Magnesium loss in cyclosporine-treated patients is related to renal epidermal growth factor downregulation

Kristien J. Ledeganck¹, Benedicte Y. De Winter¹, Annelies Van den Driessche², Angelika Jürgens¹, Jean-Louis Bosmans¹,², Marie M. Couttenye² and Gert A. Verpooten¹,²

¹Laboratory of Experimental Medicine and Pediatrics, University of Antwerp, Antwerp, Belgium and ²Department of Nephrology, Antwerp University Hospital, Edegem, Belgium

Correspondence and offprint requests to: Kristien Ledeganck; E-mail: kristien.ledeganck@uantwerpen.be

ORIGINAL ARTICLE

ABSTRACT

Background. Cyclosporine (CsA) treatment is associated with hypomagnesaemia due to a renal Mg²⁺ leak. In animal studies a role for the Mg²⁺ channel TRPM6 localized in the distal convoluted tubule and stimulated by epidermal growth factor (EGF) is suggested. We hypothesize that CsA-induced hypomagnesaemia is due to a renal magnesium leak, also in patients, resulting from a downregulation of the renal EGF production, thereby inhibiting the activation of TRPM6.

Methods. Renal transplant patients treated with CsA (n = 55) and 35 chronic kidney disease (CKD) patients were included. At three time points, with an interval of at least 1 month, blood and urine samples were taken to determine creatinine, Mg²⁺, sodium and EGF.

Results. Serum Mg²⁺ was significantly lower in the CsA group versus the CKD group with significantly more CsA-treated patients developing hypomagnesaemia. Although the fractional excretion (FE) Mg²⁺ did not differ significantly between the two groups, subanalysis of the patients with hypomagnesaemia showed a significantly higher FE Mg²⁺ in CsA-treated patients compared with CKD patients (P = 0.05). The urinary EGF excretion was significantly decreased in the CsA group and was a predictor of the FE Mg²⁺ in the two groups. Serum sodium was significantly decreased in the CsA group simultaneously with an increased FE Na⁺.

Conclusions. In CsA-treated patients, the association of a low urinary EGF excretion and a decreased renal Mg²⁺ reabsorption is in accordance with in vitro and animal studies. In the whole study population, log urinary EGF excretion is an independent predictor of the FE Mg²⁺, supporting the role of EGF in magnesium reabsorption.

Keywords: cyclosporine, epidermal growth factor, fractional excretion, magnesium, sodium

INTRODUCTION

Cyclosporine (CsA) is a calcineurin inhibitor (CNI), inhibiting T-cell activation by blocking the transcription of cytokine genes [1]. Besides acute and chronic nephrotoxicity, CsA also induces hypertension and ion homeostasis disturbances such as hypomagnesaemia and Mg²⁺ wasting, hyperkalaemia, hyponatraemia, hyperchloaemic metabolic acidosis and hyperuricaemia [2–5].

It has been reported that ~60% of patients develop hypomagnesaemia after treatment with CsA, however, presenting as a wide range of incidences from 1.5 to 100%. CsA-induced hypomagnesaemia is often asymptomatic: of the 200 published cases, only 25 patients were symptomatic [6]. Severe magnesium depletion after CsA treatment is rare but has been reported and may include clinical manifestations such as confusion, muscle weakness, tremor, dysphagia, tetany and general convulsions [6, 7].

In physiological conditions, magnesium depletion leads to a decrease in the renal magnesium excretion. Renal magnesium wasting is defined as a fractional excretion of magnesium (FE Mg²⁺) ≥2% in a subject with normal renal function [7]. Hypomagnesaemia caused by CNI is related to a renal Mg²⁺ loss accompanied by an increased FE Mg²⁺ [8–10]. Recently, two ion channels that play an important role in the Mg²⁺...
homeostasis were identified, TRPM6 and TRPM7. In the kidney, TRPM6 is expressed in the distal convoluted tubule (DCT), known as the main site of active transcellular Mg2+ reabsorption along the nephron. TRPM7 is ubiquitously expressed and implicated in cellular Mg2+ homeostasis [11]. Recently, it was shown that epidermal growth factor (EGF) stimulates Mg2+ reabsorption in the DCT. After EGF binds to the EGF receptor (EGFR) in the basolateral membrane, the EGFR pathway is stimulated, thereby increasing TRPM6 activity in the apical membrane [12, 13]. Ikari et al. [14] found that CsA decreased the TRPM6 expression in vitro in a cell culture of rat kidney epithelial cells (NRK-52E). Our laboratory confirmed these in vitro findings in an in vivo rat model of CsA nephrotoxicity where TRPM6 mRNA was downregulated simultaneously with EGF and as a consequence, the renal magnesium excretion increased [15].

In humans and in rats, CsA also causes hyponatraemia due to renal Na+ loss [5, 15, 16]. In a CsA rat model, we showed that a decreased sodium chloride channel (NCC) expression and an inactivated renin-angiotensin-aldosterone system (RAAS) could explain the increased FE Na+ [15].

The aim of this study was to verify whether we could demonstrate in humans a similar mechanism of CsA-induced downregulation of the EGF/TRPM6 pathway as described in rats. The urinary EGF concentration was measured and FE Mg2+ was used as a proxy of TRPM6 activity.

**SUBJECTS AND METHODS**

Ninety patients were recruited in this study. Patients were included between November 2009 and July 2011 at the Antwerp University Hospital. Fifty-five renal transplant patients treated with CsA (called the CsA group) were recruited into the study. The control group consisted of patients with a chronic kidney disease (called the CKD group, n = 35). Since decreasing kidney function is associated with a decrease in magnesium excretion [17] and urinary EGF excretion [18], the control group was matched for eGFR. Patients treated with diuretics, aminoglycosides or cisplatin and patients with an eGFR below 20 mL/min/1.73 m², diabetes mellitus or an active cystitis were excluded from the study. The aetiology of the renal disease was taken from the patient records and classified according to the Kidney Disease Outcome Quality Initiative (KDOQI) guidelines.

At three time points with an interval of at least 1 month, blood and urine samples were collected from each patient to determine creatinine, magnesium, EGF (urine) and sodium.

The study was conducted in accordance with the Declaration of Helsinki and the principles of Good Clinical Practice. The study protocol was approved by the Ethics Committee of the Antwerp University Hospital (file number 9/44/231). All patients gave a written informed consent.

**Determination of creatinine, magnesium, sodium and CsA levels**

Serum and urine creatinine and magnesium were analysed with the Vitros 5.1 FS analyser, using a Vitros creatinine and magnesium slide, respectively. FE Mg2+ was calculated using the following equation: $\text{FE}_{\text{Mg}} = 100 \times \frac{(U_{\text{Mg}} \times S_{\text{Cr}})}{(0.7 \times S_{\text{Mg}}) \times U_{\text{Cr}}}$, with $U_{\text{Mg}}$ urinary excretion of Mg2+ (mg/dL), $S_{\text{Mg}}$ serum magnesium (mg/dL), $S_{\text{Cr}}$ serum creatinine (mg/dL), $U_{\text{Cr}}$ urinary excretion of creatinine (mg/dL). The serum Mg2+ concentration was multiplied by 0.7, since only 70% of the serum Mg2+ is freely filtered by the glomerulus, the remaining part is protein-bound [7].

Serum and urine sodium levels were measured using an indirect potentiometric method with a dimension Vista 1500 System (Siemens Healthcare Diagnostics, Deerfield, IL, USA). The FE Na+ was calculated using the following equation: $\text{FENa} = 100 \times \frac{(U_{\text{Na}} \times S_{\text{Cr}})}{(S_{\text{Na}} \times U_{\text{Cr}})}$ with $U_{\text{Na}}$ urinary excretion of Na+ (mmol/L), $S_{\text{Cr}}$ serum creatinine (mg/dL), $S_{\text{Na}}$ serum Na+ (mmol/L) and $U_{\text{Cr}}$ urinary excretion of creatinine (mg/dL).

CsA levels were determined in whole blood using the CsA FLEX® reagent cartridge (Siemens Healthcare Diagnostics).

**Determination of urinary EGF**

Urinary EGF was measured using an EGF human Elisa kit® (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s guidelines. The detection limit of this assay was 3.9 pg/mL.

A preliminary experiment (n = 10) was performed to test the intra- and inter-variability of the EGF human Elisa kit®, showing a mean intra-assay coefficient of variance of 9.82% and an inter-assay coefficient of variation of 9.81%.

**Statistical analysis**

All data were analysed using SAS (version 9.2). Statistical significance was predetermined as a P-value <0.05. Several blood and urine samples were collected from each patient (one to three samples per patient); therefore blood and urine samples could not be considered independent but instead ‘nested’ per patient. As such, we assumed that the $n_j$ blood samples recruited from the jth patient might share some proportion of variance in characteristics (e.g. serum and urine values) attributable to the patient. We applied two-level hierarchical linear modelling [19] of serum creatinine, eGFR, serum Mg2+, FE Mg2+, serum Na+, FE Na+ and urinary EGF concentration using mixed-effects linear regression modelling with restricted maximum likelihood estimation, thereby analysing the differences between the CKD group and the CsA group. A post hoc analysis was performed to investigate differences in FE Mg2+ in patients who developed hypomagnesaemia during at least one episode in both groups. The same statistical model was used to determine the relation between the FE Mg2+ and urinary EGF concentration, patient age, sex, eGFR, serum Mg2+ and time after transplantation. Statistical analyses were performed on the decimal log transformed urinary EGF concentration to normalize the experimental data. The influence of the urinary EGF concentration (decimal logarithm) on FE Mg2+ was expressed by the formula: $y = a_0 + a_1x_1$, where $y = \text{FE Mg}^{2+}, a_0 = \text{intercept}, a_1 = \text{parameter estimate (slope)}$ and $x_1 = \text{urinary EGF concentration (log)}$.

A power of 99.9% was calculated based on a decline of 8% of the urinary ng EGF/mg creatinine in the CsA group versus the CKD group with an estimated standard deviation of 20%, two groups of 35 patients and a P-value of 0.05 [20].
RESULTS

Population

Group characteristics are described in Table 1. The mean number of blood and urine samples per patient was 2.60. Sixty-six patients (72.5%) gave three blood and urine samples. Twenty-four patients were lost from follow-up before the end of the observation period; 12 controls and 12 patients treated with CsA. Reasons for not completing the study were a deterioration of the renal function with an estimated glomerular filtration rate (eGFR) < 20 mL/min/1.73 m² (n = 1), a return to the referring hospital (n = 4), a urinary tract infection (n = 1), the patient’s decision to quit the study (n = 3) and no follow-up consultation planned (n = 15). The frequencies of the aetiology of the renal disease are presented in Table 2. No significant differences were found between the two groups (P = 0.87).

Kidney function, magnesium, sodium, EGF and CsA levels

The results are provided in Table 3. In this study, the ‘control’ group existed of patients with a CKD, presenting with a comparable level of kidney dysfunction. In Table 3 the reference values of the measured parameters are included, clearly demonstrating abnormal values for serum creatinine, eGFR and FE Mg2+ in both groups (CKD and CsA). On the contrary, urinary EGF, serum Mg2+, serum Na+ and FE Na+ can be considered normal in the CKD group while interestingly CsA treatment showed an additional effect on top of the effect induced by chronic kidney dysfunction: while both groups showed a statistical similar impairment of creatinine and eGFR, serum Mg2+ and serum Na+ were significantly more decreased in the CsA group and FE Na+ was significantly more increased in the CsA group. On the other hand, we found no significant difference in the already increased FE Mg2+ between the CsA and the CKD group (P = 0.44). A post hoc analysis revealed that in the CsA group, 64% of the patients developed hypomagnesaemia, compared with 32% of the patients in the CKD group (P = 0.004). In addition, FE Mg2+ was lower in the patients developing hypomagnesaemia in the CKD group (4.77 ± 0.59%) compared with patients developing hypomagnesaemia in the CsA group (6.33 ± 0.56%, P = 0.05).

In the CsA group, the CsA trough level was significantly higher in the patients developing hypomagnesaemia (137 ± 6.1 ng/mL) compared with the patients not developing hypomagnesaemia (115.4 ± 6.8 ng/mL, P = 0.019).

Next we analysed the correlation between FE Mg2+ and the urinary EGF concentration: FE Mg2+ was significantly correlated with the log urinary EGF concentration (P < 0.0001). As shown in Figure 1, a low urinary EGF concentration is associated with a high FE Mg2+. The coefficient of determination ($r^2$) is 0.28, which means that 28% of the variance of FE Mg2+ can be explained by the urinary EGF level.

Predictors of FE Mg2+

Transplantation-specific factors (e.g. time after transplantation) could not be investigated in the whole study population, as non-transplant patients were included in the control group. Therefore, two statistical models were tested. The first statistical model included the entire study population (Table 4, upper panel); the second statistical model included only the CsA-treated patients (Table 4, lower panel). In both statistical models, the urinary EGF concentration (log) was an independent predictor of FE Mg2+ ($P < 0.0001$). We determined in the entire study population that FE Mg2+ can be calculated using the following formula:

\[
FE \text{Mg}^{2+} = 8.0980 - 3.6406 \times \log \text{Urinary EGF}
\]

We also analysed the effect of eGFR, serum Mg2+ value, age, sex, urine Na+ and time after transplantation as covariates; however, they were not related to the FE Mg2+.

Sodium

The serum Na+ was significantly decreased in the CsA group versus the CKD group (P = 0.0003) (Table 3). Moreover, there was a significant increase in the FE Na+ in the CsA group versus the CKD group (P = 0.01). FE Na+ is significantly correlated with the log urinary EGF concentration (P < 0.0001).

DISCUSSION

In this clinical study we found an inverse relation between the urinary EGF concentration and FE Mg2+ in both CsA and CKD patients. We confirmed a lower urinary EGF
concentration in CsA-treated patients compared with CKD patients. The serum magnesium was significantly lower in the CsA-treated transplant patients and significantly more CsA-treated patients developed hypomagnesaemia. In the patients developing hypomagnesaemia, the FE Mg$^{2+}$ was significantly higher in CsA-treated patients compared with CKD patients.

EGF is produced in the TAL, early DCT and collecting duct. Based on in vitro experiments, it has been previously suggested that urinary EGF originates from the ultrafiltrate [21]. However, in vivo, it is shown that in rats and in humans the urinary EGF is mainly produced in the kidney itself [22–24]. Therefore, in the present study, the urinary EGF excretion was used as a proxy of the renal EGF production. EGF stimulates the Mg$^{2+}$ reabsorption in the DCT in vitro: after binding the EGFR in the basolateral membrane, a specific part of the EGF pathway that increases TRPM6 activity in the apical membrane is stimulated [12, 13].

The present study showed decreased serum Mg$^{2+}$ concentrations and a decreased renal EGF production in renal transplant patients treated with CsA. In addition, the log urinary EGF correlated well with the FE Mg$^{2+}$. It has been described earlier that CsA downregulates the urinary EGF excretion in humans; however, the effect on the magnesium homeostasis was not studied [20]. In our patients, FE Mg$^{2+}$ did not significantly differ between the CsA group and the CKD group, whereas it was significantly increased compared with normal

Table 3. Urine and serum analyses

<table>
<thead>
<tr>
<th></th>
<th>CKD group ($n = 35$)</th>
<th>CsA group ($n = 55$)</th>
<th>P-value</th>
<th>Reference values (reference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum creatinine (mg/dL)</td>
<td>1.51 ± 0.06</td>
<td>1.45 ± 0.05</td>
<td>0.47</td>
<td>0.6–1.2 [34]</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m²)</td>
<td>52.4 ± 2.2</td>
<td>50.9 ± 1.6</td>
<td>0.57</td>
<td>&gt;90 [35]</td>
</tr>
<tr>
<td>Serum Mg$^{2+}$ (mg/dL)</td>
<td>1.96 ± 0.03</td>
<td>1.85 ± 0.02</td>
<td>0.007</td>
<td>1.8–3.0 [36]</td>
</tr>
<tr>
<td>FE Mg$^{2+}$ (%)</td>
<td>5.71 ± 0.41</td>
<td>6.10 ± 0.30</td>
<td>0.44</td>
<td>&lt;2% [7]</td>
</tr>
<tr>
<td>Urinary EGF (ng/mL)</td>
<td>7.36 (3.49–19.45)</td>
<td>3.65 (1.56–6.83)</td>
<td>0.004</td>
<td>11.43 ± 3.61 [37]</td>
</tr>
<tr>
<td>Serum Na$^{+}$ (meq/L)</td>
<td>139.0 ± 0.3</td>
<td>138.0 ± 0.2</td>
<td>0.0003</td>
<td>136–145 [36]</td>
</tr>
<tr>
<td>FE Na$^{+}$ (%)</td>
<td>0.8 ± 0.1$^b$</td>
<td>1.1 ± 0.09</td>
<td>0.01</td>
<td>&lt;1% [38, 39]</td>
</tr>
</tbody>
</table>

Data are presented in two groups: CKD group and the CsA group. The reference lab values of healthy subjects are displayed in the right column. The reference values are displayed as a range, except for the urinary EGF concentration, which is displayed as a mean ± SD, adapted from a research paper of Messing et al. [37], since no standard reference value is available. Normally distributed data are presented as means ± SD. Abnormally distributed data are presented as median (25th–75th percentile). Statistics were performed using mixed-effects linear regression modelling with restricted maximum likelihood estimation.

FIGURE 1: Correlation between FE Mg$^{2+}$ and urinary EGF concentration (log). Data are presented as mean per patient. FE, fractional excretion. EGF, epidermal growth factor.
reference values in both groups (FE Mg^{2+} <2%) [7, 17] as expected in relation to the degree of renal insufficiency [25, 26]. A post hoc analysis revealed that significantly more CsA-treated patients developed hypomagnesaemia. In addition, in patients developing hypomagnesaemia, the FE Mg^{2+} was higher in the CsA group compared with the CKD group. Normally one would expect that a decrease in serum Mg^{2+} would result in an increased renal Mg^{2+} reabsorption and a decreased FE of Mg^{2+} [7]. In CKD patients, a compensatory increased renal magnesium reabsorption was observed in an attempt to maintain the serum magnesium level within the normal range, while in the CsA-treated patients the kidney seems not to be able to raise the magnesium reabsorption and magnesium is lost in the urine. We hypothesize that the more pronounced decrease in EGF in the CsA group is directly related to FE Mg^{2+}. Also other studies reported hypo-Mg^{2+} due to renal Mg^{2+} loss after CsA treatment in patients [6, 9, 27]. Previously, we showed that in an CsA rat model, FE Mg^{2+} was increased simultaneously with a decrease in EGF mRNA expression and that the renal Mg^{2+} loss was due to a downregulation of the magnesium channel TRPM6 [15]. We hypothesize that the mechanism revealed in rats is also applicable in patients treated with CsA: CsA decreased the renal EGF production, thereby inhibiting the activation of TRPM6, resulting in a renal Mg^{2+} loss and hypoMg^{2+}. Moreover, a statistical model could indeed demonstrate that the urinary EGF excretion is independently related to FE Mg^{2+}, while age, sex, kidney function, serum magnesium, urine Na^+ and time after transplantation (in the CsA group) were not.

Since an increased FE Na^+ has been described in patients [5, 16] and in rats [15] after CsA treatment, we also examined sodium. In this study, CsA decreased the serum sodium concentration simultaneously with an increase in FE Na^+. In rats, we showed that CsA inactivates the RAAS thereby decreasing NCC expression [15]. However, the mechanism leading to a CsA-induced hyponatraemia and renal sodium loss in humans is still unknown. A low sodium reabsorption can be due to a CsA-induced inactivation of RAAS [28, 29]. RAAS plays a role in NCC and ENaC activation, thereby stimulating the sodium reabsorption [30–32]. In addition, also EGF influences the epithelial sodium channel (ENaC) activity [33]. In the present study, a low renal EGF production correlated with an increased urinary sodium excretion, suggesting that EGF might indeed play a role in renal sodium reabsorption. We hypothesize that, in addition to other mechanisms, a decreased renal EGF production contributes to renal sodium loss in CsA-treated transplant patients. The reduced renal EGF production leads to a decreased ENaC activation resulting in an increased urinary sodium loss in patients treated with CsA. However, the differences in serum sodium and FE Na^+ found between the two study groups are rather small; its clinical relevance needs to be confirmed.

In conclusion, in patients treated with CsA, the association between a decreased renal EGF production and a decreased renal Mg^{2+} reabsorption is in accordance with in vitro cell cultures and in vivo rodent studies. In the whole study population, renal EGF production is related to FE Mg^{2+}, thereby supporting a role for EGF in the regulation of Mg^{2+} reabsorption in humans.

<table>
<thead>
<tr>
<th>Table 4. Log EGF as a predictor of FE Mg^{2+}</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) In the whole study population</td>
</tr>
<tr>
<td>Log urinary EGF (ng/mL)</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m^2)</td>
</tr>
<tr>
<td>Gender (M = 1/F = 0)</td>
</tr>
<tr>
<td>Serum Mg^{2+} (mg/dL)</td>
</tr>
<tr>
<td>Age (Y)</td>
</tr>
<tr>
<td>(B) In the CsA-treated group</td>
</tr>
<tr>
<td>Log urinary EGF (ng/mL)</td>
</tr>
<tr>
<td>Time after Tx (Y)</td>
</tr>
<tr>
<td>Age (Y)</td>
</tr>
<tr>
<td>Serum Mg^{2+} (mg/dL)</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73 m^2)</td>
</tr>
<tr>
<td>Gender (M = 1/F = 0)</td>
</tr>
</tbody>
</table>

(A) Log urinary EGF was tested as a predictive factor of the FE Mg^{2+} in a statistical model also including other possible predictive factors in the entire study population. (B) The same statistical model was tested only in the CsA-treated group. As such, time after transplantation could be included in the model. Statistics were performed using mixed-effects linear regression modelling with restricted maximum likelihood estimation. All variables were tested in the same model.

EGF, epidermal growth factor; FE, fractional excretion; eGFR, estimated glomerular filtration rate; Tx, transplantation; M, male; F, female; Y, year; CI, confidence interval.

**ACKNOWLEDGEMENTS**

We thank Petra Aerts, Marleen Vinckx, Peter Coppens, Goedele Deplancke, Kim Meskens and Veerle Van Moer for the technical assistance. A preliminary report of this work was presented at the ASN renal week, Denver, 2010 as an abstract and at the World Congress of Nephrology, Vancouver, 2011 (poster presentation).

**CONFLICT OF INTEREST STATEMENT**

None declared.
REFERENCES


Received for publication: 15.6.2013; Accepted in revised form: 13.11.2013