Cyclosporin A tubular effects contribute to nephrotoxicity: role for Ca\(^{2+}\) and Mg\(^{2+}\) ions

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Abstract

Background. Cyclosporin A (CsA) nephrotoxicity has been attributed primarily to renal haemodynamic alterations caused by afferent arteriolar vasoconstriction. However, CsA nephropathy is also characterized by CsA-induced pre-glomerular disturbances and interstitial injury that may occur independently of haemodynamic changes. Given the high lipophilic activity of CsA, we hypothesized that direct tubular injury is likely to contribute to nephrotoxicity.

Methods. To investigate tubular toxicity of CsA, increasing concentrations of CsA (1, 2.5, 10, 25, 50, and 100 \(\mu\)g/ml) and its vehicle (cremophor) were added to isolated rat proximal tubules (PT). Cell injury was assessed by lactate dehydrogenase (LDH) release. The role of Ca\(^{2+}\) ions in tubular toxicity and the effect of calcium channel blockers on CsA toxicity were evaluated by measuring intracellular calcium using the fluorescent dye Fura-2 AM. The role of Mg\(^{2+}\) ions was assessed using high extracellular Mg\(^{2+}\) medium (2 mM).

Results. Whereas cremophor alone was not toxic to PT, CsA caused PT injury but only at the highest concentration (100 \(\mu\)g/ml). After 90 min incubation, LDH was 22.5% in control PT and 41.9% in PT treated with 100 \(\mu\)g/ml CsA (\(P < 0.001, n = 11\)). There was a transient increase in intracellular calcium ([Ca\(^{2+}\)]\(_i\)) after CsA administration. A low calcium medium (100 nM) prevented CsA injury to renal tubules. However, verapamil, but not nifedipine, enhanced cell damage. Only nifedipine completely prevented [Ca\(^{2+}\)]\(_i\) increases following CsA. Finally, a high Mg\(^{2+}\) medium attenuated CsA-induced tubular injury. Verapamil, but not nifedipine, enhanced CsA tubular toxicity. Therefore, CsA-induced tubular injury may contribute to CsA nephrotoxicity independently of haemodynamic disturbances.

Keywords: calcium channel blockers; cyclosporin A; intracellular calcium; magnesium; rat proximal tubules; toxicity

Introduction

The immunosuppressant cyclosporin A (CsA) has provided significant improvements in the clinical outcome of solid organ and bone marrow transplantations and has become a new and efficient therapeutic regimen for patients with immune-mediated diseases. However, the major complication of CsA in clinical use is nephrotoxicity. The functional nephrotoxicity of CsA includes renal haemodynamic abnormalities, and especially afferent arteriolar vasoconstriction. Several factors, such as vasoactive arachidonic acid metabolites, the renin–angiotensin system, endothelin, sympathetic hyperactivity, nitric oxide and lipid peroxidation products, may be involved in the arteriolar dysfunction [1,2]. Long-term CsA may lead to sustained vasoconstriction and chronic nephropathy, characterized by renal ischaemic alterations with local inflammatory processes and fibrosis. However, CsA may also cause interstitial fibrosis that is independent of haemodynamic alterations [3].

In addition to haemodynamic effects, CsA-induced tubular injury may also contribute to nephrotoxicity [4]. CsA may cause direct tubular injury or vascular injury and ischaemia. For example, CsA produced early sublethal injury in proximal tubule (PT) S3 segments, resulting in endoplasmic reticulum oedema [5] and increased excretion of urinary enzymes, such as
N-acetyl-β-d-glucosaminidase, a lysosomal enzyme [6]. Tubular lesions may also induce local inflammatory reactions which contribute to the development of chronic CsA nephropathy.

CsA may cause cellular injury by altering the trafficking of intracellular ions. In support of this, CsA promotes several intracellular disturbances that especially affect the calcineurin–calmodulin pathway [7]. In addition, CsA increases intracellular calcium which could enhance CsA-induced vasoconstriction or cause cell injury [8,9]. Serum magnesium is also affected by CsA administration. Clinical and experimental studies have demonstrated that CsA induces hypomagnesaemia, although the effect of this on CsA nephrotoxicity has not yet been established [10].

Given this background, the aim of the present study was to evaluate direct effects of CsA on isolated PT, occurring independently of haemodynamic alterations, which may contribute to CsA nephrotoxicity. We additionally investigated the role of Ca\(^{2+}\) and Mg\(^{2+}\) ions in mediating toxic effects of CsA on renal PT.

### Materials and methods

#### Isolation of proximal tubules

Isolated proximal tubules were harvested from male Wistar rats weighing 150–250 g [11]. Briefly, rats were anaesthetized with sodium thiopental (40 mg/kg i.p.) and kidneys were flushed with 60 ml of an oxygenated solution (solution 1), pH 7.2, 4°C, containing 112 mM NaCl, 25 mM NaHCO\(_3\), 5 mM KCl, 2 mM NaH\(_2\)PO\(_4\), 1.6 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 2.5 mM HEPES, 10 mM mannitol, 1 mM glutamine, 1 mM butyric acid, 1 mM lactic acid and 4000 IU heparin. Subsequently, perfusion was continued with solution 1 (without heparin) containing 15 mg of collagenase type V (Sigma Chemical Co., St Louis, MO) and 15 mg of hyaluronidase type III (Sigma). After perfusion, kidneys were decapsulated, digested tissue. After digestion was completed, tissues were filtered through a tea strainer, three washes were carried out to remove Percoll. Suspension was washed three times to remove Percoll. After 25 min in a shaking water bath while flasks were kept oxygenated. After 12 min and then every 3 min, digestion was interrupted for removal of the digested tissue. After digestion was completed, tissues were washed and placed for 10 min in 30 ml of ice-cold and oxygenated solution 1. Renal cortices were removed and minced on a cold Petri dish. After three washes with solution 1, tissues were incubated in 60 ml of 37°C oxygenated solution 1, containing 40 mg of collagenase and 10 mg of hyaluronidase. Digestion was performed for 25 min in a shaking water bath while flasks were kept oxygenated. After 12 min and then every 3 min, digestion was interrupted for removal of the digested tissue. After digestion was completed, tissues were washed and placed for 10 min in 30 ml of ice-cold solution 1 containing 1 g of fatty acid-free albumin (Sigma). After filtering the tissue through a tea strainer, three washes were carried out to remove albumin. Tubules were separated using a 45% Percoll gradient. After centrifugation for 10 min (11000 g), PT were recovered from the lowest band. Light microscopy revealed that this band contained no glomeruli and was composed primarily (95%) of PT. The tubule suspension was washed three times to remove Percoll. Thereafter, tubules were incubated in an oxygenated solution (solution 2) at pH 7.2 and 4°C, containing 106 mM NaCl, 18 mM NaHCO\(_3\), 5 mM KCl, 5 mM CaCl\(_2\), 2 mM NaH\(_2\)PO\(_4\), 1 mM MgSO\(_4\), 5 mM glucose, 2.5 mM HEPES, 2 mM glutamine, 10 mM butyric acid and 4 mM lactic acid.

Aliquots of 6 ml (containing 1–1.5 mg of protein/ml) were placed in siliconized Erlenmeyer flasks and gassed on ice for 5 min with 95% O\(_2\)/5% CO\(_2\), followed by a 10 min period at room temperature. At this moment, tubules were ready for the experiment.

#### Effect of CsA on isolated proximal tubules

CsA (Sandimmune\(^{®}\), Sandoz) was dissolved in vehicle (cremophor, Sigma) to a concentration of 50 mg/ml. For some experiments, cremophor dissolved in ethanol (33%) was also used. CsA was diluted to the following concentrations: 1, 2.5, 25, 50 and 100 μg/ml. CsA and vehicle were added to oxygenated PT suspensions 15 min before baseline measurements.

#### Effect of low extracellular calcium on CsA injury

At baseline, EGTA (1 mM) was added to a modified solution 2 (containing 0.7 mM calcium) to obtain a final calcium concentration of 100 nM, as previously described [11]. CsA (100 μg/ml) was added to normoxic tubules placed in this medium.

#### Effect of verapamil and nifedipine on CsA-induced injury

Verapamil (Knoll, 120 μM) was added to the oxygenated tubule suspension that had been incubated previously with 10, 50 and 100 μg/ml CsA. Nifedipine (Sigma, 200 μM) was added to control tubules and to tubule suspensions containing CsA 100 μg/ml. Drug concentrations were based on previous studies [11,12].

#### Assessment of cell injury

Cell injury was assessed by lactate dehydrogenase (LDH) release according to the method of Bergmeyer [13]. This release was measured by removal of 1 ml samples from tubule suspensions at baseline, 60 min and 90 min into the experiment. LDH release was calculated by dividing supernatant LDH by total LDH (supernate + pellet) and was expressed as a percentage of total LDH [11].

#### Intracellular calcium measurements

Intracellular calcium ([Ca\(^{2+}\)]) was measured using the fluorescent dye, Fura-2 acetoxyxymethylene (AM) (Molecular Probes, USA), dissolved in dimethyl sulfoxide (DMSO; Sigma). Tubule suspensions were incubated with Fura-2 plus pluronic acid for 60 min. Tubes were washed once with solution 2 and placed in a fluorimeter (SPEX, USA). PT suspensions were kept at 37°C and were stirred continuously. Fluorescence was measured at dual wavelength excitation.
light (340 and 380 nm) with emission at 505 nm for 5 min. Intracellular calcium concentrations were calculated according to Grynkiewicz [14]: [Ca\(^{2+}\)] = K_d \times [(R - R_{min})/(R_{max} - R)] \times B where K_d, the dissociation constant of fura-2 for calcium, equals 224 nm at 37°C, R_{min} and R_{max} are values of R at zero and saturating calcium concentrations, respectively, and B was determined from the rate of fluorescence of fura-2 in saturating conditions and free calcium.

The following groups were studied: (i) control; (ii) vehicle: addition of cremophor; (iii) CsA: addition of CsA (200 μg/ml); (iv) V: addition of verapamil (120 μM); (v) N: addition of nifedipine (200 μM); (vi) V + CsA: addition of V (120 μM) followed by CsA (200 μg/ml) after 100 s; and (vii) N + CsA: addition of N (200 μM) followed by CsA (200 μg/ml) after 100 s.

Because of the short duration of these experiments (5 min), a higher concentration of CsA (200 μg/ml) was utilized.

**Statistical analysis**

Data are expressed as means ± SDs. Statistical analysis was performed using ANOVA with Student Newman–Keuls *post hoc* tests. Statistical significance was set at \( P < 0.05 \).

**Results**

**Effect of vehicle and CsA on oxygenated PT**

The vehicle for CsA did not exert toxic effects on PT. CsA caused injury to oxygenated PT only at the highest concentration (100 μg/ml). After 60 min, LDH averaged 17.6% in control PT vs 33.3% in CsA 100 (\( P < 0.001 \)) (Table 1), and similar results were obtained after 90 min. Only CsA 100 caused tubular injury (22.5% in control PT vs 41.9% in CsA 100, \( P < 0.001 \)) (Figure 1). As shown in Table 2, the low calcium medium (100 mM) prevented CsA (100 μg/ml) tubular injury. After 60 min in the low calcium medium, LDH was 20.6% in control PT vs 20.4% in CsA-treated PT (NS, \( n = 7 \)). Furthermore, LDH release in CsA-treated PT with low calcium was significantly lower than CsA-treated PT in regular calcium medium (20.4% vs 33.3%, \( P < 0.001 \)). The addition of verapamil to PT incubated with CsA enhanced cell injury (Table 3), even when non-toxic concentrations of CsA were tested (10 and 50 μg/ml) (Table 4).

In contrast, the addition of nifedipine did not affect CsA-induced cell injury (100 μg/ml). After 60 min of incubation, LDH release was 29.7% in CsA-treated PT and 32.1% in the CsA + N-treated PT (NS), and similar results were observed after 90 min (Table 5).

Higher concentrations of Mg\(^{2+}\) (2 mM) caused an attenuation of CsA- (100 μg/ml) induced tubular injury (Table 6). After 60 min, LDH was 32.9% in CsA PT with control medium compared with 27.1% in the double Mg\(^{2+}\) medium (\( P < 0.05 \)). After 90 min, responses were similar with LDH release at 56.8% in control medium vs 45.0% in the double Mg\(^{2+}\) medium (\( P < 0.05 \)).

**Effect of CsA on PT intracellular calcium**

The addition of CsA (200 μg/ml) to PT induced a transient increase in [Ca\(^{2+}\)] from a baseline value of 265 ± 25 nM to 351 ± 29 nM (\( P < 0.001 \)) after 100 s, and calcium returned to 285 ± 29 nM after 200 s (\( P < 0.001 \) vs 100 s, \( n = 15 \)) (Figure 2). Time control PT segments did not show significant [Ca\(^{2+}\)] increases over the same 200 s period. Vehicle did not increase [Ca\(^{2+}\)]. The addition of verapamil to PT prior to CsA attenuation [Ca\(^{2+}\)] increases at 100 s, and nifedipine completely prevented CsA-induced [Ca\(^{2+}\)] increases (Figure 3).

**Discussion**

The nephrotoxicity of CsA remains the major limitation of this widely used immunosuppressant, and haemodynamic alterations are clearly involved in these toxic effects. CsA nephrotoxicity is histologically verified in oxygenated PT incubated with increasing concentrations of CsA (1, 2.5, 5, 10, and 100 μg/ml) and vehicle (cremophor, 100 μg/ml) on LDH release (as a percentage of total cell LDH) in normoxic tubules after 90 min of incubation. *\( P < 0.001 \) vs control.

![Fig. 1. Effect of CsA (1, 2.5, 5, 10, and 100 μg/ml) and vehicle (cremophor, 100 μg/ml) on LDH release (as a percentage of total cell LDH) in normoxic tubules after 90 min of incubation. *\( P < 0.001 \) vs control.](image)

| Table 1. LDH release (% of total LDH) in oxygenated PT incubated with increasing concentrations of CsA |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Control (\( n = 11 \)) | CsA (μg/ml) | 1 (\( n = 5 \)) | 2.5 (\( n = 7 \)) | 5 (\( n = 7 \)) | 10 (\( n = 9 \)) |
| Baseline | 10.0 ± 2.6 | 8.3 ± 2.1 | 8.8 ± 2.5 | 12.5 ± 2.0 | 13.6 ± 4.0 | 15.3 ± 2.9 |
| 60 min | 17.6 ± 4.0 | 16.0 ± 4.9 | 16.2 ± 2.8 | 22.4 ± 8.4 | 25.0 ± 5.0 | 33.3 ± 7.4* |
| 90 min | 22.5 ± 4.0 | 22.5 ± 12.7 | 19.0 ± 2.2 | 23.7 ± 6.9 | 31.6 ± 7.4 | 41.9 ± 10.2* |
characterized by interstitial fibrosis and afferent arteriole hyalinosis. Much of CsA-induced injury has been ascribed to sustained renal vasoconstriction and to decreased afferent arteriole lumen diameter, leading to pre-glomerular ischaemia. However, Vieira et al. [3] demonstrated that low but clinically relevant doses of CsA may generate interstitial fibrosis without causing decreases in renal blood flow or alterations in arteriole anatomy. In addition, CsA has been reported to cause direct tubular injury, but the mechanisms of damage are

<table>
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<td>60 min</td>
<td>17.6 ± 4.0</td>
<td>33.3 ± 7.4*</td>
<td>20.6 ± 6.7</td>
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*P < 0.001 vs control – Ca²⁺ (1 mM), †P < 0.001 vs CsA – Ca²⁺ (1 mM), ANOVA with Student–Newman–Keuls post hoc comparisons.

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*P < 0.001 vs control; †P < 0.001 vs CsA, ANOVA with Student–Newman–Keuls post hoc comparisons.

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<td>90 min</td>
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<td>43.0 ± 13.2*</td>
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*P < 0.001 vs control, ANOVA with Student–Newman–Keuls post hoc comparisons.

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<td>Baseline</td>
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<td>7.7 ± 1.2</td>
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<tr>
<td>60 min</td>
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<td>32.9 ± 8.4*</td>
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<td>90 min</td>
<td>24.1 ± 2.9</td>
<td>26.1 ± 3.9</td>
<td>56.8 ± 12.3*</td>
<td>45.0 ± 13.3*</td>
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*P < 0.001 vs control; †P < 0.001 vs CsA (100), ANOVA with Student–Newman–Keuls post hoc comparisons.
still unclear. Thus, this type of injury may contribute to CsA nephrotoxicity independently of haemodynamic disturbances. In the present study, utilization of isolated PT allowed the evaluation of direct tubular effects of CsA without interference from haemodynamic factors.

We added varying concentrations of CsA to normoxic PT suspensions. Tubular cell injury was obtained only with the highest concentration of CsA (100 μg/ml) (Figure 1). When given clinically, CsA nephrotoxicity seems to correlate with CsA blood levels, which are kept within a narrow therapeutic range. However, renal toxicity may develop in patients with normal CsA trough levels, and there is increasing acceptance that CsA area under the curve rather than trough levels represents true drug exposure and may correlate better with nephrotoxicity. In addition, peak levels may reach concentrations 10- to 20-fold higher than trough levels and renal tissues may accumulate CsA at levels ~20 times greater than blood levels. Therefore, very high tissue concentrations similar to those used in the present study may occur in patients receiving CsA. Nevertheless, it should be recalled that these are experimental studies using in vitro PT isolated from rats that are more resistant to CsA toxicity than are humans. Myers et al. [4] proposed that tubular epithelium is the primary site of CsA-induced injury because severe tubular lesions were observed next to preserved glomeruli in cardiac transplant kidney biopsies. Cunningham et al. [15] correlated renal histology with blood levels of CsA in rats treated with CsA at 25 mg/kg/day. After 49 days of observation, the CsA blood concentration was 10 μg/ml, which probably corresponded to a renal tissue concentration of 200 μg/ml. Histological evaluation revealed injured proximal tubules with cytoplasm vacuolization [15].

It is noteworthy that another calcineurin inhibitor, tacrolimus, causes similar toxicity to rat renal PT. When testing tacrolimus using the same experimental preparation, we found that the drug showed a toxicity to PT that was similar to CsA but only in higher (3- to 6-fold) concentrations (data not published). Although both drugs are calcineurin inhibitors, this difference in toxicity was expected since they have different properties and potencies.

In previous studies, CsA increased intracellular calcium in many cell types [8,9]. Furthermore, CsA tubular toxicity may be secondary to such alterations in intracellular calcium concentration. However, the mechanisms of these calcium increments are not clear. Possible pathways for CsA-induced increases include plasma membrane alterations [16] and increased inositol phosphate production [17].

In the present study, PT toxicity to CsA was completely prevented by reducing extracellular calcium (Table 2), suggesting a role for this ion in CsA cytotoxicity. We therefore evaluated the effect of the calcium channel blockers, verapamil and nifedipine, on CsA-induced tubular toxicity. Even though these two channel blockers have been shown to act on renal tubules [11,18,19], they were not able to prevent CsA tubular toxicity. In fact, verapamil enhanced CsA toxicity (Table 3), even at non-toxic CsA concentrations (Table 4), while nifedipine was ineffective when assessed by LDH release (Table 5). These drugs act on L-type calcium channels which are controlled by voltage and calcium influx. The negative feedback induced by calcium is essential for cell homeostasis. However, once calcium channels are phosphorylated, they remain open. Schuhmann et al. [20] demonstrated that CsA prevented calcium-dependent inhibition of L-channels in smooth muscle cells from human umbilical vein. Prior incubation of these cells with CsA resulted in constant channel activity regardless of the calcium rise. This effect was probably due to calcineurin inhibition by CsA, which is responsible for channel dephosphorylation [20]. Therefore, the CsA-induced [Ca<sup>2+</sup>]<sub>i</sub> rise in PT may be secondary to increased permeability of the plasma cell membrane and to calcineurin inhibition, which may further restrict the action of calcium channel blockers. To verify these possibilities, we measured intracellular calcium in PT. We found that addition of CsA to PT caused a transient increase in intracellular calcium (Figure 2). Although the mechanisms responsible for this increase are not fully

![Fig. 2. CsA (200 μg/ml) induced a transient [Ca<sup>2+</sup>]<sub>i</sub> increase in proximal tubules at 100 s (P < 0.001 vs baseline) with recovery at 200 s (NS vs baseline).](image-url)
understood, CsA interacts intracellularly with calcium-modulating proteins, such as cyclophilins and calcineurin, that could cause either intracellular calcium mobilization or extracellular influx. Whereas both calcium channel blockers were ineffective in preventing CsA tubular injury, nifedipine completely prevented CsA-induced [Ca\textsuperscript{2+}]\textsubscript{i} increases, contrasting with abolished [Ca\textsuperscript{2+}]\textsubscript{i} rise, while verapamil partly attenuated [Ca\textsuperscript{2+}]\textsubscript{i} elevations (Figure 3). However, despite this initial inhibition of [Ca\textsuperscript{2+}]\textsubscript{i} rise, we found that CsA still caused tubular injury. Whether this initial effect (100s) was not capable of affording sustained protection or whether these channel blockers are actually ineffective against CsA tubular toxicity remains to be elucidated.

Our finding that verapamil potentiated CsA-induced tubular injury combined with attenuation of [Ca\textsuperscript{2+}]\textsubscript{i} increases, contrasting with abolished [Ca\textsuperscript{2+}]\textsubscript{i} increases using nifedipine, may be ascribed to specific effects of verapamil on intracellular organelles, especially the mitochondria [21]. However, the effect of verapamil on mitochondria could be deleterious to CsA-exposed cells because verapamil is able to block calcium entry in these organelles, which act as the most important calcium buffering system in tubular cells. Consequently, this may result in [Ca\textsuperscript{2+}]\textsubscript{i} increases, leading to activation of several enzymes, such as proteases and caspases, which may cause cell injury and enhance CsA toxicity.

Verapamil may also enhance CsA-induced injury by increasing CsA cell concentrations due to effects on P-glycoprotein. CsA is a substrate for P-glycoprotein which acts as a counter-transport pump, actively transporting drugs back into the cell. Verapamil can modulate this mechanism [22], resulting in increased cell concentrations of CsA and cytotoxicity.

Hypomagnesaemia is a common disturbance during CsA therapy. In the present study, we found that increasing extracellular magnesium from 1 to 2 mM resulted in an attenuation of CsA-induced tubular injury (Table 6). Magnesium is an intracellular ion that is bound to organic molecules and only a small portion represents its free ionized form. Intracellular magnesium levels are maintained in equilibrium by influx, outflow and tamponade. Magnesium is important for the activities of both Na\textsuperscript{+}/K\textsuperscript{+} ATPase and Ca\textsuperscript{2+} ATPase. Thus, low intracellular Mg\textsuperscript{2+} may affect these enzymes activities, resulting in [Ca\textsuperscript{2+}]\textsubscript{i} overload. Furthermore, magnesium is important for mitochondrial calcium regulation and may, under some circumstances, reduce calcium entry into the cell, thus acting as a calcium channel blocker.

In conclusion, we demonstrated that high concentrations of CsA caused direct tubular toxicity, and this effect was dependent on extracellular calcium and magnesium levels. Verapamil, but not nifedipine, enhanced CsA-induced tubular injury even though these drugs attenuated increases in intracellular calcium caused by CsA. These tubular effects of CsA may contribute to CsA nephrotoxicity and therefore participate in inflammation and fibrosis independently of ischaemic alterations.

Conflict of interest statement. None declared.

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