Mineralocorticoid receptor blockade confers renoprotection in preexisting chronic cyclosporine nephrotoxicity

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1Molecular Physiology Unit, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México and 2Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico; and 3Renal Division, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts

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Pérez-Rojas J, Blanco JA, Cruz C, Trujillo J, Vaidya VS, Uribe N, Bonventre JV, Gamba G, Bobadilla NA. Mineralocorticoid receptor blockade confers renoprotection in preexisting chronic cyclosporine nephrotoxicity. Am J Physiol Renal Physiol 292: F131–F139, 2007. First published July 11, 2006; doi:10.1152/ajprenal.00147.2006.—Recent studies from our laboratory have shown that the mineralocorticoid receptor (MR) blockade with spironolactone (Sp) prevented renal dysfunction and reduced renal injury in both acute and chronic cyclosporine (CsA) nephrotoxicity. This study was designed to evaluate whether Sp administration reduces functional and structural renal damage associated in the setting of preexisting chronic CsA nephrotoxicity. Twenty-eight male Wistar rats were fed a low-sodium diet. Fourteen received vehicle (V) and the others were treated with CsA (15 mg/kg sc). After 18 days one half of each group received Sp (20 mg/kg po) for the subsequent 18 days. Creatinine clearance, arteriolar pathology, tubulointerstitial fibrosis, arteriolar thickening, glomerular diameter, apoptosis index and TGF-β, procaspase-3, and kidney injury molecule 1 (Kim-1) mRNA levels as well as Kim-1 shedding in urine were evaluated. Sp reduced the progression of renal dysfunction and tubulointerstitial fibrosis in preexisting chronic CsA nephrotoxicity. There was a significant reduction of arteriolar thickening in the CsA+Sp group that was associated with greater glomerular diameter and reduction of apoptosis index. These renoprotective effects were associated with reduction of TGF-β, procaspase-3, and Kim-1 mRNA levels as well as Kim-1 shedding into the urine. In conclusion, MR blockade with Sp prevented the progression of renal injury in preexisting chronic CsA nephropathy. These results suggest that Sp may reduce CsA-induced established nephropathy in patients.

IN RECENT YEARS THERE HAS BEEN growing interest in the role of aldosterone and mineralocorticoid receptors (MR) in the pathophysiology of cardiovascular and renal diseases. The role of aldosterone in promoting cardiovascular injury is underlined by the randomized Aldactone evaluation study (RALES) (32) and the Eplerenone Post-Acute Myocardial Infarction Heart Failure Efficacy and Survival Study (EPHESUS) (31) trials. In these studies it was demonstrated that addition of the MR blockers spironolactone or eplerenone to standard therapy in heart failure patients and patients with myocardial infarction resulted in reduced cardiac mortality that could not be explained solely by blood pressure reduction. In addition, it has been reported that aldosterone infusion in hypertensive patients can induce endothelial dysfunction (10).

Although the role of ANG II in mediating progressive renal disease has been documented extensively, recent clinical and experimental evidence has supported the role of aldosterone/MR in the progression of renal injury that is independent of ANG II. Patients with primary hyperaldosteronism exhibit a higher prevalence of proteinuria (8, 28) and severe arteriolar sclerosis and interstitial fibrosis was observed in 50% of 32 renal biopsies from patients with aldosterone-producing adenomas (13). In animals on high sodium intake, renal lesions of malignant nephrosclerosis are observed following unilateral nephrectomy and prolonged treatment with DOCA (22). In addition, the effectiveness of MR antagonism in ameliorating renal injury has also been documented. Pilot studies in humans showed that addition of spironolactone to ACE inhibitors had no hemodynamic effects but markedly reduced proteinuria in patients with renal failure (4) and in patients with type 2 diabetes (39). The protective effect of spironolactone in patients with mild renal insufficiency has recently been corroborated by a large double blind, placebo-controlled randomized trial (7). In rats, MR antagonists had no effect on systemic blood pressure but markedly ameliorated glomerular and/or tubulointerstitial injury in several models of nephropathy including spontaneously hypertensive stroke-prone rats (33, 34), ANG II and nitric oxide synthase inhibitor-treated rats (35), aldosterone-treated rats (16), and in a model of unilateral ureteral obstruction model (46). Moreover, Aldigier et al. (1) reported that MR blockade not only reduced the development of glomerulosclerosis but also induced regression of existing glomerulosclerosis in rats after 5/6 nephrectomy. All these studies together emphasize the beneficial effect of MR antagonism in progressive renal diseases.

We previously observed that aldosterone also plays an important role in the toxicity induced by the immunosuppressant cyclosporine A (CsA), an agent that is extensively used for the prevention of allograft rejection (11, 29). The therapeutic benefits of CsA for transplantation and autoimmune diseases have been limited by the occurrence of acute and chronic nephrotoxicity. Acute CsA nephrotoxicity is characterized by renal vasoconstriction induced by an imbalance of vasoactive substance release which, in turn, produced a fall in renal function. This form of toxicity is reversible. In contrast, chronic CsA nephrotoxicity is characterized by both renal vasoconstriction and the development of arteriolopathy and scarring tubulointerstitial fibrosis that are irreversible (2, 21,

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Both forms of nephrotoxicity can be reproduced in the rat by administration of repeated doses of CsA. However, to induce the chronic model, in addition to CsA administration, the animals should be fed a low-sodium diet (2, 9, 11, 19, 29, 41).

In recent studies from our laboratory, we observed that MR blockade with spironolactone completely prevented renal dysfunction induced by CsA in both acute and chronic CsA nephrotoxicity and significantly reduced renal structural damage (11, 29). Because acute kidney injury induced by CsA is associated with afferent arteriole vasoconstriction, these data suggested that aldosterone is not only implicated in chronic structural renal damage, but also in the regulation of vascular tone (29). In these studies, spironolactone was administered simultaneously with CsA from the first day of the experimental period. Thus, these observations indicate that spironolactone is an effective prophylactic agent to prevent the development of CsA nephrotoxicity. It is not known, however, if MR blockade can contribute to prevent the progression of the already existing tubulointerstitial injury and renal dysfunction in the model of chronic CsA nephropathy.

In the present study, we show that spironolactone reduced the progression of existing tubulointerstitial injury and arteriolar thickening in a model of nephropathy in which chronic CsA nephrotoxicity was already established. The renoprotection was associated with a significant reduction of CsA-induced apoptosis, TGF-β, procaspase-3, and kidney injury molecule 1 (Kim-1) expression.

METHODS

Four groups of seven male Wistar rats each weighing 300–330 g were used. All groups were fed a low-salt diet (0.02%). As shown in Fig. 1, one group received 0.1 ml sc per day of olive oil as vehicle (V); another group received CsA 15 mg kg⁻¹ day⁻¹ sc for 36 days (CsA); this dose of CsA and the percentage of sodium in the diet used in this study have been previously demonstrated to induce chronic CsA nephrotoxicity in the rat (2, 9, 11, 19, 29, 41). To test the effect of spironolactone in preexisting chronic CsA nephrotoxicity, two additional groups were included. One group received vehicle for 18 days and then vehicle plus spironolactone at 20 mg kg⁻¹ day⁻¹ by gastric gavage for another 18 days (Sp) and the other group was treated with CsA for 18 days followed by CsA plus spironolactone for another 18 days (CsA + Sp). The dose of spironolactone has been proved to be enough to blockade MR in the rat (5, 12, 25, 49).

Because CsA-treated animals reduced their food consumption and lost body weight, control animals were pair fed with their corresponding CsA group. To confirm the presence of chronic CsA nephrotoxicity at the middle of the study, an additional group of six animals was treated with CsA for 18 days and killed to evaluate the renal structural damage. All animal procedures followed were in accordance with our institutional guidelines.

Functional and histological studies. At the beginning, middle, and end of the study (0, 18, and 36 days), rats were placed in metabolic cages and urine that was spontaneously voided during 24 h was collected. Serum and urine creatinine concentration were measured with an autoanalyzer (Technicon RA-1000, Bayer Tarrytown, NY). Renal creatinine clearances were calculated by the standard formula C = U/V/P, where U is the concentration in urine, V is urine flow rate, and P is plasma concentration. Serum potassium levels were measured at the end of the study.

At the end of the study, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg kg ip) and placed on a homeothermic table to maintain core body temperature at 37°C, by means of a rectal probe attached to a temperature regulator which is, in turn, attached to a homeothermic blanket. Trachea, jugular veins, and femoral arteries were cannulated with polyethylene tubing PE-240 and PE-50. Mean arterial pressure (MAP) was monitored with a pressure transducer (model p23 db, Gould, Puerto Rico) and recorded on a polygraph (Grass Instruments, Quincy, MA). A midline laparotomy was made, right renal artery was ligated, and the right kidney was excised, macroscopically divided into cortex and medulla, frozen in liquid nitrogen, and kept at −80°C until used. The left kidney was perfused through the femoral catheter with phosphate buffer preserving the MAP of each animal. Following blanching of the kidney, the perfusate was replaced by 10% freshly prepared formalin buffer and the perfusion was continued until fixation was completed. After appropriate dehydration, kidney slices were embedded in paraffin, sectioned at 4 μm, and stained with routine methods: hematoxylin/eosin, periodic acid-Schiff, and Masson trichromic. The slides were analyzed blindly. In at least 50 arterioles per rat, arteriolaropathy was quantified as a characteristic lesion of this model. Arteriolaropathy was counted as present or nonpresent (dichotomic variable), thus results are expressed as percentage of affected arterioles over total number of arterioles. Arteriolar thickening was evaluated in at least 25 arterioles per animal from recorded digital microphotographs, using a digital camera incorporated in a Nikon microscope. Three different measurements in each arteriole were made by using eclipse net software (magnification ×400). In addition, the glomerulus size was evaluated by measuring glomerular diameter. For this purpose, 10 to 15 images of different renal cortex fields were recorded and the diameter of at least 500 glomeruli was measured in the digitalized microphotographs (magnification ×100). Moreover, in Masson-stained sections 10 subcortical periglomerular fields per section were randomly selected in kidneys from the different groups to evaluate the degree of tubulointerstitial fibrosis by morphometry. Tubulointerstitial fibrosis consisted of extracellular matrix expansion with or without collagen deposition together with distortion and collapse of the tubules; fibrosis was evidenced by blue coloration in Masson stain. Fifteen images were recorded, the affected area was delimited, and the percentage of tubulointerstitial fibrosis was calculated by dividing the fibrotic area by the total field area, excluding the glomerular area.

TUNEL assay. Apoptosis in kidney sections was determined by TUNEL assay using the ApopTag in situ apoptosis detection kit (S7101, Chemicon International, Temecula, CA). The slides were prepared following the procedures described previously (50). A minimum of 10 fields per kidney were evaluated and all kidney tissues were examined (magnification ×400). Only tubular cells that contained TUNEL-positive nuclei with the characteristic morphology of apoptosis, including nuclear fragmentation and nuclear condensation, were quantified. The number of TUNEL-positive tubules was counted.

Fig. 1. Experimental protocol. Male Wistar rats fed a low-sodium diet (0.02%) were included and 4 groups were formed: vehicle (V), cyclosporine CsA, vehicle plus spironolactone (Sp), and CsA plus Sp (CsA + Sp). Vehicle and CsA were given during 36 days. In the Sp and CsA + Sp groups, spironolactone was started at the middle of the protocol (day 18) and continued to the end of the study.
**Table 1.** Effect of CsA and MR blockade on BW, mean arterial pressure and potassium levels

<table>
<thead>
<tr>
<th>Group</th>
<th>BW, g 0 Days</th>
<th>BW, g 18 Days</th>
<th>BW, g 36 Days</th>
<th>MAP, mmHg</th>
<th>K⁺, meq/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>313±11</td>
<td>306±7</td>
<td>270±8*</td>
<td>110±6.2</td>
<td>4.7±0.3</td>
</tr>
<tr>
<td>Sp</td>
<td>310±13</td>
<td>314±11</td>
<td>289±8*</td>
<td>95.2±6.6</td>
<td>5.4±0.2</td>
</tr>
<tr>
<td>CsA</td>
<td>321±6</td>
<td>269±11</td>
<td>261±13*</td>
<td>91.2±2.7</td>
<td>5.8±0.5</td>
</tr>
<tr>
<td>CsA+Sp</td>
<td>319±4</td>
<td>268±12</td>
<td>271±16*</td>
<td>97.6±6.1</td>
<td>5.4±0.4</td>
</tr>
</tbody>
</table>

Values are means ± SE. V, vehicle; Sp, spironolactone; CsA, cyclosporine; MR, mineralocorticoid receptor; BW, body weight; MAP, mean arterial pressure; K⁺, serum potassium levels. *P < 0.05 vs. their respective groups at the beginning of the study.

and the results were expressed as the number of TUNEL-positive nuclei per square millimeter.

RNA isolation. Total RNA was isolated from each renal cortex following the guanidine isothiocyanate-cesium chloride method (38). Integrity of isolated total RNA was examined by 1% agarose gel electrophoresis and RNA concentration was determined by UV-light absorbance at 260 nm (Beckman DU640, Brea, CA). Reverse transcription (RT) was carried out using 2.5 μg of total RNA from renal cortex of each rat. RT was performed at 37°C for 60 min in a total volume of 20 μl using 200 U of the Moloney murine leukemia virus reverse transcriptase (Invitrogen), 100 pmol of random hexamers (Invitrogen), 0.5 mM of each dNTP (Sigma, St. Louis, MO), and 1× RT buffer (75 mM KCl, 50 mM Tris·HCl, 3 mM MgCl₂, 10 mM DTT, pH 8.3).

Real-time PCR. The mRNA levels of TGF-β, Kim-1, and procaspase-3 were quantified by real-time PCR on the ABI Prism 7300 Sequence Detection System (TaqMan, Applied Biosystems ABI, Foster City, CA). FAM or VIC dye-labeled probes were used as fluorescent reporter dies to detect amplification products. For Kim-1, procaspase-3 and TGF-β expression FAM probes and for eukaryotic 18S rRNA expression VIC probe were used. The FAM (6-carboxyfluorescein) and VIC were specifically used to detect and quantify cDNA sequences without the Applied Biosystems Assays-on-Demand ABI product line and Foster City, CA). FAM or VIC dye-labeled probes were selected from TaqMan, Applied Biosystems ABI, Sequence Detection System (ABI). Validation of amplification efficiency was performed on a serial template dilutions from 0.187 through 187 ng. PCR was carried out in 96-well plates on cDNA equivalent to 3.5 ng of total RNA and the results were expressed as the number of TUNEL-positive nuclei per square millimeter.

Statistical analysis. Results are presented as means ± SE. The significance of the differences among groups was tested by ANOVA comparison using Bonferroni’s correction for multiple comparisons. The differences in the ranks of glomerular diameters among the groups were evaluated by a contingency analysis and the differences were tested by χ²-test with Yates correction. Statistical significance was defined when P value was < 0.05.

**RESULTS**

Animal body weight during the study as well as MAP and serum potassium levels at the end of the study are shown in Table 1. At the beginning, all rats had similar body weight, but it was significantly reduced at the end of the study, because of the little food consumption of CsA rats and the pair feeding of the control groups. No differences among the groups were observed in MAP, thus spironolactone effect was pressure independent. Serum potassium levels tended to be higher in CsA group, but the difference was not statistically different and it was not enhanced by spironolactone administration.

The percentage of animals surviving after 36 days of treatment was 90% in the CsA+Sp-treated group vs. 67% in the CsA group (Fig. 2A). Thus, spironolactone administration improved survival rate despite the delay in administration to
CsA-treated animals. The creatinine clearance for the four groups studied at 0, 18, and 36 days is shown in Fig. 2B. At the beginning, all groups had similar normal renal function that was not modified in both control groups along the study. Rats receiving CsA showed a significant and similar reduction of creatinine clearance at 18 days. Creatinine clearance was 0.7 ± 0.1 and 0.8 ± 0.1 ml/min in CsA and CsA+Sp groups, respectively. From this day, the spironolactone administration reduced the rate of progression of renal dysfunction. At the end of the study, the creatinine clearance in CsA-treated animals further decreased to 0.4 ± 0.1 ml/min (*P = 0.006 vs. CsA alone at day 18). In contrast, creatinine clearance in the CsA+Sp group was unchanged at 0.9 ± 0.1 ml/min at day 36 (†P = NS vs. CsA+Sp at day 18).

After 18 days of CsA administration, six rats were killed and their kidneys were fixed to determine the presence of arteriolopathy and tubulointerstitial fibrosis thereby evaluating the presence of chronic CsA nephropathy at this time of the study. We observed that this group presented structural renal injury with arteriolopathy in 20.4 ± 1.6% of arterioles. In addition, 20.2% of the analyzed area was affected by tubulointerstitial fibrosis. These results together with the measurement of creatinine clearance after 18 days of CsA administration corroborated that at this time, the rats had already established chronic CsA nephrotoxicity. When spironolactone was started at day 18 in the CsA+Sp group, the chronic CsA nephropathy did not progress. Represented in Fig. 3 is the percentage of the area affected by fibrosis, together with representative photomicrographs, at the end of the study. In rats that received CsA, the percentage area affected by fibrosis was enhanced at 36 days to a value of 45.6 ± 3.8% (*P < 0.05 vs. 18 days). In contrast, the progression of renal fibrosis was reduced in rats that received spironolactone at the middle of the study (30.8 ± 2.3% in CsA+Sp vs. 45.6 ± 3.8% in CsA, *P < 0.005). In Fig. 4 are representative images of arteriolopathy in CsA and CsA+Sp groups together with the analysis of the percentage of arteriolopathy and arteriolar thickening in these groups. In the CsA+Sp group, arteriolopathy percentage tended to be minor, but the difference did not reach statistical significance. In contrast, MR blockade produced a significant reduction of arteriolar thickening, since mean values for CsA group were 9.3 ± 0.3 µm vs. those of CsA+Sp group that were 8.1 ± 0.3 µm (†P < 0.05).

The glomerular diameters were evaluated in rats that received vehicle, CsA, and CsA+Sp. In digitalized images, the diameter size was evaluated in at least 80 glomeruli per rat and distributed by rank. Figure 5 shows the normal diameter size distribution of the control group, in which it is possible to appreciate that most of the glomeruli are in the range of 101 to 125 µm (56.9%) and a minor proportion are in the range of 76 to 100 µm (25.7%) and 126 to 150 µm (13.9%). The histogram in control group presents a typical bell-shaped Gaussian distribution. In contrast, in chronic CsA-treated rats, the distribution of diameter size was shifted to the left, reflecting glomerular constriction. Accordingly, the glomerular diameters were smaller: 13.8% from 50 to 75 µm and 36.8% from 76 to 100 µm (25.7%). In addition, a lower proportion of glomeruli were in the diameter ranges from 101 to 125 µm (40.9%) and 126 to 150 µm (7.1%). All these differences were statistically significant by using a contingency analysis. The MR blockade was associated with less glomerular constriction in progressive chronic CsA nephrotoxicity (CsA+Sp), evidenced by a glomerular size distribution that was similar to control group. CsA+Sp group exhibited a greater percentage of glomerular diameters in the range from 101 to 125 µm (49.3%) and lesser proportion in the range from 50 to 75 µm (4.2%). Both differences were statistically significant when compared with the group treated with CsA alone.

Because it has been suggested that apoptosis plays a role in aldosterone-induced target organ damage (30), we evaluated
whether aldosterone receptor blockade modifies the apoptosis that is known to be induced by CsA during chronic nephropathy (43, 45). The presence of apoptotic cells in renal cortex in the CsA and CsA+Sp groups was evaluated with the TUNEL technique. Representative microphotographs of TUNEL staining in a rat treated with CsA for 36 days and a rat that received spironolactone after day 18 of CsA treatment up to the end of the study are shown in Fig. 6, A and B, respectively. About 60 digitalized images from renal cortex sections from CsA and CsA+Sp groups were obtained using Eclipse net software and

Fig. 4. A: percentage of arteriolopathy. B: arteriolar thickening in CsA (filled bars) and CsA+Sp groups (gray bars). C and D: representative images of arteriolopathy showing arteriolar thickening in CsA and CsA+Sp groups, respectively (magnification ×400). *P < 0.05 vs. CsA group.

Fig. 5. Glomerular diameter distribution in vehicle group represented by open bars, CsA-treated rats in filled bars, and CsA+Sp in gray bars. The significance was tested by contingency analysis and χ². *P < 0.05 vs. the same rank in V group. ** P < 0.05 vs. the same rank in CsA-treated rats.

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the positive nuclei per square millimeter per kidney were quantified. The results obtained are graphically expressed in Fig. 6C. Consistent with previous observations (43, 45), after 36 days of CsA treatment there was evident tubular cell apoptosis degree quantified as 698 ± 196 positive nuclei/mm². A significant reduction in the number of apoptotic cells (174 ± 31 positive nuclei/mm²) was observed in rats treated with CsA in which the MR was blocked starting on day 18 (CsA+/Sp group). In addition to these findings, we observed that CsA-treated rats exhibited a 2.5-fold increase in renal cortex procaspase 3 mRNA expression when compared with control group (Fig. 6D). Spironolactone administration also significantly reduced the expression of procaspase 3. These results revealed that CsA induced significant degree of apoptosis in the kidney that was reduced by MR blockade.

The renoprotective effect of spironolactone was also corroborated with the evaluation of the expression of Kim-1 that we demonstrated to be a sensitive marker of renal injury (48). Figure 7, A and B, shows cortical mRNA levels and urinary protein Kim-1, respectively. Chronic CsA nephrotoxicity was associated with a marked increase in Kim-1 mRNA and Kim-1 protein levels by 40- and 8-fold, respectively. The Kim-1 upregulation was partially prevented by the use of a MR inhibitor. An inversely proportional and significant correlation between Kim-1 urine levels and creatinine clearance was observed ($r = 0.8441$), thus, with greater Kim-1 expression is associated with a greater reduction in creatinine clearance.

We previously showed that prevention of chronic CsA toxicity when spironolactone was administered since the first day of treatment with CsA was associated with significant reduction in the CsA-induced upregulation of TGF-β-mRNA (11). As shown in Fig. 8, the chronic CsA-treated group exhibited a threefold increase in TGF-β mRNA levels normalized to 18S (3.1 ± 0.2), compared with control group. Administration of spironolactone to rats with existing chronic CsA toxicity was also corroborated with the evaluation of the expression of Kim-1 that we demonstrated to be a sensitive marker of renal injury (48). Figure 7, A and B, shows cortical mRNA levels and urinary protein Kim-1, respectively. Chronic CsA nephrotoxicity was associated with a marked increase in Kim-1 mRNA and Kim-1 protein levels by 40- and 8-fold, respectively. The Kim-1 upregulation was partially prevented by the use of a MR inhibitor. An inversely proportional and significant correlation between Kim-1 urine levels and creatinine clearance was observed ($r = 0.8441$), thus, with greater Kim-1 expression is associated with a greater reduction in creatinine clearance.

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nephrotoxicity partially prevented the increase in TGF-β mRNA levels (1.9 ± 0.1, P < 0.05).

**DISCUSSION**

In the present study, we found that MR blockade with spironolactone increases rat survival and prevents the progression of renal dysfunction, tubulointerstitial fibrosis, and arteriolar thickening. Furthermore, MR blockade reduces the amounts of apoptotic cells seen with preexisting chronic CsA nephrotoxicity. MR blockade mitigates the reduction of glomerular diameter and reduction of TGF-β and procaspase-3 mRNA levels, as well as Kim-1 expression seen with continued CsA treatment.

Distal convoluted tubule cells and principal cells of collecting duct were considered for many years to be the main cellular target of aldosterone actions. In the last decade, however, the localization of MR in several epithelial and nonepithelial tissues has provided evidence that aldosterone plays a role in a variety of physiological and pathophysiological processes (4, 14, 20, 31–37, 40). These studies support the hypothesis that in addition to its physiological role in maintaining extracellular salt, potassium and water homeostasis, aldosterone seems to play an additional role as a profibrotic hormone, thus contributing to organ damage when produced in excess. For example, it has been reported that treatment of patients with chronic heart disease with spironolactone or eplerenone improves the survival rates due, at least in part, to the reduction in myocardial fibrosis (5, 32). In this regard, recent studies from our laboratory have also demonstrated that aldosterone plays a pivotal role in both renal functional and structural toxic effects of CsA (11, 29). Our previous studies, however, were designed to assess the prevention of renal injury with MR blockade, rather than to examine possible prevention of progression and/or regression of already established renal injury.

Once chronic CsA was established after 18 days of treatment and confirmed through renal function and structural analysis, one group of rats received simultaneously CsA and spironolactone administration during 18 days more and was compared with the group that received only CsA for 36 days. We observed that renal dysfunction already established in CsA-treated rats was not reverted by MR blockade. However, spironolactone administration beginning on day 18 of CsA treatment avoided further renal function deterioration. At the structural level, spironolactone produced significant renal protection associated with a reduction of arteriolar thickening, apoptosis, and the area affected by tubulointerstitial fibrosis, despite the continuous administration of CsA. Because it is known that tubulointerstitial fibrosis is associated with higher rates of progression of renal disease (for a review, see Ref. 26), the reduction observed in fibrosis in the CsA+Sp group could be responsible for the reduction in renal injury.

In the present study, we observed that the degree of tubulointerstitial fibrosis but not arteriolopathy was lower after MR blockade, suggesting that spironolactone beneficial effect was more evident for tubulointerstitial fibrosis. However, in rats treated only with CsA, the degree of arteriolopathy observed at 18 days was similar to that at 36 days. That is, the percentage of arteriolopathy did not increase after 18 days. In contrast, the degree of fibrosis at 18 days was lower than at 36 days. Thus, the area affected by fibrosis progressed but the percentage of arteriolopathy did not. In this regard, however, it is worth that although reduction of arteriolopathy induced by spironolactone was not significant, there was a significant decrease in arteriolar thickening in the CsA+Sp group that was associated with greater glomerular size. We observed that distribution of glomerular diameters by rank in the CsA+Sp group was similar to control group, while CsA alone-treated animals exhibited a reduced glomerular size distribution, suggesting that MR blockade maintains a better renal perfusion state, counteracting CsA-inducing vasoconstriction. In this regard, we previously observed that spironolactone completely prevented the renal dysfunction and reduction of renal blood flow induced by CsA during acute nephropathy in which afferent arteriolar vasoconstriction is an important component (29), suggesting that aldosterone plays a role in the regulation of vascular tone. The mechanism of aldosterone-inducing vasoconstriction in CsA nephrotoxicity is not well understood. We have observed that CsA upregulates the expression of prorenin and modulates the expression of certain receptors that mediate vasoconstrictor or vasodilator actions that together could be responsible for renal vasoconstriction. These effects of CsA were partially prevented by spironolactone (29).

In the heart, it has been recognized that MR activation contributes to the pathophysiology of heart failure by inducing cardiomyocyte apoptosis that can be prevented by MR blockade, a finding that may explain the beneficial effects of MR blockade on ventricular remodeling following myocardial infarction (6, 24). Moreover, in recent studies, Burniston et al. (6) and Williams (51) demonstrated that aldosterone increased apoptosis also in skeletal muscle and human vascular endothelial cells through a mechanism that implicated activation of caspase-3. In the present study, we extended these findings to the kidney. Chronic CsA administration in rats fed with a low-sodium diet produced a significant increase in procaspase-3 mRNA levels and cellular apoptosis. These effects were reduced by MR blockade, suggesting that aldosterone promotes cell death by apoptosis in chronic CsA toxicity.

Previous studies from us and others have shown that chronic toxicity induced by CsA is associated with TGF-β overexpression and pharmacological agents that reduced the expression of this profibrotic cytokine-reduced renal injury (11, 42, 44, 52). Therefore, these studies emphasize the role of TGF-β in promoting renal structural injury. Indeed, renal protection conferred by spironolactone in preexisting chronic CsA nephrotoxicity was associated with reduction of TGF-β mRNA levels, confirming our previous finding that TGF-β upregulation by
cyclosporine is at least partially responsible for renal injury observed in this model (11). In this regard, Juknevicius et al. (20) observed that aldosterone infusion to normal rats resulted in twofold increase of TGF-β urinary excretion without changes at transcriptional level. Thus, in the model of CsA administration plus low-salt diet, two independent stimuli to increase TGF-β expression concur and the fact that spironolactone prevented the increase in TGF-β mRNA suggests that the mechanism by which CsA induced this upregulation involves the activity of the MR receptor.

Kim-1, a recently discovered type 1 transmembrane glycoprotein, is undetectable in normal kidneys but is upregulated ~10- to 100-fold following renal proximal tubular damage in humans, rats, and mice (3, 15, 17, 18, 48). The function of Kim-1 is unclear, but it is implicated in damage processes and it has been proposed as a kidney injury marker. The Kim-1 ectodomain is cleaved by metalloproteinases and is detectable in urine. In chronic CsA nephropathy characterized by tubular injury, we observed a significant Kim-1 overexpression, evidenced by a 40-fold increase of Kim-1 mRNA levels and an 8-fold increase in Kim-1 ectodomain amount detected in the urine. Interestingly, the renal structural protection conferred by spironolactone was associated with reduction of urinary Kim-1, supporting the notion that Kim-1 could also be a marker of renal injury in chronic nephropathies.

In summary, our data show that the aldosterone/MR pathway plays an important role in the pathophysiology of chronic CsA nephropathy and reveal that spironolactone administration decreases the slope of renal function deterioration and structural damage in preexisting chronic CsA nephrotoxicity, despite continuous CsA administration. Our data suggest the potential use of spironolactone to reduce chronic nephropathy in patients that are receiving this immunosuppressive agent and suggest that MR blockade could also be useful in other chronic nephropathies in which the insult cannot be avoided.

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