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Therapeutic benefit of spironolactone in experimental chronic cyclosporine A nephrotoxicity

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Therapeutic benefit of spironolactone in experimental chronic cyclosporine A nephrotoxicity.

Background. Cyclosporine A (CsA) is an immunosuppressive drug used to prevent tissue allograft rejection. However, its long-term utilization is limited due to chronic nephrotoxicity for which no prevention is available. This study evaluated the effect of spironolactone on renal functional and structural alterations induced by CsA, and assessed whether the protective effect was associated with a reduction of transforming growth factor-β (TGF-β) and the change of extracellular matrix protein mRNA level.

Methods. Male Wistar rats fed with low sodium diet were divided in four treatment groups: vehicle, CsA (30 mg/kg), spironolactone (20 mg/kg), or CsA+spironolactone. After 21 days, creatinine clearance (C_{Cr}), blood CsA, arteriolopathy in renal tissue, and TGF-β, collagen I, collagen IV, fibronectin, and epidermal growth factor (EGF) mRNA levels in renal cortex were determined.

Results. CsA reduced the C_{Cr} and up-regulated TGF-β, collagen I and fibronectin mRNA expression with a significant development of arteriolopathy, and reduced EGF mRNA levels. In contrast, spironolactone administration prevented the fall in renal function and TGF-β, collagen I, and fibronectin up-regulation, together with a reduction of arteriolopathy and tubulointerstitial fibrosis.

Conclusion. Our data show that aldosterone plays an important role as a mediator of renal injury induced by CsA. Thus, mineralocorticoid receptor blockade may be a potential strategy to prevent CsA nephrotoxicity.

Long-term cyclosporine (CsA) immunosuppressive therapy in allograft recipients and in patients with autoimmune diseases has been limited due to the concomitant development of chronic nephrotoxicity [1], which is characterized by derangement of renal function and architecture. Renal dysfunction seems to be the result of enhanced vasoconstrictor factors release, particularly due to increased endothelin production and to the activation of the renin-angiotensin-aldosterone system (RAAS) [2–5], while the structural damage, such as arteriolopathy and tubulointerstitial fibrosis, appears to be the consequence of several mechanisms including hypoxia, free radical production and up-regulation of transforming growth factor-β1 (TGF β1) synthesis [6–11]. The increase in this profibrotic cytokine, TGF-β1, results in the activation of extracellular matrix protein synthesis, such as collagen I and fibronectin [10, 12] as well as an increase in the renal apoptosis index [7, 13, 14], which are known to be associated with the development of kidney injury. In addition, it has been shown recently that epidermal growth factor (EGF), which mediates regeneration in tubular cells after renal damage, is significantly reduced during chronic CsA nephrotoxicity [14]. Thus, the reduction in EGF might contribute to enhance the structural changes observed in this condition.

Considerable attention has been directed to angiotensin II (Ang II) as a mediator of renal damage progression observed in several nephropathies, including chronic CsA toxicity. As a consequence, a therapeutic advantage has been observed with the use of angiotensin-enzyme-converting (ACE) inhibitors or Ang II receptor antagonists to retard the progression rate of renal failure in the clinical setting, including the chronic nephropathy associated with diabetes mellitus [15–17]. Although aldosterone is part of the renin-angiotensin-aldosterone axis, little attention has been addressed to this mineralocorticoid as a potential key molecule mediating renal damage. Recent studies suggest that aldosterone could play an important role in the progression of renal disease. Continuous infusion of aldosterone in normal rats induced up-regulation of TGF-β mRNA in the kidney (abstract;

Key words: aldosterone, arteriolopathy, interstitial fibrosis, TGF-β, renal function, low sodium diet, mineralocorticoid therapy.

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Juknevicius et al, *J Am Soc Nephrol* 11:622A, 2000) and the inhibition of aldosterone actions with the aldosterone receptor blocker, spironolactone, reduced renal damage without lowering blood pressure in both the renal ablation model and the stroke-prone spontaneously hypertensive rat [18–20]. Hostetter et al speculated that the mechanism by which mineralocorticoids promote renal injury in these models could be explained by the up-regulation of TGF-β1 induced by aldosterone [21]. In this regard, the structural injury that is observed in the chronic CsA nephropathy model in the rat (induced by administration of CsA in animals fed a low salt diet) has been associated with an increase in TGF-β1 expression [9, 10], which can be partially abrogated by the concomitant administration of the Ang II receptor antagonist losartan [9]. However, because losartan administration also will result in a reduction of aldosterone release, it is unknown if the protective effect of losartan is due to blockade of the Ang II receptors in the kidney, by the reduction of aldosterone secretion, or by a combination of both mechanisms.

Our present study used a model of chronic CsA nephrotoxicity and showed that spironolactone administration effectively prevented renal dysfunction and up-regulation of TGF-β1 and extracellular matrix proteins mRNA, and reduced the structural damage induced by CsA. These data suggest that aldosterone is a key molecule in the pathogenesis of chronic CsA nephropathy.

METHODS

The present study utilized the chronic CsA nephrotoxicity model in the rat that is produced by the administration of CsA and a low sodium diet. Four groups of twelve male Wistar rats, weighing 350 g and fed with low salt diet (0.02%), were included in the study. Group I (V) animals received 0.1 mL SC of olive oil as the vehicle every 24 hours. Group II (CsA) rats were treated with a daily dose of CsA 30 mg/kg SC. Group III (SPIRO) received 20 mg/kg/day of spironolactone by gastric gavage, and group IV comprised rats treated with CsA+SPIRO. All animals were treated during 21 days. The V and SPIRO groups were pair-fed with the CsA and CsA+SPIRO groups, respectively.

Functional studies

Systolic blood pressure was measured by a non-invasive tail cuff method at the end of the experimental protocol (Model 179, IITC Life Science, Woodland Hills, CA, USA). All animals were placed in metabolic cages and urine that was spontaneously voided during every 24 hours was collected. Serum and urine creatinine concentration were measured with an autoanalyzer (Technicon RA-1000; Bayer Co. Tarrytown, NY, USA). Renal creatinine clearances were calculated by the standard formula $C_{Cr} = U \cdot V/P$, where U is the concentration in urine, V is the urine flow rate and P is plasma concentration. In all studied groups urinary protein excretion was determined using the trichloroacetic acid (TCA) turbidimetric method [22], and serum aldosterone and potassium levels were determined by radioimmunoassay (RIA) and with a NOVA4 electrolyte analyzer (NOVA Biomedical, Waltham, MA, USA), respectively. Blood CsA concentration also was determined by monoclonal radioimmunoassay kit (TDx/TDxFLx; Abbott Laboratories, Abbot Park, IL, USA) in groups receiving CsA.

Kidneys were obtained from six rats of each group for histological study. Rats were anesthetized by intraperitoneal injection of sodium pentobarbital and their kidneys were excised, macroscopically divided into renal cortex and medulla, frozen in liquid nitrogen, and kept at −80°C until used.

Histological studies

Before renal cortex separation, one half of the left kidney was taken and fixed in alcoholic Bouin's solution for light microcopy studies. After an appropriate dehydration, kidney slices were embedded in paraffin, sectioned at 3 μ, and periodic acid-Schiff (PAS), Van Gieson and trichromic stains were performed. Arteriolopathy percentage was determined by counting at least 100 afferent arterioles showing the characteristic lesion of this model. Arteriolopathy was counted as present or nonpresent (dichotomic variable). Thus, the results are expressed as the percentage of affected arterioles over total number of arterioles. The degree of tubulointerstitial fibrosis was evaluated by morphometry. For this purpose, ten subcortical periglomerular fields per Van Gieson stained section (magnification, ×200) were randomly selected in kidneys from the different groups. The images were recorded and the affected areas were delimited and semiquantified using the Leica processing and analysis system Ltd., (Leica Imaging System Ltd., Cambridge, UK). Finally, the proportion of fibrosis was calculated dividing the interstitial fibrosis by total area. The histological analysis was performed without knowing the group at which each kidney belonged.

Molecular studies

RNA isolation. Total RNA was isolated from each renal cortex or medulla following the guanidine isothyocianate-cesium chloride method [23]. 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, USA).

Northern blot analysis. Aliquots of 20 μg of total RNA from each renal cortex sample were separated by 0.9% agarose/formaldehyde gel electrophoresis and transferred to a nylon membrane (Biotrans; ICN, East Hills, NY, USA), and fixed by UV cross linking (Stratalinker; Stratagene, La Jolla, CA, USA). The probes used were TGF-β1,

collagen alpha I chain and collagen alpha IV chain obtained from mouse, as well as fibronectin and glyceraldehyde-3-phosphate dehydrogenase (GADPH) from rat. Labeling of cDNA probes was done using the Random Primer Labeling Kit (Boehringer, Mannheim, Germany) and [α^{32}P]-dCTP (Amersham, Little Chalfont, Buckinghamshire, UK) accordingly to the manufacturer's instructions. Hybridization was performed overnight at 42°C in 5 × standard sodium citrate (SSC), 50 mmol/L sodium phosphate (pH 6.8), 50% formamide, 5 × Denharts solution, 0.1% sodium dodecyl sulfate (SDS), and 250 μg/mL of Torula yeast total RNA. Membranes were washed three times for 10 minutes at room temperature in 2 × SSC, 0.1% SDS, and twice for 45 minutes at 68°C in 0.1 × SSC, 0.1% SDS. Autoradiographs were scanned and the hybridization bands were measured by densitometric analysis.

Semiquantitative RT-PCR. The relative level of EGF and GAPDH mRNA expression was assessed in the renal cortex by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR), as previously described [24, 25]. Briefly, primer sequences [26, 27] were custom obtained from Invitrogen (Gaithersburg, MD, USA).

Reverse transcription (RT) was carried out using 10 μg of total RNA from the renal cortex of each rat. RT was performed at 37°C for 60 minutes 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 mmol/L of each dNTP (Sigma Chemical Co., St. Louis MO, USA), and 1× RT buffer [75 mmol/L KCl; 50 mmol/L Tris-HCl; 3 mmol/L $MgCl_2$; 10 mmol/L dithiothreitol (DTT), pH 8.3]. One tenth of the RT from each individual sample was used for EGF, and GAPDH amplification in 20 μL final volume reactions containing 0.2 μCi of [α^{32}P]-dCTP (~3000 Ci/mmol, 9.25 MBq, 250 μCi). PCR cycles were performed in a DNA thermal cycler (M.J. Research, Watertown, MA, USA). The control gene was amplified simultaneously in each reaction. Amplification kinetics for EGF and the housekeeping gene GAPDH in renal cortex total RNA, and the optimal number of cycles for each primer pair were assessed as we previously reported [24, 25]. To analyze the PCR products, one-half of each reaction was electrophoresed in a 5% acrylamide gel. Bands were ethidium bromide stained and visualized under UV light, cut out, suspended in 1 mL of scintillation cocktail (Ecolume; ICN, Aurora, OH, USA), and counted by liquid scintillation (Beckman LS6500). All reactions were performed individually from each cortex total RNA in duplicate. Genomic DNA contamination was checked by treating all RNA samples with RNAse-free DNAase I and by carrying samples through PCR procedure without adding reverse transcriptase.

Statistical analysis

The results are presented as mean ± SEM. The significance of the differences between groups was tested by analysis of variance (ANOVA) comparison using Bonferroni's correction for multiple comparisons. Statistical significance was defined as two-tailed $P < 0.05$.

RESULTS

Physiological and functional studies

All treatments were well tolerated by all rats along the study. The CsA and CsA+SPIRO groups lost body weight gradually over 21 days of treatment. Mean body weights for these groups were 355.4 ± 6.2 and 353.6 ± 1.68 g, respectively, at the beginning of the experiment and 276.2 ± 4.1 and 294.0 ± 7.4 g, respectively, at the end. In contrast, the V and V+SPIRO groups that were pair-fed showed a slight gain in body weight during the study. The initial body weights were 358.5 ± 4.2 and 356.3 ± 6.0 g, respectively, and final body weights were 384.75 ± 5.8 and 369.1 ± 7.1 g, respectively.

Figure 1 depicts the most important physiological parameters that were evaluated in control and experimental groups. After 21 days, systolic blood pressure (SBP) was measured. As has been previously reported by several authors [7, 12, 14, 28], CsA administration in this experimental model is not associated with hypertension. SBP in the control group was 125.5 ± 3.4 whereas it was 132.6 ± 8.6 mm Hg in CsA treated rats (P = NS). Spironolactone also had no effect in SBP (123.5 ± 3.1 mm Hg). Thus, all of the rats studied were normotensive. As shown in Figure 1B, the amount of proteins in urine was not different among the groups, and although the proteinuria levels were less in CsA treated rats, as reported previously [12], this difference was not statistically different. In order to evaluate the effect of cyclosporine and spironolactone on the well known increase in serum potassium levels produced by these compounds, serum potassium was determined in all four groups. As Figure 1C shows, CsA administration resulted in hyperkalemia since the serum potassium in this group was 4.3 ± 0.3 mEq/L, which is significantly different to the control group (3.4 ± 0.2 mEq/L, $P < 0.05$). Serum potassium in the CsA+SPIRO group was 4.4 ± 0.2 mEq/L. Thus, spironolactone administration had no further effect on serum potassium levels. Finally, it is well known that a low sodium diet activates aldosterone production. Figure 1D shows a significant increase in aldosterone levels in all of the groups studied. Although values were slightly higher in rats receiving spironolactone, the differences were not significant.

To evaluate the effect of aldosterone inhibition with spironolactone on renal function, creatinine clearance (C_{Cr}), as an index of glomerular filtration rate, was mea-

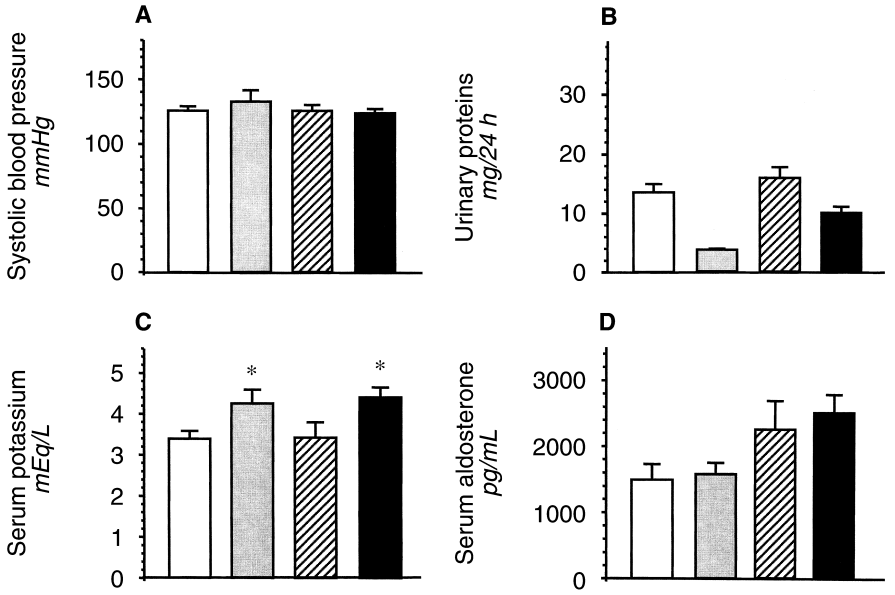

Fig. 1. (*A*) Systolic blood pressure, (*B*) urinary protein excretion, (*C*) serum potassium, and (*D*) serum aldosterone levels in the vehicle group (□), CsA treated animals (■), SPIRO group (▨) and CsA+SPIRO treated rats (■). $^*P < 0.05$ vs. V, SPIRO. Although not shown, serum aldosterone levels in rat fed with normal salt diet are around 100 pg/mL.

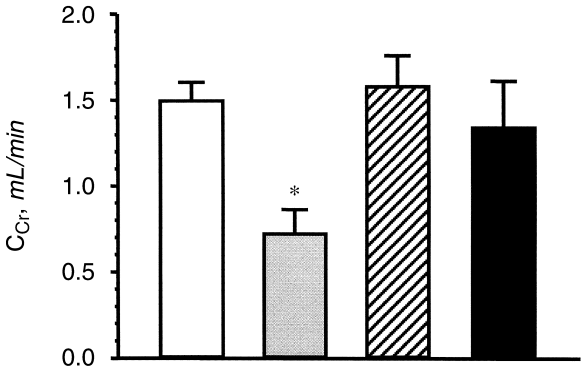

Fig. 2. Glomerular filtration rate estimated by creatinine clearance in vehicle group (□), animals treated with CsA during 21 days with 30 mg/kg (■), SPIRO group (▨) and CsA+SPIRO treated rats (■) maintaining on low sodium diet, $^*P < 0.05$ vs. V, SPIRO, and CsA+SPIRO groups.

sured at the end of the 21 days of treatment in the four groups of rats. As shown in Figure 2, the administration of CsA resulted in a significant reduction in the glomerular filtration rate, from 1.49 ± 0.1 mL/min in the V group to 0.72 ± 0.1 mL/min in CsA group, whereas inhibition of aldosterone with spironolactone in the V+SPIRO group had no effect on this parameter (1.58 ± 0.2 mL/min). Interestingly, spironolactone administration prevented the fall in renal function induced by CsA treatment, since the C_{Cr} in the CsA+SPIRO treated rats was 1.34 ± 03 mL/min. This C_{Cr} value was not statistically different from values observed in V and V+SPIRO control groups. To differentiate if the protective effect of spironolactone was related to a decrease in CsA blood levels, we assessed the CsA concentration in blood from CsA and CsA+SPIRO groups. The value observed in CsA+SPIRO rats of 3594 ± 133 ng/mL was 14% lower than the value of 4199 ± 99 ng/mL obtained in rats treated with CsA alone. Although the reduction was statistically significant ($P < 0.05$), it does not explain the protection conferred by spironolactone in CsA-treated animals, since the blood CsA concentration was still within the toxic range reported by other investigators [10, 11, 14]. In addition, it is known that one tenth of CsA is metabolized in the kidney. Accordingly, it is feasible that the small reduction in CsA blood levels observed in CsA+SPIRO group could be secondary to the restoration of renal function.

Histological studies

To establish if aldosterone receptor blockade with spironolactone also reduces or prevents the structural damage induced by CsA administration, the degree of afferent arteriolopathy and tubulointerstitial fibrosis was quantified. The percentage of arteriolopathy was obtained by counting at least 100 preglomerular afferent arterioles in each rat. A representative light microscopy image of the characteristic arteriolar lesion of CsA nephropathy is shown in Figure 3A. This alteration was mainly located in the preglomerular afferent arteriole and extended several microns upstream, consisting of an enlargement of the smooth muscle cells and resulting in a partial or total narrowing of the arteriolar lumen. The affected cell cytoplasm showed eosinophylic, PAS positive, homogeneous or granular degeneration. Endothelial cells looked prominent and perivascular cells seemed proliferated. The percentage of afferent arterioles showing this kind of lesion is shown in Figure 3B. As we previously reported [29], in the control group treated with vehicle alone, a few afferent arterioles were shown

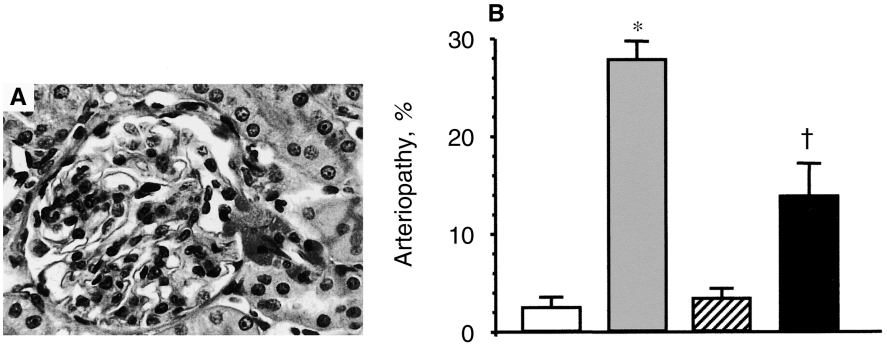

Fig. 3. (*A*) Afferent arteriolopathy in CsA treated rat during 21 days (PAS stain ×350). (*B*) Percentage of afferent arterioles injured, which was estimated in at least 100 preglomerular afferent arterioles from vehicle group (□), CsA treated animals (■), SPIRO group (▨) and CsA+SPIRO treated rats (■). *$P <$ 0.05 vs. V, SPIRO and CsA+SPIRO groups, and †$P < 0.05$ vs. V, SPIRO and CsA groups.

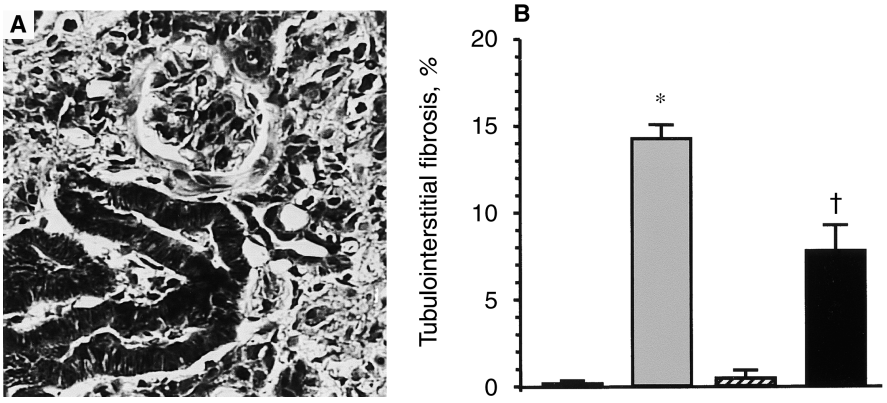

Fig. 4. (*A*) Tubulointerstitial fibrosis observed in a salt depleted rat treated with CsA (Van Gieson stain ×200). (*B*) Mean percentage of injured area obtained in at least ten fields. *$P <$ 0.05 vs. V, SPIRO and CsA+SPIRO groups and †$P < 0.05$ vs. V, SPIRO and CsA groups.

to have arteriolopathy (2.6%), possibly as a result of activation of the RAAS system induced by a low sodium diet. This low frequency of arteriolopathy was similar in the SPIRO group. In contrast, as expected, in the chronic CsA group, arteriolar lesion was present in as much as 28% of glomeruli, whereas this percentage of arteriolopathy was reduced to 14% in the CsA+SPIRO group.

The photomicrograph presented in Figure 4A is a representative example of the tubulointerstitial fibrosis observed in CsA sodium-depleted animals. The morphometric analysis is shown in Figure 4B. As expected, significant tubulointerstitial fibrosis was observed in the group receiving CsA. In contrast, spironolactone was able to reduce this alteration. These observations suggest that at least part of the CsA-induced arteriolar and tubulointerstitial lesions are mediated by aldosterone, and demonstrates that spironolactone not only prevents the negative effects of CsA at the functional, but also at the structural level.

Molecular studies

To begin to understand the mechanisms by which spironolactone protects the kidney from CsA toxicity, we evaluated the gene expression level of extracellular matrix proteins that are known to be involved in the pathogenesis of chronic renal injury. The autoradiographs of the Northern blot analysis of total RNA from renal cortex using specific probes for TGF-β, collagen I, collagen IV, and fibronectin are shown in Figure 5, together with the Northern blot analysis of the housekeeping gene GAPDH that was used to control each experiment. The densitometric analysis of TGF-β1 is shown in Figure 6. In contrast to vehicle-treated animals, CsA administration in sodium-depleted rats produced a marked increase in TGF-β1 mRNA levels. The TGF-β/GAPDH ratio was 1.1 ± 0.1 in the vehicle group and 4.5 ± 0.5 in the CsA group ($P < 0.01$). The administration of aldosterone antagonist spironolactone had no effect on TGF-β1 mRNA expression, since the TGF-β/GAPDH ratio in the SPIRO group was 1.6 ± 0.1. In contrast, in CsA+SPIRO group the TGF-β/GAPDH ratio was 2.0 ± 0.4. This value was significantly lower than the expression observed in the CsA group (2.0 ± 0.4 vs. 4.5 ± 0.5, $P < 0.05$), but not different to the value observed in the SPIRO group (2.0 ± 0.4 vs. 1.6 ± 0.1, $P =$ NS). Accordingly, spironolactone administration prevented the TGF-β1 up-regulation induced by CsA.

The effects of CsA and spironolactone on the expression of extracellular matrix proteins such as collagen I, collagen IV, and fibronectin mRNAs in renal cortex are shown in Figure 7. Chronic CsA nephrotoxity was associated with a fivefold up-regulation of collagen I. The ratio

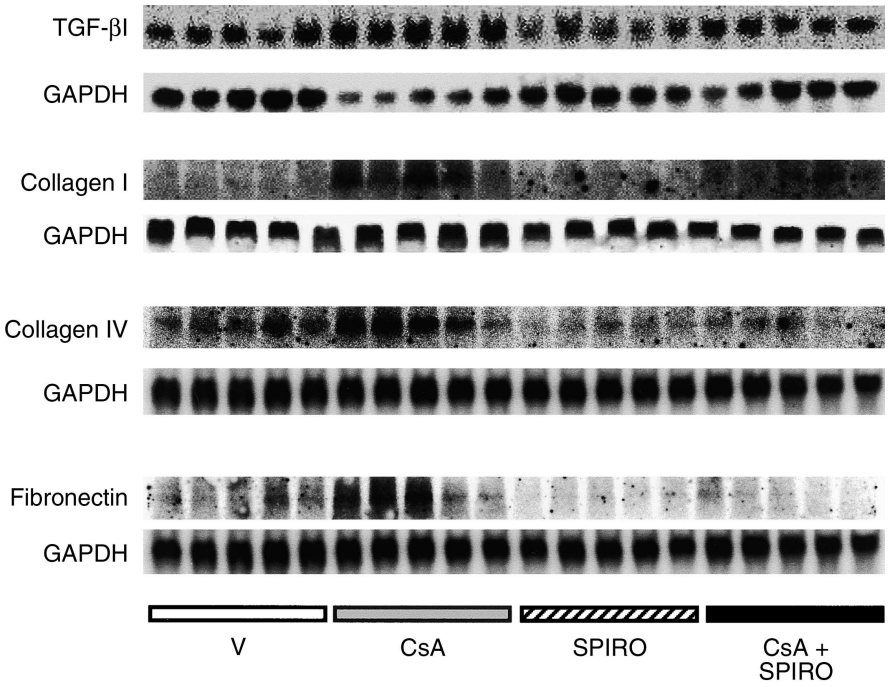

Fig. 5. Northern blot analysis of mRNA from renal cortex of the four study groups using specific probes for TGF-β1, collagen I, collagen IV, fibronectin and GAPDH. Each lane was loaded with 20 μg of total RNA from a different rat, corresponding to each group as stated.

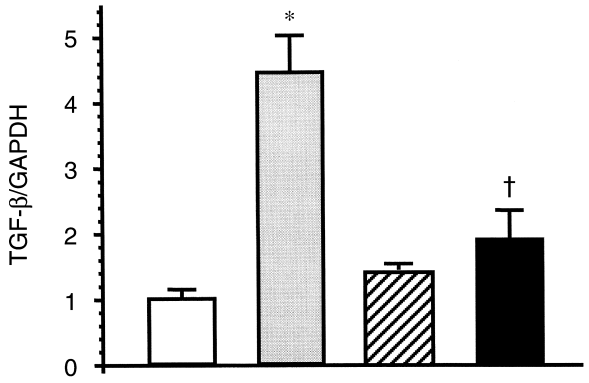

Fig. 6. TGF-β1 mRNA levels in renal cortex are represented as the ratio between TGF-β1 and GAPDH bands densitometry in vehicle group (□), CsA-treated animals (▩), SPIRO group (▨) and CsA+SPIRO treated rats (■). *$P < 0.05$ vs. V, SPIRO and CsA+SPIRO groups and †$P < 0.05$ vs. CsA group.

of collagen I/GAPDH in the CsA group was 3.8 ± 0.9, whereas in the V group it was 0.6 ± 0.05, $P < 0.01$. This up-regulation of collagen I induced by CsA was abrogated by spironolactone, since the ratio in CsA+SPIRO group was 1.6 ± 0.3. A similar observation was obtained in the analysis of fibronectin mRNA expression that is shown in Figure 7B. The ratio of fibronectin/GAPDH increased in CsA treated rats and this up-regulation was prevented by spironolactone. The values of the ratio between collagen IV and GAPDH are shown in Figure 7C. The expression of collagen IV in the CsA group was increased, but the difference with the V group did not reach significance with ANOVA for multiple comparisons (V group 0.36 ± 0.01 vs. CsA group 0.51 ± 0.07, $t = 2.77$, $P > 0.05$). However, the ratio of collagen IV/GAPDH observed in the CsA+SPIRO group (0.18 ± 0.03) was significantly lower than the ratio in the CsA treated group (0.51 ± 0.07, $t = 6.1$, $P < 0.01$). Thus, CsA induced up-regulation of extracellular matrix proteins mRNA in renal cortex, and this effect of CsA was prevented when spironolactone was simultaneously administered.

It has been suggested previously that down-regulation of the EGF contributes to the structural damage induced by toxic agents, including CsA [14, 30], since EGF is a potent mitogenic agent and its expression has been associated with renal repair following ischemic injury. Thus, we analyzed the effect of CsA and spironolactone on the EGF mRNA levels in renal cortex following a semiquantitative RT-PCR strategy. The upper panel of Figure 8 shows the PCR products obtained for each rat and the lower panel shows the mean ratio between EGF and GAPDH of each group. CsA treatment induced a significant reduction in the expression of EGF as compared to the V group (1.5 ± 0.1 vs. 3.9 ± 0.1, respectively, $P < 0.01$). Interestingly, spironolactone alone also induced a slight decrease in EGF mRNA levels (3.0 ± 0.1 SPIRO, $P < 0.05$ vs. V group). However, when spironolactone was administered with CsA, the ratio of EGF/GAPDH was 2.2 ± 0.3. This value was significantly higher than the value observed in the CsA group, suggesting that spironolactone partially reversed the down-regulation of EGF induced by chronic CsA toxicity.

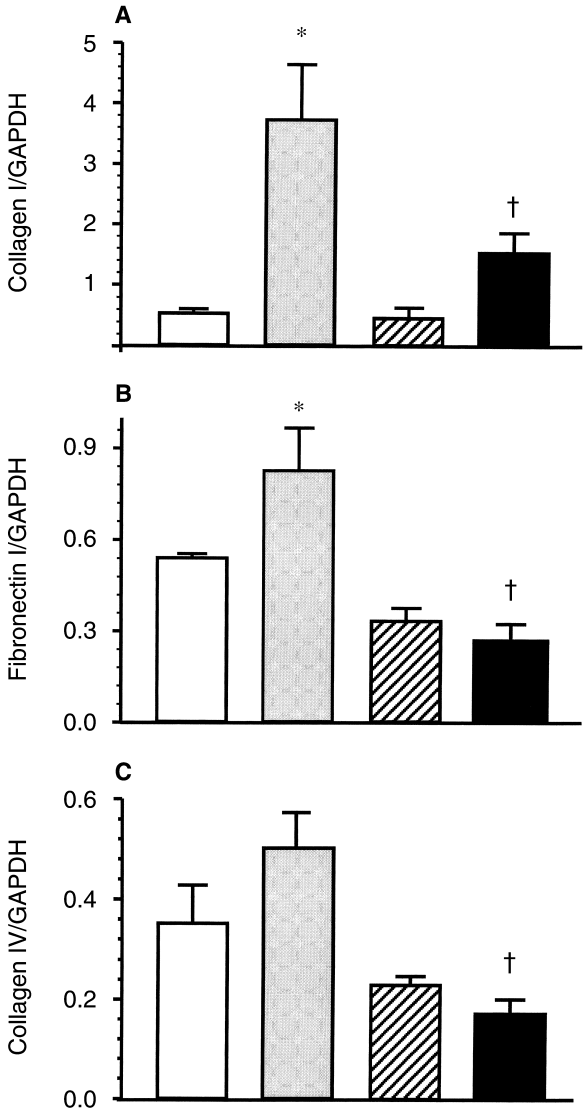

Fig. 7. Effects of CsA and SPIRO on the expression of extracellular matrix proteins. (*A*) Collagen I, (*B*) fibronectin and (*C*) collagen IV in vehicle group (□), CsA-treated animals (■), SPIRO group (▨) and CsA+SPIRO treated rats (■). *$P < 0.05$ vs. V, SPIRO and CsA+SPIRO groups and †$P < 0.05$ vs. CsA-treated rats.

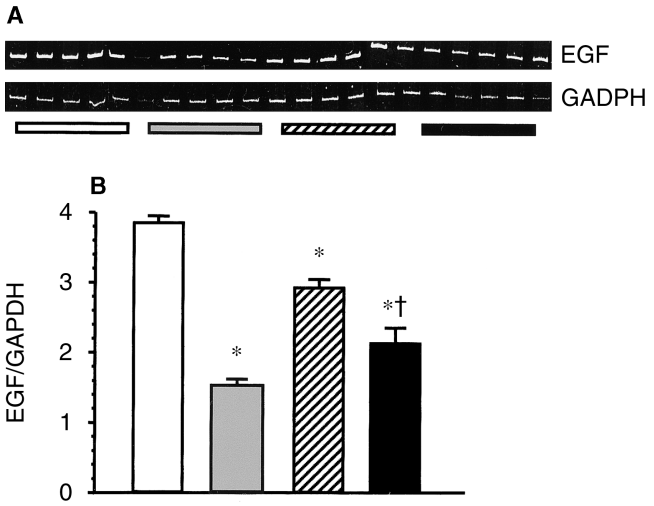

Fig. 8. Epidermal growth factor mRNA levels in renal cortex assessed by RT-PCR. (*A*) An acrylamide gel showing EGF and GAPDH amplification bands for all of the study groups. Each lane was loaded with 10 μL of the RT-PCR reaction from different animals. (*B*) Graphic representation of the mean ratio between EGF and GAPDH for the Vehicle group (□), CsA-treated animals (■), SPIRO group (▨) and CsA+SPIRO treated rats (■). *$P < 0.05$ vs. V and †$P < 0.05$ vs. CsA group.

DISCUSSION

Using a model of chronic CsA nephrotoxicity in the rat, our present study shows that aldosterone receptor blockade with spironolactone effectively prevents the fall in renal function, as well as reduces the arteriolopathy and tubulointerstitial fibrosis induced by CsA. The protective effect of spironolactone was associated with prevention of the CsA-induced up-regulation of TGF-β, collagen I, collagen IV, and fibronectin, and partial restoration of CsA-inducing down-regulation of EGF mRNA levels in renal cortex.

A chronic CsA nephrotoxicity model induced by CsA administration in rats fed with a low salt diet is characterized by renal failure accompanied by severe renal vasoconstriction, which is due at least in part to activation of the renin-angiotensin-aldosterone system (RAAS). Our data show that C_{Cr} of the rats treated with spironolactone and CsA was similar to the vehicle-treated animals and significantly higher than the C_{Cr} observed in the CsA group (Fig. 2). Therefore, aldosterone receptor blockade completely prevented the decrease in glomerular filtration rate induced by CsA. It is unlikely that this spironolactone-conferred protection was due to the effect of this diuretic agent on the blood pressure levels, since this model is not associated with the development of hypertension. SBP was similar among all of the study groups. It is also unlikely that protection conferred by spironolactone was secondary to the small reduction on CsA blood levels observed in the CsA+SPIRO group. It has been shown that CsA levels above 1000 pg/mL are enough to produce renal damage [10, 11, 14]. In addition, because it is known that one tenth of CsA is metabolized in the kidney, the small reduction in CsA blood levels observed in CsA+SPIRO group was probably secondary to the restoration of renal function.

This beneficial effect of spironolactone was associated with a significant reduction of arteriolopathy and tubulointerstitial fibrosis, which is the structural hallmark of CsA injury in the kidney. Several therapeutic agents such as endothelin [31] and angiotensin inhibitors [9, 14, 31], vascular endothelial growth factor (VEGF) [28], polysulphate pentosan [29] and L-arginine [32, 33] have been investigated as possible tools to reduce chronic CsA nephrotoxicity. However, most of these treatments have not effectively reduced both functional and structural

changes induced by CsA in the kidney. Although administration of losartan [9, 14], VEGF [28], and polysulfate pentosan [29] partially prevented the structural damage induced by CsA, these agents did not improve the renal dysfunction during chronic CsA nephrotoxicity.

The mechanism for arteriolopathy and chronic renal damage to the kidney induced by CsA is unknown and, thus, the mechanism of protection by spironolactone is not clear. Acute experimental CsA nephrotoxicity is directly related to renal vasoconstriction and this form of nephrotoxicity can be completely prevented by coadministration of dexamethasone [34], arginine [35, 36], or glycine [6] as well as enalapril and spironolactone [4]. Thomson et al [4] and Iacona et al [37] observed that spironolactone improved renal function during acute CsA nephrotoxicity in the rat. In contrast, chronic CsA toxicity not only involves renal injury due to hypoxia, but also is due to up-regulation of TGF-β and extracellular matrix proteins [9, 10] as well as an increase in apoptosis [7]. Interestingly, Viera et al have shown up-regulation of TGF-β expression within the afferent arterioles, suggesting that this cytokine not only mediates interstitial lesions, but also might be a mediator of hypertrophy observed in juxtaglomerular arterioles [11]. Shihab et al showed that chronic CsA toxicity in the rat is associated with up-regulation of TGF-β expression, and that this effect could be prevented by Ang II blockade, either with the enzyme converting inhibitor enalapril or the angiotensin II receptor antagonist losartan [9]. The use of these compounds, however, did not prevent renal dysfunction, suggesting that up-regulation of TGF-β during CsA toxicity is independent of renal hemodynamics, and is mediated, at least in part, directly by Ang II. With our current data, however, we propose that CsA-induced overexpression of TGF-β is not mediated by Ang II. Instead, it is mediated by aldosterone, since it can be completely prevented by spironolactone (Fig. 6). In this regard, preliminary evidence shows that aldosterone infusion in normal rats increased TGF-β mRNA levels in the kidney (abstract; Juknevicius et al, *J Am Soc Nephrol* 11:622A, 2000). The chronic model for CsA toxicity is produced in rats that are fed with low salt diet. Hence, the activity of the renin-angiotensin system is enhanced and this also includes the increased release of aldosterone. Therefore, the beneficial effect of losartan or enalapril on TGF-β expression observed by Shihab et al [9] is probably the result of decreasing aldosterone release that is expected to occur when angiotensin blockers are used. In contrast to Shihab et al, we observed that spironolactone not only prevented the increase in the expression of TGF-β in the renal cortex, but also the functional and structural damage induced by CsA. Thus, spironolactone appears to be a helpful agent that effectively offers a therapeutic benefit in chronic CsA nephrotoxicity in the rat.

Increased attention has arisen on aldosterone as a potentially important mediator of chronic heart failure and renal disease. The use of spironolactone improves the survival rate in patients with chronic heart disease [38]. Moreover, studies in two different experimental models of renal disease, one due to hypertension and the other due to renal ablation, has shown that aldosterone infusion abrogates the renal protection conferred by Ang II inhibition either with ACE inhibitors, or Ang II receptor antagonists. In the model of severe hypertension in stroke-prone spontaneously hypertensive rats that were fed with high salt diet, Rocha et al observed that glomerular and vascular lesions were prevented with spironolactone or captopril, and the effect of captopril was reversed when rats were treated with aldosterone [19, 20]. In the renal ablation model, Greene, Kren and Hostetter provided evidence that aldosterone contributes to the development of hypertension and renal injury [18]. In these rats with 5/6 nephrectomy, losartan and enalapril reduces proteinuria and nephrosclerosis, but the protection conferred by these agents was completely avoided when aldosterone was re-infused to maintain levels comparable to untreated rats with reduced renal mass. In addition, selective blockade of aldosterone with eplerone reduced proteinuria and glomerulosclerosis in L-NAME hypertensive rats [39]. Thus, aldosterone seems to play an important role in the progression of renal diseases. In this regard, recently it has been suggested that patients with diabetic nephropathy and already treated with enalapril may obtain further benefit by adding spironolactone to their daily treatment [40].

The exact mechanisms by which spironolactone protects the kidney from CsA toxicity are not clear from our current data and further studies will be necessary to clarify this issue. However, our observation that renal function was completely restored by spironolactone administration in CsA-treated rats suggests that, in addition to its effect on TGF-β expression, aldosterone blockade counterbalances the renal vasoconstriction induced by CsA. Although the mechanisms for this effect remain to be elucidated, it has been proposed that aldosterone modulates the vascular tone [41], possibly through increased vasoconstrictive effects of catecholamines [42], impaired vasodilation in response to acetylcholine [43], up-regulation of β-adrenergic and Ang II receptors [44–46], and a direct aldosterone effect, which could be mediated by non-genomic mechanisms [41]. The fact that arginine administration also prevented renal dysfunction and partially reversed the structural injury during chronic CsA toxicity [10] suggests that the possible vasodilatory action of spironolactone administration is responsible, at least in part, for the renal protection observed in our study.

Epidermal growth factor (EGF) is a 53-amino acid polypeptide that is known to be important as mediator of renal repair following injury. Recently, Yang et al showed

that chronic CsA nephrotoxicity is associated with a reduction of EGF protein levels in the kidney, which correlates with the degree of fibrosis and apoptosis [14]. Because these effects were reversed with losartan administration, the authors proposed that Ang II is the principal mediator of tubular injury through its effect on EGF. The present study confirms a down-regulation of EGF mRNA levels in the renal cortex of CsA treated rats. We also observed partial restoration of EGF expression when spironolactone was concomitantly administered with CsA. Thus, our data together with observations by Yang et al [14] show that blockade of either Ang II or aldosterone partially abrogates the EGF down-regulation observed in CsA-treated animals, and that this effect was associated with a significant reduction of tubulointerstitial damage.

In conclusion, our findings show that aldosterone plays a key role in producing functional and structural changes associated with CsA nephrotoxicity, and points to mineralocorticoid blockade as a potential treatment for reducing renal toxicity induced by CsA.

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