induced morphological lesions.

Animals were fed with a normal-Mg2+-, low-sodium diet (0.05% sodium, Harlan Laboratories, Horst, The Netherlands), a necessary constituent of the CsA nephrotoxicity rat model: salt depletion accelerates CsA-induced morphological lesions.

Animals were fed with a normal-Mg2+-, low-sodium diet (0.05% sodium, Harlan Laboratories, Horst, The Netherlands), a necessary constituent of the CsA nephrotoxicity rat model: salt depletion accelerates CsA-induced morphological lesions.

Experimental set-up. Rats were divided into four groups (each containing n = 12) and treated for 28 days as follows.

Control rats received a daily subcutaneous injection of saline/PBS in a volume equal to the EGF- and/or CsA-treated groups.

Control rats received a daily subcutaneous injection of EGF (150 µg·kg⁻¹·day⁻¹) and saline in a volume equal to the CsA-treated groups.

CsA-treated rats received a daily subcutaneous injection of EGF (150 µg·kg⁻¹·day⁻¹) and saline in a volume equal to the CsA-treated groups.

CsA-treated rats received a daily subcutaneous injection of saline/PBS in a volume equal to the EGF- and/or CsA-treated groups.

Control rats received a daily subcutaneous injection of EGF (150 µg·kg⁻¹·day⁻¹) and saline in a volume equal to the CsA-treated groups.

CsA-treated rats received a daily subcutaneous injection of CsA (15 µg·kg⁻¹·day⁻¹) and PBS in a volume equal to the EGF-treated groups.

CsA-treated rats received a daily subcutaneous injection of CsA (15 µg·kg⁻¹·day⁻¹) and PBS in a volume equal to the EGF-treated groups.

CsA-treated+EGF rats received a daily subcutaneous injection of CsA (15 µg·kg⁻¹·day⁻¹) and EGF (150 µg·kg⁻¹·day⁻¹).

CsA-treated+EGF rats received a daily subcutaneous injection of CsA (15 µg·kg⁻¹·day⁻¹) and EGF (150 µg·kg⁻¹·day⁻¹).

CsA-treated+EGF rats received a daily subcutaneous injection of CsA (15 µg·kg⁻¹·day⁻¹) and EGF (150 µg·kg⁻¹·day⁻¹).

CsA-treated+EGF rats received a daily subcutaneous injection of CsA (15 µg·kg⁻¹·day⁻¹) and EGF (150 µg·kg⁻¹·day⁻¹).

CsA treated rats received a daily subcutaneous injection of CsA (15 µg·kg⁻¹·day⁻¹) and saline in a volume equal to the CsA-treated groups.

CsA treated rats received a daily subcutaneous injection of CsA (15 µg·kg⁻¹·day⁻¹) and saline in a volume equal to the CsA-treated groups.

CsA treated rats received a daily subcutaneous injection of CsA (15 µg·kg⁻¹·day⁻¹) and saline in a volume equal to the CsA-treated groups.

CsA treated rats received a daily subcutaneous injection of CsA (15 µg·kg⁻¹·day⁻¹) and saline in a volume equal to the CsA-treated groups.

Sample collection. One week before the first administration, the rats were caged individually in a metabolic cage to obtain a 24-h urine collection. Also, 0.9 ml of blood was collected from the tail vein.

After 28 days of treatment, according to the study design, the rats were caged individually to obtain a second 24-h urine collection. Meanwhile, the rats were housed four per cage. Afterward, they were euthanized by an overdose of pentobarbital sodium (100–150 mg/kg) intraperitoneally. Blood samples were taken from the vena cava inferior at the time of death. The kidneys were quickly prelevated and decapsulated. The right kidney was snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction was performed. The left kidney was cut into 2-mm-thick transverse slices and processed for further histological analysis using different fixation procedures (described below).

Serum and urine creatinine, Mg2+, Na+, K+, and CsA levels. Serum and urine creatinine and Mg2+ were analyzed with the Vitros 5.1 FS analyzer, using a Vitros creatinine and Mg2+ slide, respectively.

Serum Na+ was calculated as 

\[ \text{FE Na} = 100 \times \left( \frac{\text{UNa}}{\text{PCr}} \right) \left( \frac{\mu l}{l} \right) \]

Serum and urine K+ levels were measured using an indirect potentiometric method with a dimension Vista 1500 System (Siemens Healthcare Diagnostics, Deerfield, IL).

Serum aldosterone levels. Serum aldosterone levels were measured using a rat aldosterone ELISA Kit (Hölzel Diagnostika Handels, Cologne, Germany). Samples were diluted in a sample diluent supplied with the kit (1:100). The detection range was 31.2–2,000 pg/ml.

CsA-induced morphological lesions. For light microscopy, the rat kidney slices were fixed in formol for 24 h, embedded in paraffin, sectioned at 5 µm, and stained with periodic acid-Schiff reagent or Sirius red stained stain. The tissue samples were evaluated for tubular injuries and juxtaglomerular apparatus (JGA) hyperplasia by three observers blinded to the treatment groups. The percentage of dilated or vacuolized tubules was counted in at least 28 cortical fields (×250 magnification). The following score was used: 0, no tubular injury; 1, <10% of tubules injured; 2, 10–25% of tubules injured; 3, 25–50% of tubules injured; 4, 50–75% of tubules injured; and 5, >75% of tubules injured (28). JGA hyperplasia was counted in all glomeruli and expressed as the percentage of total number of glomeruli. Sirius red-stained sections were scanned with an Olympus BX61 Motorized Research Microscope (Olympus, Tokyo, Japan) equipped with analysis pro 5.0 software (Olympus). Because there was no visible fibrosis, the amount of collagen was measured. After processing of the image, the total amount of collagen was measured and expressed as the percentage of the total cortex.

Phenotyping of inflammatory cells. The formol-fixed, paraffin-embedded renal tissue was stained with the ED1 monoclonal antibody (Acris Antibodies, Hidenhausen, Germany) directed to an antigen of tissue macrophages and peripheral blood granulocytes. Infiltration was quantified by counting immunoreactive cells in 28 randomly chosen areas (magnification ×200) in the cortex (11). Thirty glomeruli per renal section were examined for infiltrating cells. Inflammatory cells contained within large blood vessels or surrounded by erythrocytes were excluded from all counts. The results were expressed as absolute numbers of immunoreactive cells per square millimeter or per glomerular cross section. Sections were evaluated blinded for the subgroup division of the rats.
mRNA isolation. Total RNA was extracted from the kidneys using a column-based technique (RNeasy Minikit, Qiagen, KJ Venlo, The Netherlands). Purified total RNAs were treated with DNase to obtain DNA-free RNA (Turbo DNase free, Ambion, Applied Biosystems, Lennik, Belgium). cDNA was synthesized using a Transcripter First Strand cDNA Synthesis Kit (AMV, Roche Applied Science, Indianapolis, IN).

Real-time RT-PCR. To examine the mRNA expression of TRPM6, the NCC, EGFR, EGF, and renin, quantitative real-time RT-PCR was performed using LightCycler Taqman Master (Roche, Vilvoorde, Belgium). The PCR reaction was performed in a 20-μl reaction volume containing 5 μl of cDNA sample, 4 μl of MasterMix, and a forward primer, reverse primer, and probe. TGF-β, PAI-1, TRPM7, claudin-16, and claudin-19 mRNA expression was examined using LightCycler FastStart DNA Master plus SYBR green I (Roche).

NCC mRNA isolation. NCC staining was performed on formal-fixed, paraffin-embedded renal tissue using a rabbit anti-thiazide-sensitive NCC polyclonal antibody (Millipore, Brussels, Belgium). Before the immunohistochemical procedures, endogenous peroxidase activity in the rat kidney was blocked by H2O2 (3% in H2O, 5 min). The tissue was trypsinized for 10 min at 37°C (50 mg trypsin/10 ml H2O). Just before being covered with a coverslip, sections were stained with hematoxylin and eosin.

AEC-stained sections were scanned with an Olympus BX61 Motorized Research Microscope equipped with analysis pro 5.0 software. After processing of the image, the total amount of AEC was measured and expressed as the percentage of the total cortex. The gray value of the AEC was also measured. A number from 0 to 155 was given to each slide, where 0 was black (chromogen) and 155 was white (background).

STATISTICAL ANALYSIS
Results are presented as means ± SD. Differences between groups were investigated with one-way and two-way ANOVA followed by group-to-group comparisons with the post hoc Student-Newman-Keuls test. Nonparametric data were investigated with a Kruskal-Wallis test (tubular injury, claudin-19, claudin-16). Significant differences were assumed at the level of P < 0.05. Statistical analysis was computed using SPSS (version 18.0) for Windows.

RESULTS

Our rat model showed the expected functional and morphological characteristics of CsA nephrotoxicity as shown below.

Functional parameters. No rats died during the experiment. At the start of the study, there was no difference among the four groups in body weight or in laboratory values, i.e., serum creatinine, creatinine clearance, serum Mg2+, FE Mg2+, serum Na+, and FE Na+ (data not shown). At the time of death, the mean body weight in both vehicle-treated groups was significantly higher than in the CsA-treated groups (P < 0.05) (Table 2). Serum creatinine values were significantly higher in the CsA-treated groups after 4 wk of treatment (P < 0.001). The serum Mg2+ was significantly higher in the CsA-treated group with EGF vs. the controls (P = 0.016). The FE Mg2+ was significantly higher in the CsA-treated groups vs. the controls treated with EGF (P < 0.001). The serum Na+ was comparable in the four groups, but the FE Na+ was significantly increased in the CsA-treated groups vs. the control groups (P < 0.01). The potassium levels were slightly increased in the CsA-treated groups, although no significance was reached (P = 0.136). The FE K+ was comparable in the four groups (P = 0.297). The mean blood CsA level was 3,360.5 ± 198.1 ng/ml in the CsA-treated group and 3,296.5 ± 240.7 in the CsA + EGF-treated group.

Serum aldosterone levels. Serum aldosterone levels were significantly lower in the CsA-treated rats (33.01 ± 8.06 ng/ml) vs. controls (45.86 ± 10.41 ng/ml, P = 0.003).

CSA-induced morphological lesions. Three independent researchers agreed on the results. In the experimental groups, CsA caused mild tubular injury in the cortical areas. The mean score of tubular injury was 1.75 ± 0.45 in CsA-treated rats (P = 0.01 vs. controls) and 2.00 ± 0.43 in CsA-treated + EGF rats (P < 0.0001 vs. controls and P = 0.005 vs. control + EGF). In control rats, the mean score was 1.08 ± 0.29 and in

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

<table>
<thead>
<tr>
<th>Genes</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TRPM6</strong></td>
<td>Forward: 5′-AACCATATCTGACAGGGTATCAGC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-CTCTTCAATGAAAGATGTCG-3′</td>
</tr>
<tr>
<td></td>
<td>Probe: 5′-GGGGCATCACACTGTGACGTTG-3′</td>
</tr>
<tr>
<td><strong>TRPM7</strong></td>
<td>Forward: 5′-ATTTGGCCGTATCGGACGC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-GACTGGAATTTTGTGGAACG-3′</td>
</tr>
<tr>
<td></td>
<td>Probe: 5′-ATCTCGTGGATGTTGATGGGTG-3′</td>
</tr>
<tr>
<td><strong>EGFR</strong></td>
<td>Forward: 5′-AGGGAAGGACACATGATGACG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-CTGGGCGGTAATGGATAC-3′</td>
</tr>
<tr>
<td></td>
<td>Probe: 5′-AAACCAATCTCTCCAAAAAGGC-3′</td>
</tr>
<tr>
<td><strong>EGF</strong></td>
<td>Forward: 5′-CATGCGCTGAAGTACGCTAC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-ATCAGCAGCTGTCACGATG-3′</td>
</tr>
<tr>
<td></td>
<td>Probe: 5′-TCTGCGCTGAAGGCTATAC-3′</td>
</tr>
<tr>
<td><strong>NCC</strong></td>
<td>Forward: 5′-ACACACCTACATGTGGATG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-GGCAGCAGTACAGTGGCG-3′</td>
</tr>
<tr>
<td></td>
<td>Probe: 5′-GGGCCATACCTGGCTGACTGCA-3′</td>
</tr>
<tr>
<td><strong>Claudin-16</strong></td>
<td>Forward: 5′-ATCTTTTCTGATACATGTCG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-GACAGTAATAGACTGTCGCC-3′</td>
</tr>
<tr>
<td></td>
<td>Forward: 5′-TGCTGAAGGACACCCATCTG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-TGCTGCTTGTGATGAAAGTC-3′</td>
</tr>
<tr>
<td><strong>Preprorenin</strong></td>
<td>Forward: 5′-GGTGCCCTCTTACCAAGATGT-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-GACAGTGACCTGTCGTAATG-3′</td>
</tr>
<tr>
<td></td>
<td>Forward: 5′-TGCTGCTTGTGATGAAAGTC-3′</td>
</tr>
<tr>
<td><strong>TGF-β</strong></td>
<td>Forward: 5′-TGGCTCTCACAGGATGGATGACC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-CGTCTGATGAAAATGTCACAC-3′</td>
</tr>
<tr>
<td></td>
<td>Forward: 5′-CAGAGCTGTATGGAGGATCCC-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-CCCGAGGATGGAATCAGTGT-3′</td>
</tr>
<tr>
<td><strong>PAI-1</strong></td>
<td>Forward: 5′-ACAAGATGTTGAGATTGTTG-3′</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5′-AGAAGGCAGCCTGGTGAAC-3′</td>
</tr>
<tr>
<td></td>
<td>Probe: 5′-GGTTGATGTTGATGAAAGTC-3′</td>
</tr>
</tbody>
</table>
control+EGF rats the mean score was 1.25 ± 0.45. CsA caused JGA hyperplasia (19 ± 7%). There was no effect of EGF administration on the prevalence of JGA hyperplasia in CsA-treated rats (20 ± 9%). In control rats, no JGA hyperplasia was observed. The four groups showed no difference in interstitial fibrosis: the mean score of interstitial fibrosis was 10.10 ± 1.38% in CsA-treated rats and 11.21 ± 1.80% in CsA+EGF rats. In control rats, the mean score was 10.59 ± 2.61%, and in control+EGF rats the mean score was 11.32 ± 1.78%.

**Phenotyping of inflammatory cells.** In the control rats and the control rats treated with EGF, very few ED1-positive cells were detected in the total cortex area (Fig. 1). There was a significant infiltration of ED1-positive cells in the cortex of the rats treated with CsA (P = 0.001). In the rats treated with CsA and EGF, a higher number of ED1-positive cells were detected than in control rats and rats treated with CsA alone, although this difference was not significant. There was no difference among the four groups regarding ED1-positive cells infiltrating the glomeruli (data not shown). The mean number of ED1-positive cells per glomerulus was 0.3 ± 0.49.

**Real-time RT-PCR.** The renal mRNA levels of TRPM6 (Fig. 2) were significantly downregulated in the CsA-treated rats [normalized ratio (NR) 1.25 ± 0.32, P = 0.029] and CsA+EGF-treated rats (NR 0.99 ± 0.13, P < 0.0001) vs. the controls (NR 1.48 ± 0.24). TRPM6 was significantly upregulated in the controls+EGF rats (NR 1.77 ± 0.25, P = 0.005).

TRPM7 renal mRNA (Fig. 2) was significantly downregulated in both CsA-treated groups (NR CsA-treated rats: 1.35 ± 0.40, P = 0.035 and NR CsA+EGF-treated rats: 0.99 ± 0.23, P < 0.0001) vs. controls (NR 1.62 ± 0.22).

The four groups showed no difference in renal mRNA expression of the EGFR (Fig. 2). The renal mRNA expression of EGF (Fig. 2) significantly decreased in the CsA-treated groups (P < 0.0001) vs. both control groups. NR was 1.74 ± 0.45 in the control group, 1.95 ± 0.28 in the controls+EGF group, 0.51 ± 0.39 in the CsA-treated group, and 0.49 ± 0.14 in the CsA+EGF-treated group.

Renal mRNA of the NCC (Fig. 4) was significantly downregulated in the CsA-treated groups vs. both control groups (P < 0.01). NR was 0.91 ± 0.24 in the control group, 0.91 ± 0.26 in the controls+EGF group, 0.35 ± 0.31 in the CsA-treated group, and 0.39 ± 0.51 in the CsA+EGF-treated group.

Renin mRNA expression (Fig. 4) was significantly upregulated in both CsA-treated groups (CsA: NR 1.14 ±

---

**Table 2. Urine and serum analyses and body weight at 4 wk**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Control+EGF</th>
<th>CsA</th>
<th>CsA+EGF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>339.25 ± 36.77&lt;sup&gt;d&lt;/sup&gt;</td>
<td>355.92 ± 16.78&lt;sup&gt;d&lt;/sup&gt;</td>
<td>299.25 ± 17.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>307.83 ± 20.22&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum creatinine, mg/dl</td>
<td>0.26 ± 0.07&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.26 ± 0.06&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.45 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.49 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Creatinine clearance, ml/min</td>
<td>2.09 ± 0.51&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.92 ± 0.47&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.95 ± 0.23&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.98 ± 0.40&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum Mg&lt;sup&gt;2+&lt;/sup&gt;, mg/dl</td>
<td>1.81 ± 0.33</td>
<td>1.79 ± 0.16&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.00 ± 0.29</td>
<td>2.09 ± 0.20&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Serum Na&lt;sup&gt;+&lt;/sup&gt;, mmol/l</td>
<td>148.33 ± 8.06</td>
<td>146.72 ± 3.86</td>
<td>146.23 ± 8.98</td>
<td>143.99 ± 4.07</td>
</tr>
<tr>
<td>Serum K&lt;sup&gt;+&lt;/sup&gt;, mmol/l</td>
<td>4.93 ± 0.48</td>
<td>4.66 ± 0.39</td>
<td>5.29 ± 0.96</td>
<td>5.13 ± 0.62</td>
</tr>
<tr>
<td>FE Mg&lt;sup&gt;2+&lt;/sup&gt;, %</td>
<td>8.90 ± 4.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.49 ± 2.34&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>12.02 ± 3.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.23 ± 4.23</td>
</tr>
<tr>
<td>FE Na&lt;sup&gt;+&lt;/sup&gt;, %</td>
<td>0.09 ± 0.06&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.07 ± 0.04&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.17 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.22 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FE K&lt;sup&gt;+&lt;/sup&gt;, %</td>
<td>6.07 ± 1.91</td>
<td>6.29 ± 1.99</td>
<td>7.58 ± 1.97</td>
<td>7.20 ± 2.41</td>
</tr>
</tbody>
</table>

Values are means ± SD and are presented for 4 groups: control animals receiving vehicle only, control+EGF (150 μg·kg<sup>−1</sup>·day<sup>−1</sup>), cyclosporine (CsA; 15 mg·kg<sup>−1</sup>·day<sup>−1</sup>), and CsA+EGF. Statistics were performed using 2-way ANOVA with a post hoc Student-Newman-Keuls test. FE, fractional excretion. *P < 0.05 vs. control. †P < 0.05 vs. control+EGF. ‡P < 0.05 vs. CsA. §P < 0.05 vs. CsA+EGF.

---

![Image](http://ajprenal.physiology.org/)
Renal mRNA levels of TGF-β were significantly increased in the CsA-treated rats (NR 1.61 ± 0.16, control rats + EGF (NR 1.10 ± 0.19), and CsA + EGF-treated rats (NR 1.12 ± 0.25). The mRNA levels of PAI-1 were the lowest in the control rats (NR 1.25 ± 0.73). They were increased in the controls + EGF (NR 1.65 ± 0.67) and the CsA-treated rats (NR 1.83 ± 1.25), although not significantly. The mRNA of PAI-1 was significantly increased in the CsA + EGF-treated rats (NR 2.20 ± 0.19) vs. the controls (P = 0.03), but not vs. both other groups.

**NCC immunohistochemical staining.** To assess whether the decreased mRNA levels corresponded with reduced protein expression, protein levels were semiquantified by immunohistochemistry. Table 3 shows that CsA significantly reduced the protein expression of the NCC. The NCC was significantly upregulated in the control rats vs. the three other groups.

1.82, CsA + EGF: NR 1.19 ± 2.15) vs. the control groups (controls: NR 0.50 ± 1.00, controls + EGF: NR 0.43 ± 0.83, P < 0.01).

Fig. 2. Effect of CsA on renal mRNA expression levels of Mg²⁺ transport proteins TRPM6 and TRPM7 (top) and the EGF receptor (EGFR) and EGF in the rat kidney (bottom). Values are means ± SD; n = 12/group. Statistics were performed using 2-way ANOVA with a post hoc Student-Newman-Keuls test.

In this study, we showed in vivo that CsA downregulates EGF, TRPM6 and TRPM7, and NCC, while both FE Mg²⁺ and the FE Na⁺ increased. These results suggest that CsA treatment results in diminished renal Mg²⁺ and Na⁺ reabsorption, respectively, via downregulation of the Mg²⁺ channels TRPM6 and TRPM7 and the NCC. hEGF administration seemed to have a positive effect on Mg²⁺ reabsorption in the control rats with an increase in TRPM6 renal mRNA and a decrease in FE Mg²⁺. This protective effect of hEGF administration was not maintained in CsA-treated rats.

The rat model of CsA nephrotoxicity was characterized by an increase in serum creatinine. FE Mg²⁺ increased after 4 wk of treatment, conforming to what had been established in other studies (1, 28). Our rat model showed the pathogenic characteristics of chronic CsA nephrotoxicity: the profibrotic factors PAI-1 and TGF-β were both upregulated as previously shown by our own research group and other investigators (10, 11, 48, 50). The histological changes included tubular injury and JGA hyperplasia (47). An additional upregulation of PAI-1 in the CsA group treated with EGF was found compared with the group treated with CsA alone. PAI-1 was also upregulated in the controls treated with EGF vs. the controls alone. We assume that this upregulation of PAI-1 was an effect of EGF administration. On the other hand, TGF-β was normalized in the CsA group treated with EGF compared with the group treated with CsA alone.

This study is the first to show in vivo downregulation of renal TRPM6 mRNA in CsA nephrotoxicity. In comparison, Nijenhuis et al. (33) found decreased renal mRNA levels of TRPM6 in tacrolimus-treated rats, another calcineurin inhibitor. We hypothesize that the mechanism leading to hypomagnesemia is similar in rats treated with tacrolimus and in rats treated with CsA and that CsA-induced hypomagnesemia is related to the decreased expression of the renal Mg²⁺ channel TRPM6. This hypothesis is supported by the results of Ikari et al. (21) showing that CsA decreased TRPM6 expression and inhibited Mg²⁺ influx in renal tubular epithelial NRK-52E cells.

**DISCUSSION**

In this study, we showed in vivo that CsA downregulates EGF, TRPM6 and TRPM7, and NCC, while both FE Mg²⁺ and the FE Na⁺ increased. These results suggest that CsA treatment results in diminished renal Mg²⁺ and Na⁺ reabsorption, respectively, via downregulation of the Mg²⁺ channels TRPM6 and TRPM7 and the NCC. hEGF administration seemed to have a positive effect on Mg²⁺ reabsorption in the control rats with an increase in TRPM6 renal mRNA and a decrease in FE Mg²⁺. This protective effect of hEGF administration was not maintained in CsA-treated rats.

The rat model of CsA nephrotoxicity was characterized by an increase in serum creatinine. FE Mg²⁺ increased after 4 wk of treatment, conforming to what had been established in other studies (1, 28). Our rat model showed the pathogenic characteristics of chronic CsA nephrotoxicity: the profibrotic factors PAI-1 and TGF-β were both upregulated as previously shown by our own research group and other investigators (10, 11, 48, 50). The histological changes included tubular injury and JGA hyperplasia (47). An additional upregulation of PAI-1 in the CsA group treated with EGF was found compared with the group treated with CsA alone. PAI-1 was also upregulated in the controls treated with EGF vs. the controls alone. We assume that this upregulation of PAI-1 was an effect of EGF administration. On the other hand, TGF-β was normalized in the CsA group treated with EGF compared with the group treated with CsA alone.

This study is the first to show in vivo downregulation of renal TRPM6 mRNA in CsA nephrotoxicity. In comparison, Nijenhuis et al. (33) found decreased renal mRNA levels of TRPM6 in tacrolimus-treated rats, another calcineurin inhibitor. We hypothesize that the mechanism leading to hypomagnesemia is similar in rats treated with tacrolimus and in rats treated with CsA and that CsA-induced hypomagnesemia is related to the decreased expression of the renal Mg²⁺ channel TRPM6. This hypothesis is supported by the results of Ikari et al. (21) showing that CsA decreased TRPM6 expression and inhibited Mg²⁺ influx in renal tubular epithelial NRK-52E cells.
TRPM6 is very homologous to TRPM6 (=50% homology) and is also responsible for cellular Mg$^{2+}$ homeostasis (6, 31). It was shown that TRPM6 specifically interacts with TRPM7 to form a functional ion channel complex at the cell surface of human embryonic kidney 293 cells (8, 25). In our experiment, TRPM7 was downregulated in both experimental groups, even more in the EGF-treated CsA group. In contrast, Ikari et al. (21) did not find a CsA-induced downregulation of TRPM7 in vitro. However, since TRPM7 plays an important role in Mg$^{2+}$ homeostasis and since it forms functional complexes with TRPM6 in kidney cells, we hypothesize that this downregulation of TRPM7 could be important in CsA-induced renal Mg$^{2+}$ loss. It is not clear why this downregulation of TRPM7 was not detected in vitro. However, it remains unclear whether TRPM6 and TRPM7 are expressed on the DCT in rat kidneys, similar to the situation in mice and humans (38, 49). In our hands, immunocytochemical stainings for both channels gave no results due to the unavailability of appropriate primary antibodies for the use in rats.

Simultaneously with the decrease in renal expression of TRPM6 and TRPM7, we found reduced expression of EGF in rats treated with CsA. This in vivo finding is in accordance with a mechanism which was previously revealed in vitro. EGF stimulates Mg$^{2+}$ reabsorption in the DCT via its receptor on the basolateral membrane and via activation of TRPM6 in the apical membrane in vitro (16, 41). On the contrary, there was no difference in the expression of EGFR mRNA. This can be explained by the fact that the EGFR is expressed not only in the DCT but also in the proximal tubule (14, 23). Since we have isolated total renal mRNA, we have also measured the EGFR in the proximal tubule. In this rat model, the effect of CsA seems to be predominantly directed against the DCT, with a downregulation of transporters which are only expressed in the DCT, namely, TRPM6 and the NCC, while mRNA expression of both claudin-16 and claudin-19 did not change, in contrast to what Chang et al. (7) found in cultured cortical TAL cells.

Until now, no relationship between TRPM7 and EGF has been described. We hypothesize that through the downregulation of EGF and TRPM6, TRPM7 is also affected in the DCT.

This study also addressed the CsA effect on NCC expression. Fe Na$^{+}$ was increased together with a downregulation of the NCC in rats treated with CsA. An increased Fe Na$^{+}$ after CsA treatment was found earlier in animal models without unraveling the underlying mechanisms (12, 26). Also, in humans CsA causes hyponatremia or an increased Fe Na$^{+}$ after liver or renal transplantation (18, 51). The downregulation of the NCC, the most important sodium transporter in the final adjustment of renal sodium excretion, could explain the increase in Fe Na$^{+}$ (15, 27). This downregulation can be explained by the inactivation of the RAAS, which plays a role in NCC activation. ANG II is necessary to translocate the NCC to the plasma membrane, while both ANG II and aldosterone independently phosphorylate and thereby activate the NCC. ANG II is necessary to translocate the NCC to the plasma membrane, while both ANG II and aldosterone independently phosphorylate and thereby activate the NCC (37, 44). In our experiments, renal renin mRNA expression levels increased while serum aldosterone decreased in CsA-treated rats. Simultaneous with these findings, serum potassium levels slightly increased, as did Fe Na$^{+}$ while NCC expression decreased. Our results can be explained by an inhibition of the

Table 3. Immunohistochemical staining of NCC

<table>
<thead>
<tr>
<th>Group</th>
<th>Controls (n = 6)</th>
<th>Controls + EGF (n = 5)</th>
<th>CsA (n = 5)</th>
<th>CsA + EGF (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>%Total cortex</td>
<td>2.83 ± 0.58$^{b,c,d}$</td>
<td>3.96 ± 0.67$^{a,c,d}$</td>
<td>2.09 ± 0.35$^{b}$</td>
<td>1.51 ± 0.63$^{b}$</td>
</tr>
<tr>
<td>Gray value of AEC</td>
<td>101.37 ± 4.75$^{b,c,d}$</td>
<td>109.58 ± 4.50$^{a}$</td>
<td>112.14 ± 5.39$^{a}$</td>
<td>112.96 ± 9.20$^{a}$</td>
</tr>
</tbody>
</table>

Values are means ± SD and are presented for 4 groups: control animals receiving vehicle only, control + EGF, CsA, and CsA + EGF. The gray value of 3-aminophenylcarbazole (AEC) was measured, and a number from 0 to 155 was given to each slice, where 0 was black (chromogen) and 155 was white (background). Statistics were performed using 2-way ANOVA with a post hoc Student-Newman-Keuls test. $^{a}$P < 0.05 vs. control. $^{b}$P < 0.05 vs. control + EGF. $^{c}$P < 0.05 vs. CsA. $^{d}$P < 0.05 vs. CsA + EGF.
conversion of inactive prorenin to active renin, resulting in an inactivation of the RAAS with low aldosterone secretion, which is comparable to data in humans (2, 30). However, our study is the first to report a decrease in RAAS activity in rats treated with CsA whereas several groups have demonstrated that CsA is an activator of the RAAS in animals (24, 36); one study reports that CsA has no influence on renal renin mRNA expression (43).

This study also investigated the effect of EGF on Mg\textsuperscript{2+} and Na\textsuperscript{+} homeostasis and DCT transporters after CsA treatment. In the control rats receiving hEGF, TRPM6 mRNA was significantly upregulated and FE Mg\textsuperscript{2+} was decreased. These findings suggest that administration of hEGF has a positive effect on Mg\textsuperscript{2+} homeostasis in control rats. In contrast, this positive effect was not maintained in CsA-treated rats. As expected, this beneficial phenomenon was not found for FE Na\textsuperscript{+} nor for the NCC and therefore was specific for Mg\textsuperscript{2+} homeostasis. Claudin-16 significantly upregulated in CsA+EGF-treated rats vs. controls + EGF. This unexpected data should be confirmed in other experiments to determine its significance.

What factors could explain the differential effect of hEGF administration in controls and CsA-treated rats? First, a dose-related effect of EGF could be important. Possibly, the dose of EGF was too low in the CsA-treated rats to compensate for the related effect of EGF. Possibly, the dose of CsA inhibits the signaling pathway activated by EGF. EGF administration had no effect on the expression of the paracellular proteins claudin-16 and claudin-19. This study indicates that CsA treatment undermines EGF expression and the RAAS, which results in decreased ion channel expression leading to hypomagnesemia and hypernatremia.

ACKNOWLEDGMENTS

We thank Angelika Jürgens and André Van Daele for scoring the renal slices and Petra Aerts and Dirk Borgenjon for technical assistance. A preliminary report of this work was presented at the 2009 World Congress of Nephrology (Milan, Italy) and at the ASN 2010 Renal Week (Denver, CO) as an abstract (poster presentation).

GRANTS

This study was supported by a research grant to K. J. Ledeganck from the University of Antwerp.

DISCLOSURES

G. Verpooten received grants and/or consultancy fees from Novartis Belgium and Roche Belgium.

REFERENCES


