Renocortical mRNA expression of vasoactive factors during spironolactone protective effect in chronic cyclosporine nephrotoxicity

Jazmin M. Pérez-Rojas, Stephanie Derive, Jorge A. Blanco, Cristino Cruz, Lilía Martínez de La Maza, Gerardo Gamba, and Norma A. Bobadilla

Department of Molecular Physiology, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, and Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, Mexico City, Mexico

Submitted 20 April 2005; accepted in final form 28 June 2005

Abstract

Renocortical mRNA expression of vasoactive factors during spironolactone protective effect in chronic cyclosporine nephrotoxicity. Am J Physiol Renal Physiol 289: F1020–F1030, 2005.—We showed that spironolactone reduced structural damage and prevented renal dysfunction in chronic cyclosporine (CsA) nephrotoxicity. These findings evidenced an aldosterone renal vascular effect under this condition. To investigate aldosterone’s role in modulating renal vascular tone, renocortical vasoactive pathways mRNA levels in chronic CsA nephrotoxicity as well as spironolactone’s effect on renal function in acute CsA nephrotoxicity were evaluated. Two experimental sets were designed. For chronic nephrotoxicity, rats fed with low-sodium diet were divided into groups receiving vehicle, spironolactone (Sp), CsA, and CsA+Sp, for 21 days. Creatinine clearance, survival percentage, and renocortical mRNA levels of pro-renin, angiotensinogen (Ang), angiotensin receptors (AT1A, AT1B, and AT2), preproendothelin, endothelin receptors (ETα, ETβ), cyclooxygenase-2 (COX-2), and adenosine receptors (Ad1, Ad2A, Ad2B, and Ad3) were analyzed. For acute nephrotoxicity, similar groups fed with a standard chow diet for 7 days were included. Serum potassium and sodium, glomerular filtration rate (GFR), and renal blood flow (RBF) were determined. In chronic model, CsA produced pro-renin and ET upregulation, altered adenosine receptors expression, and reduced Ang, AT1A, AT1B, ETβ, and COX-2 mRNA levels. Spironolactone protective effect in chronic nephrotoxicity was associated with prevention of pro-renin upregulation and increased AT2, together with ETβ reduction. In acute nephrotoxicity, spironolactone completely prevented GFR and RBF reduction induced by CsA. Our results suggest that aldosterone contributes to renal vasoconstriction observed in CsA nephrotoxicity and that renoprotection conferred by spironolactone was related to modification of renocortical vasoactive pathways expression, in which pro-renin normalization was the most evident change in chronic nephropathy. Finally, our data point to spironolactone as a potential treatment to reduce CsA nephrotoxicity in transplant patients.

Renin; AT1 receptors; COX-2; acute CsA nephrotoxicity

Cyclosporine A (CsA) is a widely used immunosuppressive drug to prevent tissue allograft rejection. However, its use for long periods is limited due to its nephrotoxic effect, which is characterized by renal vasoconstriction and the development of histological lesions such as arteriolopathy and scarring tubulointerstitial fibrosis (12), which in turn are associated with loss of renal function and the development of end-stage renal disease. In a large study of diabetic recipients of a kidney-pancreas transplant, Nankivell et al. (40) observed that after 10 yr of immunosuppression with calcineurin inhibitors, all patients eventually developed nephropathy.

Because CsA is considered as an excellent immunosuppressive drug (12), several therapeutic agents have been studied in animal models of chronic CsA nephrotoxicity to prevent or decrease CsA-inducing toxic renal effects. The tested agents include endothelin (32) and angiotensin inhibitors (32, 53, 66), vascular endothelial growth factor (VEGF) (29), polysulphate pentosan (52), 1-arginine (2, 67), gene therapy with hepatocyte growth factor (HGF) (39), and pravastatin (34). Most of these treatments have effectively reduced either functional or structural damage induced by CsA in the kidney. For instance, although administration of losartan (53, 66), VEGF (29), HGF (39), and pentosan polysulphate (52) partially prevented the structural damage induced by CsA, these agents did not improve renal function. In contrast, blockade of the endothelin A/B receptor was effective in improving renal function as measured by creatinine clearance, but had no positive effect on CsA-induced arteriolopathy and tubulointerstitial fibrosis (32). The hypocholesterolemic drug pravastatin, which also possesses anti-inflammatory and antifibrotic effects, partially reduced both structural damage and renal dysfunction induced by CsA (34), suggesting that this agent may have an unknown effect on renal vasoconstriction.

Although angiotensin II has received the greatest consideration as a mediator of cardiovascular and renal injury, there is enough evidence pointing to aldosterone as a deleterious component of the renin-angiotensin-aldosterone system in cerebral, cardiovascular, and kidney tissues. Some studies have shown that the detrimental actions of aldosterone in the kidney include hypertrophy, remodeling, and fibrosis that could be reverted by aldosterone receptor blockade (for a review, see Refs. 22 and 41). In this regard, we have shown in chronic CsA nephrotoxicity in the rat that spironolactone effectively reduced arteriolopathy and fibrosis and completely prevented the reduction of the glomerular filtration rate, suggesting that aldosterone is an important mediator of both functional and structural injury in this model of nephropathy (13). The fact that spironolactone improved the glomerular filtration rate suggests that in this model aldosterone participates in promoting renal vasoconstriction. Possible mechanisms include impaired vasodilatation in response to acetylcholine (35, 55, 59), upregulation of the angiotensin II receptor (AT1) (51, 64, 65), increased vasoconstrictor effects of catecholamines (62), and a direct aldosterone...
effect that could be mediated through genomic and non-
genomic mechanisms (4, 63). To evaluate whether aldosterone’s vascular effect is through modifying the expression of vasoactive pathways, in the present study we assessed the mRNA renocortical expression of the main renal vasoactive pathways during the spironolactone’s protective effect in chronic CsA nephrotoxicity.

Because chronic CsA toxicity results from a combination of vasoconstriction and structural damage, the improvement of renal function in spironolactone-treated CsA rats could result from either vasoactivation and/or reduction of structural injury. In the present study, we hypothesized that if aldosterone is playing a major role in CsA-inducing renal vasoconstriction, then spironolactone should also be an effective drug to prevent acute CsA nephrotoxicity, a reversible condition in which it is known that tissue changes have not yet been established and renal vasoconstriction is the main protagonist. Thus to investigate the role of aldosterone as a modulator of renal vascular tone in CsA nephrotoxicity, we also evaluated the effect of aldosterone blockade in an acute model of nephrotoxicity.

METHODS

Chronic cyclosporine nephrotoxicity. Four groups with six male Wistar rats each weighing 330–370 g were used for the study. To induce chronic nephrotoxicity, the animals were treated for 21 days and fed a low-salt diet (0.02%). Group I received 0.1 ml subcutaneously (sc) of olive oil as vehicle every 24 h (LS); group II received 20 mg·kg⁻¹·day⁻¹ of spironolactone (LS+Sp) by gastric gavage; group III was treated with a daily dose of CsA (30 mg/kg sc; LS+CsA); and group IV was formed by rats treated with cyclosporin and spironolactone (LS+CsA+Sp). The LS and LS+Sp groups were pair fed with the LS+CsA and LS+CsA+Sp groups, respectively. In addition, six control rats fed with standard chow diet were included (C). At the end of the study (21 days), rats were placed in metabolic cages and urine that was spontaneously voided during every 24 h was collected. Serum and urine creatinine concentrations 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. After urine collection, rats were anesthetized by an intraperitoneal injection of pentobarbital sodium and their kidneys were excised, macroscopically divided into renal cortex and medulla, frozen in liquid nitrogen, and kept at −80°C until used.

RNA isolation. Total RNA was isolated from each renal cortex or medulla following the guanidine isothiocyanate-cesium chloride method (50). 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).

Semiquantitative RT-PCR. The relative level of pro-renin, angiotensinogen, angiotensin receptors AT₁A, AT₁B, and AT₂, preproendothelin, endothelin receptor ETA and ETB, cyclooxygenase-2 (COX-2), adenosine receptors A₁D, A₂D, A₂B, and A₃, and β-actin mRNA expression was assessed in the renal cortex by semiquantitative RT-PCR, as previously described (5, 6, 13, 45, 57). Primer sequences are detailed in Table 1 and were custom obtained from Invitrogen (Gaithersburg, MD).

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 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, and 10 mM DTT, pH 8.3). One-tenth of the RT from each individual sample was used for each PCR in 20-μl final volume reactions containing 0.2 μCi of [α³²P]dCTP (~ 3,000 Ci/mmol, 9.25 MBq, 250 μCi). PCR cycles were performed in a DNA thermal cycler (Whatman Biometra, Goettingen, Germany). The control gene was amplified simultaneously in each reaction.

Table 1. Oligonucleotide sequence to amplify specific PCR fragments

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<thead>
<tr>
<th>Gene</th>
<th>Oligonucleotide Sequence</th>
<th>Size, bp</th>
<th>Reference No.</th>
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<td>COX-2</td>
<td>5’-ATC ACT GTC TCT CAT TCG GAG TG-3’</td>
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<tr>
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<tr>
<td>Adenosine 2A receptor</td>
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<tr>
<td>Adenosine 3 receptor</td>
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Amplification kinetics for pro-renin, angiotensinogen, angiotensin receptors AT$_1$, AT$_2$, and AT$_3$, preproendothelin, endothelin receptors ET$_A$ and ET$_B$, COX-2, adenosine receptors Ad$_1$, Ad$_2A$, Ad$_2B$, and Ad$_3$, and $\beta$-actin in renal cortex. The optimal number of cycles for each primer pair was assessed through kinetic amplification determination. 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), and counted by liquid scintillation (Beckman LS6500, Fullerton, CA). All reactions were performed individually from each cortex’s total RNA in duplicate. Genomic DNA contamination was checked by treating all RNA samples with RNAse-free DNAase I and by carrying samples through the PCR procedure without adding RT.

Protocol 2

Acute cyclosporine nephrotoxicity. Four groups of 10 male Wistar rats weighing 330–370 g fed a standard chow diet were included. Animals were subjected to the following daily treatments for 7 days: group I included rats that received 0.1 ml olive oil as vehicle (V); group II consisted of rats treated with 20 mg•kg$^{-1}$•day$^{-1}$ of spironolactone (Sp) by gastric gavage; group III was formed by rats that received CsA 30 mg/kg sc (CsA); and group IV was formed by rats treated with CsA and spironolactone (CsA+Sp). V and Sp groups were pair fed. All procedures were in accordance with our institutional guidelines for animal care.

Functional studies. At the end of the study (7 days), rats from protocol 2 were anesthetized with an intraperitoneal injection of pentobarbital sodium (30 mg/kg) and placed on a homeothermic table to maintain the core body temperature at 37°C by means of a rectal probe attached to a temperature regulator that is, in turn, attached to a homeothermic blanket. The trachea, both jugular veins, and femoral arteries were catheterized with PE-240 and PE-50 polyethylene tubing. The bladder was cannulated with PE-50. Rats were maintained under euvolesic conditions by infusing 10 ml•h$^{-1}$ isotonic rat plasma during surgery, followed by an infusion of 5% polyfructosan, (Inutest, Laevosan-Gesellschaft, Linz, Austria) at 2.2 ml/h. Mean arterial pressure was monitored with a pressure transducer (model p23 db, Gould) and recorded on a polygraph (Grass Instruments, Quincy, MA). Via a midline abdominal incision, the left renal artery was exposed. An ultrasound transit-time flow probe (1RB, Transonic, Ithaca, NY) was placed around the left renal artery and filled with ultrasonic coupling gel (HR Lubricating Jelly, Carter-Wallace, New York, NY) for recording renal blood flow (RBF). After an equilibration period of 60 min, care was taken to avoid dead space in the bladder, urine was drained from the bladder by gravity and collected for 30–60 min, and blood samples were taken at the beginning and at the end of each urine collection period. Inutest concentrations in urine and plasma were determined by the Davidson et al. technique (9).

Statistical Analysis

Results are presented as means ± SE. The significance of the differences between groups was tested by ANOVA using Bonferroni’s correction for multiple comparisons. Statistical significance was defined when the $P$ value was <0.05.

RESULTS

Chronic CsA Nephrotoxicity

Figure 1 shows the effect of spironolactone administration on survival percentage and body weight of the rats chronically treated with cyclosporine and fed a low-sodium diet. LS-CsA rats exhibited a significant reduction in survival rate by 50% (Fig. 1A). The CsA-inducing mortality was completely prevented with the simultaneous administration of spironolactone, because the survival of LS-CsA+Sp group was 100%. Figure 1B shows the evolution of body weight. LS-CsA induced a progressive body weight loss from day 2 to day 10 of treatment. Subsequently, body weight was maintained until the end of the study. The rats that received LS-CsA+Sp presented a similar pattern of body weight loss in the first 8 days; however, after day 10, these animals gained weight. These results suggest that aldosterone receptor blockade maintains LS-CsA+Sp-treated rats in better general health conditions than LS-CsA.

Figure 2 depicts creatinine clearance determined in the five groups at the end of the study. Sodium restriction or sodium restriction and spironolactone did not produce a significant change in renal function compared with control animals. As we previously reported (13), rats receiving CsA treatment presented a significant reduction of renal function by 59% compared with vehicle-treated rats. This reduction was completely prevented by spironolactone administration, because creatinine clearance in the LS-CsA-Sp group was not different from that shown in the control groups. We have previously shown (13) that the protective effect of spironolactone is not associated with a significant increase in serum potassium. In this study, similar results were obtained. Serum potassium levels in CsA-treated rats were 5.5 ± 0.3 meq/l and in CsA+spironolactone-

Fig. 1. Administration of spironolactone to rats in chronic cyclosporine (CsA) nephrotoxicity improved survival percentage (A) and body weight gain (B) during study. Low-salt diet plus cyclosporine (LS+CsA)-treated group is represented by filled circles and low-salt diet plus CsA plus spironolactone (LS+CsA+Sp)-treated group by open circles. *$P < 0.05$ vs. LS+CsA group.
treated animals were 5.5 ± 0.2 meq/l (P = not significant). In addition, we (13) and others (10, 15, 42, 68) have previously shown that CsA nephrotoxicity is not associated with changes in urinary protein excretion.

Fig. 2. Mineralocorticoid receptor blockade prevented renal dysfunction in rats with chronic CsA nephrotoxicity. All groups were fed a low-sodium diet, except the control group (C), which was fed with standard chow diet (open bar). In this figure and in Figs. 4–7, vehicle-treated rats are represented by a light gray bar (LS), spironolactone-treated rats by a dark gray bar (LS + Sp), the group with chronic CsA nephrotoxicity by a black bar (LS + CsA), and rats that received simultaneously CsA and spironolactone by a hatched bar (LS + CsA + Sp). *P < 0.05 vs. all studied groups.

Renocortical mRNA levels of vasoconstrictor pathways were assessed by semiquantitative RT-PCR by using the specific primers detailed in Table 1. As shown in Fig. 3, the amplification kinetics for each primer pair were determined. The optimal number of cycles to amplify each cDNA fragment was calculated by using the middle point of the exponential phase, as stated in each graph. All results are shown as the ratio between each amplified cDNA fragment and β-actin, which was used as a housekeeping gene.

Angiotensin pathway. The mRNA levels of angiotensinogen, pro-renin, and angiotensin receptors AT$_{1A}$, AT$_{1B}$, and AT$_2$ in the renal cortex for the five groups studied are shown in Fig. 4. The activation of the renin-angiotensin-aldosterone system (RAAS) with a low-sodium diet induced a significant increase in mRNA levels of pro-renin (0.7 ± 0.03 vs. 0.5 ± 0.03, P < 0.01) and AT$_{1A}$ (2.9 ± 0.4 vs. 1.7 ± 0.2) and AT$_{1B}$ receptors (3.4 ± 0.1 vs. 1.6 ± 0.1). Mineralocorticoid receptor blockade in LS rats prevented AT$_{1A}$ (ratio was 1.9 ± 0.2) and, partially, AT$_{1B}$ upregulation induced by sodium restriction (ratio 2.8 ± 0.1).

Chronic CsA administration produced a significant reduction (nearly one-half) in the renocortical expression of the most components of the angiotensin pathway, compared with the LS group, except for pro-renin that, in contrast, was significantly

Fig. 3. Amplification kinetics of the vasoactive pathways studied. For each primer pair, PCR products were obtained at several cycles and resolved in acrylamide gels. Ethidium bromide-stained bands were cut, and radioactivity was determined by liquid scintillation counting. PCR product is expressed as log of the incorporated [α-$^{32}$P]dCTP in bands excised from the gel. The optimal number of cycles to amplify each cDNA fragment was calculated by using the middle point of the exponential phase as stated in each graph. AT$_{1A}$ and AT$_{1B}$, angiotensin receptors; ET$_A$ and ET$_B$, endothelin receptors; COX-2, cyclooxygenase-2; Ad$_1$, Ad$_{2A}$, Ad$_{2B}$, and Ad$_3$: adenosine receptors.
upregulated. The percent reductions for angiotensinogen, AT$_{1A}$, and AT$_{1B}$ were 50, 45, and 48%, respectively, whereas pro-renin increased more than twofold (ratio 1.6 ± 0.1 vs. 1.2 ± 0.1, respectively, $P < 0.05$). CsA administration induced a significant increase in prorenin mRNA levels by 40%, together with a reduction in the ET$_B$ receptor by 25%. Intriguingly, mineralocorticoid receptor blockade in rats with CsA nephrotoxicity did not modify endothelin upregulation but reduced ET$_A$ and normalized ET$_B$ mRNA levels. These observations together suggest that 1) during sodium restriction, the ET$_B$ receptor is regulated by renin-angiotensin activation but not by aldosterone, 2) CsA enhanced preproendothelin expression and reduced the vasodilator ET$_B$ receptor, and 3) the protective effect of spironolactone during chronic CsA nephrotoxicity was associated with a reduction in vasoconstrictor receptor (ET$_A$) and reestablishment of endothelin vasodilator receptor expression (ET$_B$) without modification of endothelin upregulation.

**Adenosine receptors.** Figure 6 shows adenosine receptor mRNA levels in the renal cortex. The activation of RAAS by sodium restriction induced a significant increase in all adenosine receptor mRNA levels compared with levels in rats fed a normal-salt diet. The percentage of increment for Ad$_{1A}$, Ad$_{2A}$,
Ad2B, and Ad3 was 58, 53, 67, and 120%, respectively. In rats fed a low-sodium diet, spironolactone prevented only Ad2A upregulation. In contrast, CsA administration produced significant changes in the expression pattern of adenosine receptors compared with rats fed with LS. In the LS-CsA group, Ad1 and Ad3 mRNA expression increased by 3.4- and 0.6-fold, respectively, because the Ad1/β-actin ratio was 2.4 ± 0.3 in the LS-CsA vs. 0.7 ± 0.1 in the LS group and the Ad3/β-actin ratio was 2.5 ± 0.2 vs. 1.6 ± 0.2, respectively. Ad2A mRNA levels were not affected by CsA. In contrast, Ad2B mRNA levels were significantly reduced in the LS-CsA group compared with rats fed a low-sodium diet, 0.6 ± 0.1 vs. 1.0 ± 0.1 (P < 0.05). All these changes in adenosine receptors induced by CsA were not modified by spironolactone, except for Ad2A, where a similar effect than in the LS group was observed. All these findings together suggest that 1) adenosine receptor mRNA expression is regulated by RAAS and, specifically, Ad2A is modulated by aldosterone, in view of the fact that spironolactone prevented Ad2A upregulation in the control group and during chronic CsA nephrotoxicity; 2) CsA nephrotoxicity was associated with upregulation of Ad1 and Ad3, accompanied by Ad2B down-regulation; and 3) the protective effect of spironolactone in chronic CsA nephrotoxicity is not related to changes in the expression pattern of adenosine receptors.

**COX-2 expression.** COX-2 mRNA levels are depicted in Fig. 7. As has been previously reported (7), sodium restriction induced an increase in COX-2 mRNA levels by 64%. Intriguingly, spironolactone administration induced a further increase in COX-2 expression by 40%. CsA administration produced a remarkable downregulation of COX-2 mRNA levels from 1.8 ± 0.1 to 0.6 ± 0.1. This reduction was not reversed by spironolactone administration. These results emphasize that 1) COX-2 is regulated by RAAS and 2) COX-2 downregulation induced by CsA was not prevented by spironolactone.

### Acute CsA Nephrotoxicity

Table 2 shows the physiological parameters of the rats that were included in protocol 2, in which aldosterone’s role in acute CsA nephrotoxicity was evaluated. At the end of the study, there was no difference in body weight, as well as in serum sodium and potassium, among the four groups studied.

#### Graphical Representations

- **Fig. 6.** Renocortical upregulation of adenosine receptors by sodium restriction and effect of chronic CsA and spironolactone administration. Values are means ± SE of Ad1/β-actin (A), Ad2A/β-actin (B), Ad2B/β-actin (C), and Ad3/β-actin (D) ratios. *P < 0.05 vs. C group. †P < 0.05 vs. LS group. ‡P < 0.05 vs. LS + CsA group.

- **Fig. 7.** Effect of a low-sodium diet, spironolactone, and chronic CsA on COX-2 mRNA levels in renal cortex assessed by RT-PCR. Each bar represents the ratio mean value ± SE of COX-2/β-actin in the 5 studied groups. *P < 0.05 vs. C group. †P < 0.05 vs. LS group.
Spironolactone did not modify renal function in control rats values corrected by body weight for the four groups studied. Table 2 shows the glomerular filtration rate (GFR), nephrotoxicity.

Table 2. Physiological parameters in acute cyclosporine nephrotoxicity

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<th>Group</th>
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<th>Serum K, meq/l</th>
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<td>V</td>
<td>305±7</td>
<td>112.6±2.8</td>
<td>0.75±0.05</td>
<td>145.8±2.4</td>
<td>3.9±0.2</td>
</tr>
<tr>
<td>Sp</td>
<td>303±12</td>
<td>112.1±6.3</td>
<td>0.71±0.07</td>
<td>152.4±5.6</td>
<td>4.2±0.1</td>
</tr>
<tr>
<td>CsA</td>
<td>311±8</td>
<td>97.2±6.5*</td>
<td>0.28±0.04*</td>
<td>142.5±2.1</td>
<td>4.3±0.3</td>
</tr>
<tr>
<td>CsA + Sp</td>
<td>324±12</td>
<td>109.9±2.3†</td>
<td>0.64±0.07</td>
<td>137.4±2.7</td>
<td>4.5±0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. BW, body wt; MAP, mean arterial pressure; GFR, glomerular filtration rate; V, vehicle; Sp, spironolactone; CsA, cyclosporine A. *P < 0.05 vs. V. †P < 0.05 vs. CsA.

CsA-treated rats presented mean arterial pressure values significantly lower than the vehicle group. This difference was not observed in rats treated with CsA + Sp.

Figure 8A depicts the mean values of the total glomerular filtration rate, and Table 2 shows the glomerular filtration rate values corrected by body weight for the four groups studied. Spironolactone did not modify renal function in control rats (2.1 ± 0.2 vs. 2.3 ± 0.1 ml/min). As expected, CsA administration for 7 days significantly reduced glomerular filtration rate (0.8 ± 0.1 ml/min). As we have shown in chronic CsA nephrotoxicity (13), spironolactone administration completely prevented glomerular filtration rate reduction in rats treated with CsA (1.9 ± 0.1 ml/min). As shown in Fig. 8B, RBF recorded in the left renal artery was 6.1 ± 0.5 ml/min in control rats and 6.0 ± 0.1 ml/min in spironolactone-treated animals. Thus control rats receiving spironolactone had RBF values similar to the control group. Because RBF was assessed only in one kidney, it is reasonable that total RBF was about twice these numbers. CsA produced an intense renal vasoconstriction as evidenced by the reduction of RBF compared with the control group, 3.1 ± 0.5 vs. 6.1 ± 0.5 ml/min, respectively (P < 0.05). This reduction was abrogated by concomitant administration of spironolactone and CsA, 5.7 ± 0.7 ml/min. Thus these findings, together with our previous observations (13), support the hypothesis that aldosterone regulates renal vascular tone in both acute and chronic CsA nephrotoxicity.

DISCUSSION

In this study we showed that mineralocorticoid receptor blockade prevents acute CsA nephrotoxicity that was evidenced by restoration of the glomerular filtration rate and RBF to normal values. In addition, we also found that spironolactone modifies the expression of certain receptors that mediate vasoconstrictor or vasodilator actions that, together with the reduction of pro-renin expression, might contribute to reduce the renal vasoconstriction observed in chronic CsA nephrotoxicity.

Cells in the distal tubule and cortical collecting duct that express mineralocorticoid receptor were considered for a long time as the unique cellular target of aldosterone in the kidney. However, it is now clear that many other cell types in the kidney and other epithelial and nonepithelial tissues are also potential targets for aldosterone. In this regard, mineralocorticoid receptor has been detected in glomerulus, afferent and efferent arterioles (3, 56), as well as in the cardiovascular system, brain, and vasculature (for a review, see Ref. 48). Moreover, clinical and experimental studies have established that aldosterone plays a major role in the pathophysiology of cardiovascular and renal disease (14, 43, 47, 48, 69). Specifically, aldosterone has been associated with the fibrotic process. Of note is the fact that induction of chronic CsA nephrotoxicity in rats requires that animals are fed a low-salt diet, a maneuver that activates the RAAS, suggesting that activation of this axis provides the proper setting for CsA to express its full potential toxicity. In this regard, our previous observation (13) that spironolactone markedly reduces the renal damage induced by CsA suggests that aldosterone, and not angiotensin II, is the main hormone involved in this mechanism.

The fact that spironolactone not only reduced structural injury in chronic CsA nephrotoxicity but also completely prevented renal dysfunction suggests that aldosterone also participates in regulating renal vascular tone (13). To address this issue, we reasoned that mineralocorticoid receptor blockade should prevent acute CsA nephrotoxicity, a condition in which renal vasoconstriction is the main protagonist. We observed in the present study, using a model for acute CsA nephrotoxicity, that spironolactone administration completely prevented the fall in glomerular filtration rate that was accompanied by reestablishment of RBF. Thus these data, together with our previous observations in chronic CsA toxicity, show that mineralocorticoid receptor blockade prevents renal vasoconstriction, suggesting that aldosterone participates in renal homodynamic effect of CsA.

Fig. 8. Effect of mineralocorticoid receptor blockade in acute cyclosporin nephrotoxicity. A: glomerular filtration rate estimated by inulin. B: renal blood flow in the left kidney recorded by a probe placed around the renal artery. Open bars, vehicle-treated rats; dark gray bars, spironolactone-treated rats; black bars, rats with acute CsA nephrotoxicity; hatched bars, rats that simultaneously received CsA and spironolactone. *P < 0.05 vs. all studied groups.
Recent reports have documented that aldosterone exerts its actions by genomic and nongenomic mechanisms. The first is dependent on the classic mineralocorticoid receptor that promotes or prevents the transcription of certain genes, whereas the second seems to be mediated by a “membrane unknown receptor” that mediates fast actions independently of gene transcription (for a review, see Ref. 41). Our results with acute and chronic CsA nephrotoxicity support the notion that aldosterone contributes to renal vasoconstriction by a conventional genomic mechanism, because spironolactone effectively prevented the fall of the glomerular filtration rate in these models. However, we cannot exclude that other mediators are implicated. In this regard, Li et al. (34) recently showed that pravastatin reduced renal damage and improved renal function in chronic CsA nephrotoxicity, evidencing that in addition to its anti-inflammatory properties, pravastatin may modify renal vasoconstriction through mechanisms that have not been defined. Although in both Li et al. (34) and the present study, CsA toxicity was associated with a significant increase in renin expression, which pravastatin did not reverse, whereas spironolactone completely prevented renin upregulation. Thus the distinctive effect of pravastatin and spironolactone on renin expression suggests that these compounds may improve renal function in CsA-treated rats through different mechanisms.

Possible mechanisms by which aldosterone could regulate vascular tone include increased catecholamine vasoconstrictor effect (62), impaired vasodilation in response to acetylcholine (35, 55, 59), and upregulation of angiotensin II receptors (51, 64, 65). Taking all these together, we reasoned that aldosterone might regulate vascular tone by altering the renal expression of vasoactive pathways, that is, by either increasing the expression of vasoconstrictor, reducing expression of vasodilator pathways, or both. Thus the effect of spironolactone on renal cortical mRNA expression of vasoactive hormones or their receptors was analyzed in chronic CsA nephrotoxicity.

Sodium restriction by itself, however, is known to modify the expression of vasoactive pathways within the kidney. Thus a control group fed a standard chow diet was included. Indeed, we observed that the rats fed a low-sodium diet developed changes in the mRNA expression pattern of several transcripts compared with rats fed a normal-sodium diet. Interestingly, some of these changes were abrogated when the animals were treated with spironolactone, suggesting that aldosterone is regulating the correspondent vasoactive pathways, as we discuss below.

Renin-Angiotensin Pathway

Angiotensin II, the major effector of the RAAS, is involved in a series of physiological and pathophysiological events, particularly in the kidney. All the components of RAAS are present within the kidney. Angiotensin II actions are mediated by stimulation of subtype receptors, namely, the AT₁ and AT₂ receptors. However, in some instances AT₂ receptor activation counteracts the effects of the AT₁ receptor. Thus the ratio between AT₁ and AT₂ receptor expression should be considered. The AT₁ receptor is a member of the superfamily of G protein-coupled receptors that stimulate the activity of protein kinase C and release of inositol triphosphate. This receptor is present in glomerular mesangial cells, proximal and distal tubular epithelia, and renal vasculature. The stimulation of the renal AT₁ receptor in the kidney induces vasoconstriction, sodium reabsorption, and cell growth. AT₂ is also a G-coupled receptor that activates protein phosphotyrosine phosphatase and is located in renal vasculature, glomeruli, juxtaglomerular apparatus, and tubules. In afferent arterioles, AT₂ receptor stimulation causes vasodilation, whereas in renal tubules it contributes to sodium excretion and pressure-natriuresis (for a review, see Ref. 54).

In this study, we corroborated a kidney-specific regulation by using a low-sodium diet of several components of the angiotensin II pathway, as has been previously described (27, 61). Sodium depletion was associated with increased mRNA levels of pro-renin, AT₁A, and AT₁B receptors but did not influence angiotensinogen and AT₂ receptor expression. Some of these changes were prevented by spironolactone, such as upregulation of the AT₁A and, partially, AT₁B receptor (Fig. 4), suggesting that aldosterone mediates the upregulation of these receptors when the RAAS is activated. Interestingly, CsA nephrotoxicity reduced most of the component expression of this pathway, except pro-renin, which was significantly upregulated. The beneficial effect of spironolactone was associated with prevention of pro-renin upregulation and increased expression of the vasodilator AT₂ receptor. In this regard, Klar et al. (30) have shown in primary cultures of mouse juxtaglomerular cells that aldosterone enhanced pro-renin mRNA levels. These observations together suggest that the prevention of renal failure, which is associated with increased RBF induced by spironolactone in CsA nephrotoxicity, is at least partially due to the reduction of pro-renin expression and thus RAAS activity, together with increased expression of the AT₂ receptor (Fig. 4), which is known to mediate renal vasodilation, thus counteracting the vasoconstrictor state induced by CsA.

Endothelin Pathway

Endothelin-1 (ET-1) is a potent endothelial-derived vasoconstrictor and sodium reabsorption-regulating peptide. In the kidney, ET-1 is synthesized in the endothelium of the renal vasculature, mesangial cells, peritubular capillaries, and in the epithelium of the proximal tubule, medullary thick ascending limb, and inner medullary collecting duct. The biological effects of ET-1 are mediated by specific membrane receptors. ETₐ receptors are predominantly distributed in the vasculature of glomeruli and medulla, whereas ETₐ receptors are present in the renal tubules and collecting ducts (for a review, see Ref. 37). Whether relaxation or constriction is predominantly elicited by endogenous ET-1 may depend on the concentration of ET-1 in vascular beds and the density of ET receptor subtypes in endothelial and smooth muscle cells (28).

In the present study, we observed that a low-salt diet induced upregulation of the ETₐ receptor in the renal cortex. This effect was not reversed by spironolactone. In this regard, similar results were observed by Vanni et al. (58) in the renal medulla, suggesting that sodium restriction modulates ETₐ expression. Several studies have pointed to the endothelin as a key molecule that promotes CsA renal vasoconstriction (8, 23, 33, 36, 44). In fact, we found that CsA administration produced a significant upregulation of preproendothelin and reduction of ETₐ receptor mRNA levels. This combination probably results in increased renal vasoconstriction induced by endothelin due, on the one hand, to an increased expression of endothelin and...
on the other hand to a reduced expression of the ETB receptor that is believed to promote vasodilation. The effect of CsA on endothelin expression was not prevented by mineralocorticoid receptor blockade, but significant changes in endothelin receptors were observed in such a way that expression of ETA that is known to mediate vasoconstriction is reduced, whereas expression of the ETB receptor is increased. Thus although increased expression of endothelin was not affected by spironolactone, it is possible that endothelin-induced vasoconstriction is reduced due to the switch in ET receptor expression.

Adenosine Pathway

Extracellular actions of adenosine are mediated by four types of G protein-coupled receptors, known as Ad1, Ad2A, Ad2B, and Ad3. In most blood vessels including the kidney vasculature, adenosine elicits marked vasodilatation, and this effect is mediated by Ad2A and Ad2B. In contrast, a clear vasoconstrictor effect is observed when adenosine binds to the Ad1 receptor. In the kidney, Ad1 is predominantly expressed in afferent arterioles, whereas Ad2A and Ad2B are present in all preglomerular vessels and in descending vasa recta (for a review, see Ref. 16). A preliminary study in acute CsA toxicity (20) suggested that the adenosine 1 receptor blocker 1,3-dipropyl-8-cyclopentylxanthine may have a protective effect in normal, diabetic, and hypertensive animals.

We observed that sodium restriction produced a significant upregulation of the adenosine receptors, but only Ad2A was prevented by spironolactone, suggesting that expression of this receptor is regulated by aldosterone. Interestingly, renal vasoconstriction induced by CsA was associated with a significant increase in Ad1 mRNA levels and reduction of the vasodilator receptor Ad2B. These changes were not corrected by the selective blockade of the mineralocorticoid receptor, suggesting that the observed changes in adenosine receptor mRNA expression during CsA toxicity are not induced by genomic actions of aldosterone.

COX-2

In renal tissue, prostaglandins are also important mediators of vascular tone, salt and water balance, and renin release. The rate-limiting enzyme cyclooxygenase initiates the metabolism of arachidonic acid to PG2 and subsequently to PGH2, which is then further metabolized by tissue-specific isomerases to diverse PGs and thromboxanes. There are at least two distinct cyclooxygenases, COX-1 and COX-2, which are the products of different genes and exhibit distinct patterns of expression and regulation. COX-2 mRNA and immunoreactive protein have been observed in cells of the macula densa and in scattered cells in the cortical thick ascending limb cells immediately adjacent to the macula densa. In human kidney, COX-2 expression has also been noted in podocytes and arteriolar smooth muscle cells (for a review, see Ref. 19). There is convincing evidence to suggest that COX-2-derived prostanooids are involved in the regulation of renin synthesis and secretion in the juxtaglomerular apparatus, as well as in tubular salt and water handling (17, 18, 26, 49). Macula densa cells regulate afferent arteriolar tone and renin release by sensing luminal chloride via the Na⁺-K⁺-2Cl⁻ cotransporter. When intraluminal salt concentration in the macula densa is low, renin synthesis and release are increased by a process in which induction of COX-2 has been proposed to be required (19), because administration of nonspecific COX inhibitors blunt increases in renin release mediated by the macula densa (19). Thus, in addition to salt depletion, high-renin states are seen with angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers, diuretic administration, or increased COX-2 expression (17, 18, 26, 49). In agreement with all these studies, we observed that sodium restriction was associated with cortical COX-2 upregulation; intriguingly, mineralocorticoid receptor blockade further increased COX-2 expression, suggesting that aldosterone downregulates COX-2 mRNA levels during sodium depletion. In contrast, CsA-treated rats presented a remarkable reduction of cortical COX-2 expression. A similar observation was made by Hocherl et al. (21). Thus this particular situation represents a state of a high renin induced by both a low-sodium diet and the direct effect of CsA, but with reduction of COX-2 mRNA levels, suggesting that renin expression could be regulated by mechanisms independent of COX-2. Indeed, CsA as an inhibitor of calcineurin phosphatase inhibits the nuclear factor of activated T cells signaling pathway. This pathway also regulates COX-2 gene expression (11, 21, 24). Thus spironolactone was not able to prevent COX-2 downregulation, suggesting that suppression of COX-2-dependent prostanoid formation is not critical for maintaining vascular tone in chronic CsA nephrotoxicity.

In summary, this study shows that the prevention of renal vasoconstriction by spironolactone could be mediated, in part, by inducing changes in the expression pattern of vasoactive pathways that might reestablish the imbalance between vasoconstrictors and vasodilators observed in chronic CsA nephrotoxicity. The major changes observed in our study of vasoactive pathways were the upregulation of pro-renin mRNA and the downregulation of ETB receptor expression induced by CsA and that spironolactone prevented both changes, suggesting that aldosterone receptor activity is involved. In addition, we observed that aldosterone participates in producing renal vasoconstriction during acute CsA nephrotoxicity. All our findings together pointed to spironolactone as a potential treatment to prevent or reduce CsA nephrotoxicity in transplant patients.

ACKNOWLEDGMENTS

We are grateful to members of the Molecular Physiology Unit for suggestions and discussion.

GRANTS

This work was supported by research grants C01–40182 and IN208602 from the Mexican Council of Science and Technology (CONACYT) and National University of Mexico (DGAPA), respectively, to N. A. Bobadilla. Part of this work was presented at the 36th Annual Meeting of the American Society of Nephrology, November 12–17, 2003, San Diego, CA, and Experimental Biology 2005, April 2–6, 2005, San Diego, CA.

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