Inhibition of mineralocorticoid receptors with eplerenone alleviates short-term cyclosporin A nephrotoxicity in conscious rats

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Abstract

Background. Recent data indicate that aldosterone aggravates cyclosporin A (CsA)-induced nephrotoxicity. We examined whether the mineralocorticoid receptor (MR) blocker eplerenone (EPL) antagonized early deterioration of renal function and blood pressure (BP) increase in CsA-treated rats.

Methods. Male Sprague-Dawley rats received CsA (15 mg/kg/day i.p.) and/or EPL (100 mg/kg/day p.o.) for 21 days. After 2 weeks, arterial, venous and urinary bladder catheters were implanted and the rats were trained to accept a restraining device allowing arterial blood sampling and direct measurement of BP and renal function. BP was measured on-line in conscious rats.

Results. CsA significantly increased systolic BP: 139 ± 4 versus 134 ± 2 mmHg, reduced body weight gain: −5 ± 6 versus 36 ± 7 g, glomerular filtration rate (GFR): 1.02 ± 0.16 versus 2.64 ± 0.27 ml/min, renal blood flow (RBF): 5.3 ± 2.4 versus 13.5 ± 2.1 ml/min and lithium clearance (C Li⁺): 0.16 ± 0.04 versus 0.26 ± 0.07 ml/min compared to controls. These changes were prevented by simultaneous EPL treatment: systolic BP: 130 ± 4 mmHg; weight gain, 53 ± 7 g; GFR, 1.67 ± 0.26 ml/min; RBF, 12.3 ± 2.1 ml/min and C Li⁺, 0.27 ± 0.03 ml/min. Analysis of kidney morphology after the CsA treatment showed hyaline vacuolization in tubules and vascular depositions in arterioles; these changes were less pronounced after combination therapy. No significant changes were seen regarding haemoglobin, haematocrit, plasma renin and vasopressin, plasma and urinary sodium, or osmolality.

Conclusions. MR blockade by EPL prevented short-term alterations in GFR, RBF and hypertension associated with CsA nephrotoxicity. We conclude that the aldosterone-MR pathway contributes markedly to the renal toxicity induced by this calcineurin inhibitor.

Keywords: cyclosporin A; eplerenone; glomerular filtration rate; mineralocorticoid receptor antagonist; nephrotoxicity

Introduction

The calcineurin inhibitor (CNI), cyclosporin A (CsA), is a potent macrolide immunosuppressive and anti-proliferative agent, which is widely used after organ transplantation and for the treatment of various immunological diseases. CsA exerts major nephrotoxic effects. This nephrotoxicity seems to involve an acute vasoconstriction related to afferent glomerular arterioles, but also a major pro-fibrotic effect in the chronic phase. Many treatment options have been examined for the purpose of countering these adverse effects, but until recently none have managed to arrest the gradual decline in renal function and the progressive renal fibrosis occurring during the CsA treatment. However, recently there have been indications that a mineralocorticoid blockade might have a preventive effect. In these studies the aldosterone antagonist spironolactone slowed the progression of renal dysfunction and reduced the morphological changes seen after the CsA treatment; the studies were conducted in anaesthetized animals after major surgery, which might alter blood pressure (BP) and parameters of renal function.

The present study was undertaken to investigate further the hypothesis that CsA nephrotoxicity might be prevented by mineralocorticoid receptor (MR) inhibition. We used the more selective aldosterone antagonist eplerenone (EPL), which has a clinically superior profile with respect to adverse effects. All measurements were taken in conscious, trained rats under steady-state conditions. This model allows direct recordings of the glomerular filtration rate (GFR), renal blood flow (RBF), segmental reabsorption by clearance techniques and BP. The plasma concentrations of renin, aldosterone and vasopressin were also determined and morphological analysis of in vivo perfused kidneys stained with HE and PAS was done. The CsA dose was chosen on the basis of the results of earlier dose-response...
studies, so that the immunosuppressive effect in this particular rat strain was fully exploited [7]. A treatment period of 21 days was used to study short-term effects.

**Subjects and methods**

All procedures conformed to Danish national guidelines for the care and handling of animals and the published guidelines from the National Institute of Health. The Danish Animal Experiments Inspectorate approved the study (2005/561-967).

**Experimental animals**

The animals were inbred, male Sprague-Dawley rats (Mol:SPRD) from Harlan Scandinavia (Harlan, Alleroed, Denmark) initially weighing 180–220 g. The rats had free access to tap water and a wet mash non-sodium-depleted diet (Altromin® Standard 1320, Lage, Germany).

**Drug preparation and treatment protocol**

The rats were treated with CsA (n = 7), EPL (n = 7), both (n = 9) or neither, thus serving as controls treated with the vehicle only (n = 7) for 21 days before the renal function tests were performed. The CsA infusion substance (Sandimmune Neoral®, Novartis Pharma AG, Basel, Switzerland) 100 mg/ml was diluted with sterile saline to a concentration of 10 mg/ml. All rats received intraperitoneal injections of saline, rats in the CsA and CsA + EPL treatment groups had CsA added and rats in all groups were given equal volumes as the dose was adjusted in step with the weight increase of the individual rat. Accordingly, control rats and rats treated with EPL alone were given similar amounts of saline without CsA. The intraperitoneal route was chosen to ensure a precise dose. EPL tablets 50 mg (Inspra®, VetaPharma, Sherburn-in-Elmet, Leeds, UK) were crushed and added to the diet for the EPL treatment groups: 1.2 g/dry food, approaching a daily dose of 100 mg/kg body weight for the individual rat [8]. The non-EPL treatment groups received a similar diet without EPL. Two days before the renal function tests, lithium chloride 10 mmol/kg dry weight of food was added.

**Catheters in artery, vein, and urinary bladder**

One week before the renal function tests the rats were anaesthetized with 3 ml/kg body weight of a combination of fentanyl 80 μg/ml, fluanisone 2.5 mg/ml (Hypnorm®, VetaPharma), Sherburn-in-Elmet, Leeds, UK) and midazolam (Dormicum®, Roche Pharmaceuticals, Basel, Switzerland) 1.25 mg/ml administered intraperitoneally and Na₂O₂; 50%. For analgesia 0.5 ml buprenorphine (Temgesic®, Schering-Plough Corporation, Kenilworth, NJ, USA) 0.3 mg/ml was administered subcutaneously. A catheter was inserted into the femoral vein and another in the femoral artery. Both catheters were closed with heparin 200 IU/ml and chymotrypsin (Worthington Biochemical Corporation, Lakewood, NJ, USA) 225 IU/ml added to glucose 50% and subcutaneously tunnelled to exit at the back of the head. The bladder was catheterized through a suprapubic incision with a specially designed stainless steel catheter that was sutured to the abdominal wall and kept closed until the day of the renal function tests. After the operation the rats were placed in a recovery cage at a temperature of 27–30°C. The animals received buprenorphine 2 ml/kg of body weight subcutaneously after the operation and on the following day. On Days 1, 2, 3, 5 and 7 the artery and vein catheters were rinsed with the heparin/glucose solution.

**Blood pressure measurements and clearance studies**

Beginning 2 days after surgery, the rats were trained for another 5 days to sit awake and relaxed in a restraining device for clearance and BP measurements. The arterial catheter was connected to a BP transducer linked to an amplifier (BLPR and BP1, World Precision Instruments, Hertfordshire, UK) and computer running custom-designed software (LabVIEW Real-Time®, version 7 Express, National Instruments™, Dublin, Ireland) for continuous recording of BP and heart rate. The venous catheter was connected to two syringes of an infusion pump, one delivering insulin (Polysorbsen S, Laevosan®, Petrone Group, Napoli, Italy) 25% in saline: a priming dose of 80 μl/kg/min over 2 min, followed by a continuous infusion of 8 μl/kg/min, and the other delivering para-aminohippuric acid (PAH) 20% in saline: a priming dose of 120 μl/kg/min over 2 min, followed by a continuous infusion of 12 μl/kg/min. One hour was allowed for reaching the steady state. Thereafter the urine was collected for the next 60 min; at the end of this period blood samples were taken from the arterial catheter for clearance determinations. Blood samples were centrifuged and plasma was collected and stored at –80°C.

**Analysis**

The CsA treatment was checked after 21 days of treatment by measuring the whole blood CsA concentrations as trough levels with a monoclonal radioimmunoassay kit (TDx/TDxFLx, Abbott Laboratories, Abbott Park, IL, USA). The blood samples were collected 22–26 h after the last administration of CsA.

Insulin in plasma and urine was analysed by the diphynylamine method [9] modified for microanalysis. By this technique, inulinase hydrolyses inulin to fructose, which is converted to sorbitol by sorbitol dehydrogenase (SDH) with the consumption of nicotinamide adenine dinucleotide (NADH). The amount of NADH used is proportional to the amount of insulin originally present in the sample, and NADH is detected by spectrophotometry at 340 nm (Versamax® Micro plate reader, Molecular Devices Corporation, Sunnyvale, CA, USA). PAH in plasma and urine was measured by a colorimetric reaction with the dimethylaminocinnamaldehyde (DACA) solution in an acidic environment [9]. The intensity of the colour generated was measured at 545 nm after 15–30 min of incubation.

The concentrations of lithium, sodium and potassium ions in plasma and urine were determined by flame photometry (ILS 943, Instrumentation Laboratory, Lexington,
Mineralocorticoid receptor inhibition alleviates cyclosporin A nephrotoxicity

Table 1. Body weight increase and blood pressure after 21 days of treatment

<table>
<thead>
<tr>
<th></th>
<th>CsA</th>
<th>CsA + EPL</th>
<th>EPL</th>
<th>Controls</th>
<th>ANOVA</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>7</td>
<td>9</td>
<td>7</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increase in BW</td>
<td>–5 ± 6</td>
<td>37** ± 7</td>
<td>41** ± 11</td>
<td>51** ± 7</td>
<td>P &lt; 0.05</td>
<td>g</td>
</tr>
<tr>
<td>SYS</td>
<td>139 ± 4</td>
<td>130** ± 4</td>
<td>138 ± 1</td>
<td>134 ± 2</td>
<td>P &lt; 0.05</td>
<td>mmHg</td>
</tr>
<tr>
<td>DIA</td>
<td>123 ± 3</td>
<td>119 ± 4</td>
<td>125 ± 2</td>
<td>124 ± 2</td>
<td>NS</td>
<td>mmHg</td>
</tr>
<tr>
<td>MAP</td>
<td>128 ± 4</td>
<td>123 ± 4</td>
<td>129 ± 1</td>
<td>127 ± 1</td>
<td>NS</td>
<td>mmHg</td>
</tr>
</tbody>
</table>

CsA: cyclosporin A, EPL: eplerenone, BW: body weight, SYS: systolic aortic pressure, DIA: diastolic aortic pressure, MAP: mean arterial pressure:
\[ \frac{1}{3} \times (\text{SYSAP} + 2 \times \text{DIAAP}) \].

\*P < 0.05 when compared to the control group.

\**P < 0.05 when compared to the CsA group.

Fig. 1. Renal function after 21 days of treatment. Inulin clearance as a measure of the glomerular filtration rate, lithium clearance as a measure of the end proximal fluid delivery and the difference between these as the absolute proximal reabsorption. CsA: cyclosporin A; EPL: eplerenone.

\*P < 0.05 when compared to the control group; \#P < 0.05 when compared to the CsA treatment group, but not different from the control group.

Fig. 2. Renal blood flow as measured by para-aminohippuric acid (PAH) clearance after 21 days of treatment. CsA: cyclosporin A; EPL: eplerenone.

\*P < 0.05 when compared to the control group; \#P < 0.05 when compared to the CsA treatment group, but not different from the control group.

MA, USA). The concentration of creatinine in urine was measured by photometric analysis (Microlab 300, Vital Scientific, Spankeren, The Netherlands). Osmolality in plasma and urine was measured immediately after centrifugation by freezing point depression (Osmomat® 030-D, Gonotec GmbH, Berlin, Germany), and haemoglobin and haematocrit were measured by an autoanlyser (Celltac® MEK-6108K, Nihon Kohden, Japan).

Renin in plasma was measured by the antibody-trapping method of Poulsen and Jorgensen [10]. Aldosterone in plasma was measured by a commercial radioimmunoassay kit (Coat-A-Count® Aldosterone, Diagnostic Products
### Table 2. Measurements of blood and urine samples after 21 days of treatment

<table>
<thead>
<tr>
<th></th>
<th>CsA</th>
<th>CsA + EPL</th>
<th>EPL</th>
<th>Controls</th>
<th>Significance</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin</td>
<td>8.2 ± 0.2*</td>
<td>8.4 ± 0.2*</td>
<td>9.1 ± 0.2</td>
<td>9.0 ± 0.2</td>
<td>*P &lt; 0.05</td>
<td>mmol/l</td>
</tr>
<tr>
<td>Haematocrit</td>
<td>41.3 ± 0.7*</td>
<td>42.7 ± 1.4*</td>
<td>44.9 ± 0.7</td>
<td>44.9 ± 0.9</td>
<td>*P &lt; 0.01</td>
<td>mmol/l</td>
</tr>
<tr>
<td>U creatinine</td>
<td>2671 ± 831</td>
<td>4356 ± 419</td>
<td>3953 ± 796</td>
<td>3712 ± 1034</td>
<td>NS</td>
<td>µmol/l</td>
</tr>
<tr>
<td>Potassium</td>
<td>4.8 ± 0.2</td>
<td>4.9 ± 0.0</td>
<td>4.9 ± 0.2</td>
<td>4.6 ± 0.1</td>
<td>NS</td>
<td>mmol/l</td>
</tr>
<tr>
<td>Sodium</td>
<td>139.7 ± 0.9</td>
<td>138.5 ± 1.5</td>
<td>139.2 ± 1.0</td>
<td>139.5 ± 0.9</td>
<td>NS</td>
<td>mmol/l</td>
</tr>
<tr>
<td>U potassium</td>
<td>10.0 ± 3.7</td>
<td>16.0 ± 0.6</td>
<td>12.0 ± 1.2</td>
<td>8.9 ± 2.1</td>
<td>NS</td>
<td>mmol/l</td>
</tr>
<tr>
<td>U sodium</td>
<td>119 ± 25</td>
<td>121 ± 29</td>
<td>116 ± 32</td>
<td>58 ± 22</td>
<td>NS</td>
<td>mmol/l</td>
</tr>
<tr>
<td>Osmolality</td>
<td>294 ± 4</td>
<td>287 ± 10</td>
<td>303 ± 13</td>
<td>296 ± 1</td>
<td>NS</td>
<td>mosmol/kg</td>
</tr>
<tr>
<td>U osmolality</td>
<td>764 ± 132</td>
<td>1151 ± 221</td>
<td>879 ± 185</td>
<td>720 ± 190</td>
<td>NS</td>
<td>mosmol/kg</td>
</tr>
<tr>
<td>Potin</td>
<td>12.0 ± 6.3</td>
<td>13.0 ± 6.6</td>
<td>9.2 ± 2.9</td>
<td>13.6 ± 6.6</td>
<td>NS</td>
<td>nl/l</td>
</tr>
<tr>
<td>Vasopressin</td>
<td>1.8 ± 0.3</td>
<td>1.2 ± 0.3*</td>
<td>2.1 ± 0.1</td>
<td>2.2 ± 0.3</td>
<td>*P &lt; 0.03</td>
<td>pg/ml</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>84 ± 5</td>
<td>169 ± 18*</td>
<td>114 ± 10*</td>
<td>96 ± 12</td>
<td>*P &lt; 0.02</td>
<td>pg/ml</td>
</tr>
</tbody>
</table>


*P < 0.05 when compared to the control group.

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**Morphology**

The rats were anaesthetized and the kidneys were fixed by formaldehyde infusion and embedded in paraffin. Kidney sections (3 µm) were stained with haematoxylin and eosin, PAS and Trichrome Masson-Goldner before blind examination by light microscopy. The following histological parameters were graded and scored with a semi-quantitative scale between 0 and 3: glomerular injury, arterial injury, interstitial fibrosis, tubular dilation, protein casts and inflammatory cell infiltrates.

**Renal clearances**

Standard formulae were used to calculate clearance. Inulin clearance was taken as a measure of the GFR and lithium clearance (C\textsubscript{Li}) was taken as a measure of the delivery of proximal tubular fluid from the end of the pars recta into the thin descending limb of Henle [12]. Absolute proximal reabsorption (APR) rate was estimated as APR = GFR - C\textsubscript{Li}. Fractional proximal reabsorption (PFR) was calculated as PFR = APR/GFR and expressed as a percentage of the GFR. The PAH clearance was taken as a measure of the renal plasma flow (RPF), calculated according to the haematocrit as RBF.

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**Fig. 3.** (A) CsA treated animal with arteriolar hyalinosis (arrow), (B) CsA + EPL treated animal with normal arteriole (arrow), PAS ×300.
is for a two-tailed test, and a $P$ value $<5\%$ was considered statistically significant.

**Results**

There were no statistically significant differences in the CsA trough concentrations of the CsA treatment groups ($3377 \pm 562$ ng/ml), as compared to the combination therapy group ($2017 \pm 416$ ng/ml). Over the 21 days of treatment the body weight of the control rats increased significantly more than that of the rats in the CsA group ($P < 0.002$) (Table 1). CsA decreased GFR, proximal fluid delivery and APR (Figure 1). In parallel, CsA reduced RBF ($P < 0.05$) (Figure 2), and the urine creatinine concentration was significantly lower in the CsA-only treated animals, as compared to the controls ($P < 0.05$) (Table 2). Interestingly, the body weight of the rats in the combination treatment group increased at a rate similar to that of the control rats (Table 1). Addition of EPL to the CsA treatment reversed all the parameters mentioned to normal or above normal; RBF increased 2.3-fold (Figure 2), whereas the EPL treatment alone did not change these values, as compared to the vehicle-treated control rats (Figure 2). The CsA treatment resulted in significant higher systolic BP compared to controls ($P < 0.05$) while the changes in diastolic BP and mean arterial BP were not significantly different (Table 1). Neither the systolic BP, the diastolic BP nor the mean arterial BP were significantly different when the combination treatment group was compared to the control group (Table 1).

Compared to that of the controls, plasma renin, plasma vasopressin, plasma and urinary osmolality, plasma potassium, plasma sodium and urinary potassium did not change during the CsA treatment (Table 2). Haemoglobin was significantly lower in the CsA group (8.2 mmol/l) than in the controls (9.0 mmol/l) ($P < 0.05$) (Table 2). This effect was not reversed by the EPL treatment, as the CsA + EPL group showed similar anaemia (haemoglobin 8.2 mmol/l). The EPL alone and the control groups had similar haemoglobin levels: 9.1 mmol/l, and a similar pattern was seen for haemocrit (Table 2). Plasma aldosterone was significantly higher in the EPL-treated groups than in the control and the CsA-only treatment groups ($P < 0.05$) (Table 2).

The paraffin-embedded, perfusion-fixed kidneys were subsequently stained with haematoxylin and eosin, PAS and Trichrome Masson-Goldner, and were analysed by light microscopy. Hyalin vacuolization in tubules and vascular deposition in arterioles mainly located at the basis of the glomeruli were seen in 70% of the animals treated with CsA (Figure 3). These semi-quantitatively scored findings were significantly different between groups ($P < 0.05$, Mann–Whitney), that is less-pronounced findings were found in the CsA + EPL group [median score 0.5 (range 0–2), $n = 7$] compared to the CsA group [median score 1 (range 0–2), $n = 7$] ($P < 0.05$, Kruskal–Wallis) and were not found at all in the control rats [median score 0 (range 0–1), $n = 5$] ($P < 0.05$ when compared to the CsA group; NS when compared to the CsA + EPL group, Kruskal–Wallis’ test). Overt fibrosis was not apparent in any kidneys.

**Discussion**

We show in conscious rats that a specific MR antagonist significantly and markedly improves the GFR and RBF in a model of chronic CsA nephrotoxicity. Numerous clinical and experimental studies report a significant nephrotoxic effect produced by the CNI treatment. This nephrotoxicity seems to have two elements. First, a reduction in GFR and $C_{\text{gt}}$, together with increases in PFR and BP are thought to be mediated by afferent glomerular vasoconstriction [1,13]. Second, a long-term fibrogenic effect occurs and this seems to be independent of the vascular effect [1,7,13,14]. Clinically, the progressive toxicity is a major problem resulting in gradual deterioration in the renal function of patients receiving CNI. Although the acute and short-term effects can be prevented to some degree, e.g., by angiotensin converting enzyme inhibitors [2], calcium channel antagonists [15] or angiotensin II receptor blockade [16], these treatments do not improve the long-term clinical outcome. Recently, preventive effects on both the acute toxicity and the fibrogenic effect of the CsA treatment were indicated in studies combining the CsA treatment with the MR antagonist, spironolactone [2–5]. These observations agree with other reports that aldosterone blockade exerts renoprotective effects in states with chronic renal injury [17].

We explored the hypothesis of MR blockade as a renoprotective measure in a conscious rat model of CsA nephrotoxicity. We used the Sprague-Dawley strain in order to directly compare our results with earlier CNI nephrotoxicity studies [1,7,13,14] and used a relatively short-term high-dose protocol of 21 days to study primarily functional changes and not structural and inflammatory secondary changes [1,7]. The intraperitoneal route was used to ensure that the individual rat received a precise dose. Measurement of whole blood CsA concentrations confirmed the presence of similar concentrations in the various groups. EPL was given in a high dose to ensure the clinically significant effect earlier established in rats [8].

The CsA-treated rats had a smaller increase in body weight than the controls had (Table 1). This agrees with earlier findings for CsA [1,3,14] and tacrolimus [13] and was seen as an effect induced by uraemia and malnutrition arising from the nephrotoxicity rather than the specific substance. Administration of EPL prevented growth impairment (Figure 1). After the treatment, GFR, RBF, $C_{\text{gt}}$, and the calculated APR were reduced in the CsA-treated animals when compared to controls in concordance with numerous publications [1,7,14]. Interestingly, all of these parameters were preserved in animals in which the CsA treatment was combined with EPL. This indicates that both the vascular and the short-term tubular effects of CsA were prevented by the MR blockade (Figures 1 and 2). It should be emphasized that the effect of EPL was seen despite a very high dosage of CsA. In the clinical setting a much lower CsA dosage is used. BP measurements were taken continuously with intra-arterial catheters for 60-min
periods and were performed 7 days after surgery to minimize postoperative pain and influence by analgesics or anaesthetic drugs. Although the catheters were regularly rinsed by an anticoagulation heparin/glucose solution, the observed small BP amplitudes might indicate a buffering of the signal through the catheter to the BP transducer. The BP was rather high in all the treatment groups and tended to stay at high levels during the observation period, perhaps because of the stress related to the restraining device, even though the animals were trained to tolerate this situation. The systolic BP in the CsA treatment group was significantly higher than in rats from the control group in haps because of the stress related to the restraining device, to stay at high levels during the observation period, per-

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