Endothelin A Receptor Mediates Functional but Not Structural Damage in Chronic Cyclosporine Nephrotoxicity

T.E. Hunley, A. Fogo, S. Iwasaki, and V. Kon

ABSTRACT

Chronic treatment with cyclosporine (CsA) is limited not only by glomerular hypofiltration but also by structural damage. The pathogenesis of these nephrotoxicities was studied in a model of chronic CsA-induced renal damage. Salt-depleted rats were treated with daily CsA (15 mg/kg sc) for ~3 weeks, at which time renal function was measured and kidneys were harvested for morphologic assessment. A separate group of rats (CsA + BQ123) were identically treated with CsA but in addition also received simultaneous treatment with a specific endothelin A (EtA) receptor antagonist, BQ123, which was continuously delivered via sc osmotic pump (1 mg/kg per hour) and maintained throughout the study. Chronic CsA treatment caused profound functional and structural damage, although blood pressure was normal (102±6 mm Hg); GFR was 0.05±0.02 mL/min per 100 g body wt, and RPF was 0.15±0.06/100 g body wt. Renal injury was scored on a scale of 0 to 4 and showed dilatation/vacuolization of 1.07±0.29 and tubulointerstitial fibrosis of 0.78±0.17. Arteriolopathy was present in 78±4% of arterioles. Chronic antagonism of the EtA receptor preserved renal function: GFR was 0.15±0.03 mL/min per 100 g body wt, and RPF was 0.32±0.08/100 g body wt (P < 0.05 for GFR versus CsA). Blood pressure was not affected: 104±8 mm Hg. Although renal function was improved by antagonism of the EtA receptor, structural damage was not. Thus, in CsA+BQ123 rats, the tubule dilatation/vacuolization score was 1.32±0.12 (P, not significant versus CsA) and arteriolopathy was present in 74±2% (P, not significant versus CsA). Remarkably, tubulointerstitial fibrosis was significantly greater in CsA + BQ123 rats, 1.20±0.11 (P < 0.05 versus CsA). These findings indicate an important role for Et-1, through the EtA receptor, in mediating glomerular dysfunction associated with chronic CsA treatment. The study further indicates that, although the EtA receptor mediates glomerular hypofiltration, factors other than EtA are responsible for structural damage and imply a dissociation of mechanisms that lead to functional versus structural damage.

Key Words: Endothelin, endothelin A receptor, cyclosporine

Nephrotoxicity is a serious and pervasive consequence of the immunosuppressant cyclosporine (CsA)(1,2). CsA nephrotoxicity is characterized by a spectrum of renal involvement, ranging from acute dysfunction, which develops within hours of initiating CsA, to chronic injury, which includes structural renal damage. All stages of CsA nephrotoxicity are characterized by vasoconstriction (1), which has been variously linked to the adrenergic nervous system, angiotensin II, and thromboxane A2 (3-6). We and others have defined a mediating role for endothelin (Et) in acute CsA nephrotoxicity: thus, CsA stimulates Et production both in vitro and in vivo (7,8). CsA increases renal Et binding, and CsA up-regulates Et receptor mRNA (9-12). Moreover, acute antagonism of Et actions, either by anti-Et-1 antibodies or by antagonism of the EtA receptor, has been shown to lessen renal vasoconstriction in response to acute CsA infusion (8,13,14). In addition to glomerular dysfunction, chronically administered CsA is also associated with tubular changes, arteriolopathy, and ultimately, interstitial fibrosis (1,2,15-18).

Currently, however, it is not known whether mechanisms similar to those producing vasoconstriction after the acute administration of CsA also lead to persistent glomerular dysfunction associated with CsA treatment. Moreover, because Et-1 affects cellular proliferation and matrix deposition (19-23), it may contribute to the structural damage. Recently, a model of chronic CsA nephrotoxicity was described that encompasses the above histologic changes (15-18). Using this model and the selective endothelin A (EtA) receptor antagonist BQ123, we investigated whether Et-1 through the EtA receptor participates in chronic CsA nephrotoxicity.
METHODS

Male Munich-Wistar rats weighing 211 to 277 g were given a single dose of furosemide (4 mg/kg body wt ip; American Regent Lab., Inc., Shirley, NY). At the same time, rats were placed on a salt-free rice diet enriched with niacin, ferric orthophosphate, and thiamin mononitrate. Rats were maintained on this diet throughout the remainder of the study as recently described (15-17). The animals were housed in climate-controlled rooms on a 12-h light/dark cycle in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and had free access to tap water. Beginning 2 wk into the diet, CsA (15 mg/kg body wt sc, Sandimmune concentrate for injection; Sandoz Pharmaceutical Corp., East Hanover, NJ) was administered daily to two groups of animals, with weekly dose adjustments made for body weight changes. The first group (CsA, N = 5) received CsA only. The second group (CsA + BQ123, N = 5) was treated identically and in addition received a selective ETA receptor antagonist (BQ123, 1 mg/kg body wt per hour) by osmotic pump (Alza Corporation, Palo Alto, CA), starting at the time of initiation of CsA treatment. BQ123 is a selective competitive antagonist of the ETA receptor and was a generous gift from Banyu Pharmaceutical Co., Ltd. (Tsukuba, Japan). It is derived from modification by amino acid substitution of a natural cyclic pentapeptide antagonist of ETA [a cyclo-(d-trp-d-asp-pro-D-val-leu-1), obtained from Streptomyces misakiiensis. It has previously been shown that BQ123 at 1, 5, and 10 mg/kg body wt antagonizes the increase in mean arterial pressure induced by exogenous ET-1 given at a dose of 1 nmol/kg body wt iv (24). Further, this dose BQ123 has been shown to antagonize the renal hemodynamic derangement in response to the acute infusion of CsA (14). Control animals (CONT, N = 4) received furosemide treatment and were maintained on the rice diet throughout the period of study, without the administration of CsA. Three animals (CONT + BQ123) were identical treated with the furosemide/rice diet regimen but additionally received BQ123 by osmotic pump, as described above. Last, a group of animals (Cre, N = 5), in addition to the furosemide/rice diet regimen, received daily sc administration of the CsA vehicle Cremophor (Sandoz Research Institute).

Renal Functional Parameters

After 3 wk, renal function was measured in CONT, CONT + BQ123, CsA, and CsA + BQ123 as previously described (9). Briefly, Inactin anesthesia was induced (70 mg/kg body wt ip; Byk, Konstanz, Germany), tracheostomy was performed, and indwelling catheters were inserted into the ureters for timed urine collections and into the femoral artery and jugular vein for blood sampling and the infusion of insulin, para-aminohippurate, and plasma to maintain euvoolemia. Mean arterial blood pressure was measured and recorded on a Gould pressure monitor (Gould Instruments, Oxnard, CA). Urine and plasma insulin and para-aminomophippurate levels were measured for the calculation of GFR and RPF. Controls, CONT + BQ123, and Cre all underwent blood and timed urine collection for the determination of plasma and urine creatinine by an automated method using the Jaffe reaction. Creatinine clearances were then calculated.

Renal Structural Parameters

Kidneys were harvested at 3 wk, were immersion fixed in 4% paraformaldehyde, and were routinely processed; 3-µm sections were stained with periodic acid-Schiff. For each animal, lesions were scored without knowledge of the protocol from 0 to 4, with 0, normal; 1, up to 25% of the field involved by the lesion; 2, 25 to 50% of the field involved; 3, 50 to 75% of the field involved; and 4, >75% of the field involved. The degree of tubulointerstitial fibrosis and the degree of tubular dilation/vacuolization were scored separately for each ×20 field of the kidney, and an average score for each parameter was then calculated for each animal by adding all scores and dividing by the number of fields examined (16-18). All arterioles were examined and scored as positive or negative for the presence of arteriolopathy, as characterized by periodic acid-Schiff-positive lesions. On average, 20 arterioles were assessed in each kidney section. Arteriolopathy score is expressed as the percentage of arterioles affected with this lesion.

Statistics

Data are given as mean ± SE. Comparisons were made by analysis of variance, followed by paired and unpaired t tests as appropriate to evaluate data for statistical significance (P < 0.05 defining significance).

RESULTS

General

Data for the systemic parameters and renal function are shown in Table 1. Body weight decreased significantly and similarly in each of the groups over the duration of the study: from 221 ± 3 to 199 ± 1 g in

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood Pressure (mm Hg)</th>
<th>Hematocrit (%)</th>
<th>GFR (ml/min per 100 g body wt)</th>
<th>RPF</th>
<th>Tubular Dilation/Vacuolization</th>
<th>Arteriolopathy (%)</th>
<th>Tubulo-Interstitial Fibrosis</th>
</tr>
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<tbody>
<tr>
<td>CsA (N = 5)</td>
<td>102 ± 6</td>
<td>39 ± 1</td>
<td>0.05 ± 0.02b</td>
<td>0.15 ± 0.06b</td>
<td>1.07 ± 0.29b</td>
<td>78 ± 4b</td>
<td>0.78 ± 0.17b</td>
</tr>
<tr>
<td>CsA + BQ123a (N = 5)</td>
<td>104 ± 8</td>
<td>37 ± 1c</td>
<td>0.15 ± 0.03b,c</td>
<td>0.32 ± 0.08b</td>
<td>1.32 ± 0.12b</td>
<td>74 ± 2b</td>
<td>1.20 ± 0.11b,c</td>
</tr>
<tr>
<td>CONT (N = 4)</td>
<td>93 ± 1</td>
<td>39 ± 3</td>
<td>0.35 ± 0.11</td>
<td>1.19 ± 0.30</td>
<td>0.06 ± 0.04</td>
<td>26 ± 6</td>
<td>0.12 ± 0.04</td>
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*a BQ123, specific ETA receptor antagonist. Specific P values are given in text.

b P < 0.05 versus CONT.

c P < 0.05 versus CsA.
CaS (P < 0.005), from 218 ± 1 to 199 ± 4 g in CaS + BQ123 (P < 0.005), and from 254 ± 8 to 217 ± 3 g in CONT (P < 0.01). Mean arterial pressure was 102 ± 6 mm Hg in CaS-treated rats and was not different in the CaS + BQ123 animals (104 ± 8 mm Hg) or CONT (93 ± 1 mm Hg) (P, not significant [NS]). Hematocrit was 39 ± 1% in CaS, 37 ± 1% in CaS + BQ123 (P < 0.01), and 39 ± 3% in CONT (P, NS, CONT versus CaS or CaS + BQ123).

Renal Function

Rats receiving CaS only demonstrated profound renal dysfunction. GFR was 0.05 ± 0.02 versus 0.35 ± 0.11 mL/min per 100 g body wt in CONT animals (P < 0.025). Similarly, CaS-treated animals exhibited profound renal hypoperfusion with RPF 0.15 ± 0.06 versus 1.19 ± 0.30 mL/min per 100 g body wt for CONT animals (P < 0.005). In contrast, significant preservation of GFR was observed in rats treated with CaS + BQ123: GFR was 0.15 ± 0.03 mL/min/100 g body wt (P < 0.025 versus CaS, < 0.05 versus CONT). RPF for CaS + BQ123 animals was more than double that for CaS at 0.32 ± 0.08 mL/min per 100 g body wt. BQ123 did not affect renal function in animals not receiving CaS. Thus, GFR in CONT + BQ123 was 0.34 ± 0.06 mL/min/100 g body wt (P, NS versus CONT) and RPF was 1.26 ± 0.12 mL/min per 100 g body wt (P, NS versus CONT). Similarly, the vehicle for CaS, Cremophor, did not affect renal function. GFR determined by creatinine clearance was 0.61 ± 0.08 mL/min/100 g body wt in Cremophor rats, 0.50 ± 0.10 mL/min per 100 g body wt in CONT, and 0.62 ± 0.07 mL/min per 100 g body wt in CONT + BQ123 (P, NS, Cremophor versus CONT; P, NS, Cremophor versus CONT + BQ123).

Renal Structure

Histologic evaluation of renal tissue from CaS animals revealed findings consistent with the nephrotoxicity recently described in this chronic CaS model (16–18) (Figure 1: Table 1). In CaS-treated rats, the tubular dilation/vacuolization score was 1.07 ± 0.29, the tubulointerstitial fibrosis score was 0.78 ± 0.17, and arteriolopathy involved 78 ± 4% of the arterioles. Mild periglomerular fibrosis was evident, a finding consistent with ischemia. Control animals had minimal tubular dilation/vacuolization and tubulointerstitial fibrosis (scores, 0.06 ± 0.04 and 0.12 ± 0.04, respectively). Both parameters were similarly negligible in both CONT + BQ123 and Cremophor animals (P, NS, CONT versus CONT + BQ123 or Cremophor for both tubular dilation/vacuolization and fibrosis). Although significantly less than in CaS, arteriolar pathology was present in CONT animals, involving 26 ± 6% (P < 0.0005, CONT versus CaS), and 32 ± 4% in Cremophor animals (P < 0.0005, CONT versus CaS; P, NS, CONT versus Cremophor). Furthermore, BQ123 exacerbated arteriolopathy in salt-depleted rats: thus, in CONT + BQ123, the arteriolopathy score was 52 ± 6% (P < 0.025 CONT + BQ123 versus CONT or Cremophor). Contrasting the positive effect afforded by BQ123 on glomerular function in rats receiving CaS, no amelioration of structural injury was observed in these rats, with some injury even exacerbated. Thus, the mean tubular dilation/vacuolization score in CaS + BQ123 was 1.32 ± 0.12 (P, NS versus CaS), and arteriolopathy was present in 74 ± 2% of arterioles (P, NS versus CaS). Remarkably, the tubulointerstitial fibrosis score in CaS + BQ123 was significantly greater than that in CaS, at 1.20 ± 0.11 (P < 0.05).

DISCUSSION

This study shows that chronic treatment with CaS causes profound renal dysfunction, characterized by glomerular hypoperfusion and hypofiltration, as well as structural lesions, which include tubular dilation, vacuolization, atrophy and interstitial fibrosis, as well as arteriolopathy. Further, the study shows that chronic antagonism of the ETA receptor subtype ameliorates the glomerular dysfunction. However, although chronic ETA antagonism improved function in these studies, structural damage was not lessened; indeed, ETA receptor antagonism worsened the interstitial fibrosis in CaS-treated rats.

Glomerular dysfunction is a prominent and constant feature across the spectrum of CaS-related nephrotoxicity (1,2). Initially, CaS treatment is associated with a reversible renal dysfunction (1,25). Thus, normal, healthy subjects develop marked but transient renal vasoconstriction and impairment of GFR after even a single dose of CaS (26). Patients with recently transplanted kidneys also demonstrate renal hypoperfusion and hypofiltration after each daily CaS dose. This dysfunction has been shown to wane within several hours (25). Longer durations of CaS treatment are associated with persistent hypoperfusion/hypofiltration, with accompanying renal fibrosis (1,2). However, even at the advanced stage, the renal vasoconstriction can be lessened by interventions, such as decreasing the CaS dose (1). This observation implies that, even in the face of structural damage, there is a functional, i.e., a reversible, component to the renal vasoconstriction.

Previously, vasoconstriction accompanying acute, rather than chronic, CaS treatment has been extensively studied and has been linked to several endogenous vasoconstricting substances, including adrenergic tone, the renin-angiotensin system, thromboxane, and most recently, Et (3–6). Thus, CaS can stimulate Et-1 in vitro and can, at least transiently, increase Et-1 in vivo (7,8). CaS-induced renal dysfunction correlates with increased urinary Et levels (25). CaS can selectively up-regulate renal binding for Et and increase gene expression for at least one of the Et receptors (EtB) (9,10). These findings support a role for Et-1 as a mediator of acute renal dysfunction after CaS. Two receptors translate the actions of Et-1, ETA and EtB. Of the two, antagonism of the ETA receptor...
Renal morphology in rats treated with CsA. Arteriolopathy, characterized by periodic acid-Schiff-positive hyaline nodular lesions (arrow) and mild periglomerular fibrosis (top; original magnification, ×400). Patchy tubular dilation, vacuolization, and early interstitial fibrosis (arrows) (bottom; original magnification ×110). Periodic acid-Schiff stain.

Figure 1. Renal morphology in rats treated with CsA. Arteriolopathy, characterized by periodic acid-Schiff-positive hyaline nodular lesions (arrow) and mild periglomerular fibrosis (top; original magnification, ×400). Patchy tubular dilation, vacuolization, and early interstitial fibrosis (arrows) (bottom; original magnification ×110). Periodic acid-Schiff stain.

has been shown to offer protection against acute CsA-induced dysfunction (13,14). EtA antagonism also lessens the CsA-induced increase in myosin light chain phosphorylation, an in vitro parameter of mesangial cell contraction (27). Taken together, these studies indicate that acute exposure to CsA causes glomerular dysfunction, which is modulated by Et through, at least in part, the EtA receptor subtype. However, whether long-standing vasoconstriction associated with CsA is linked to Et-1 has not been determined. Furthermore, whether Et-1, through its effects on cellular proliferation and matrix deposition, also modulates structural damage associated with CsA is unknown. This is of particular interest, be-
cause chronic ETA antagonism has most recently been shown to lessen structural damage associated with renal ablation (28). We therefore studied the contribution of Et, through the ETA receptor, to glomerular dysfunction and fibrosis, which characterize chronic CsA nephrotoxicity.

Glomerular dysfunction was ameliorated in this chronic CsA model by chronic antagonism of the ETA receptor (Table 1). This finding suggests that, at least over this time course, functional, i.e., reversible, ETA-mediated hypoperfusion is an important determinant of renal dysfunction with chronic CsA treatment. Moreover, this component does not depend on the structural damage. These observations complement our recent findings in heart-transplanted rats with chronic CsA nephrotoxicity (29), which were characterized by profound renal vasoconstriction without structural damage. In those animals, the acute administration of the ETA receptor antagonist promptly improved GFR (30). These studies in chronic CsA nephrotoxicity, together with our previous observations that glomerular dysfunction after acute CsA administration is lessened by the acute antagonism of the ETA receptor (13), imply that Et, through the ETA receptor, mediates glomerular dysfunction throughout the spectrum of CsA toxicity. However, because ETA antagonism did not normalize GFR, other factors affecting vasoconstriction and/or structural injury also contributed to glomerular dysfunction.

In this regard, blockade of the ETA receptor may allow more interaction of Et-1 with the ETB receptor (31,32). Contrasting the recognized functions of the ETA receptor (vasoconstriction, cellular proliferation, and collagen deposition), the function of the ETB receptor remains elusive. It has been linked to the generation of nitric oxide and prostaglandin and, therefore, has been postulated to provide counterregulation for the vasoconstricting and proliferating actions of Et (33). However, our most recent data indicate a new function for the ETB receptor, that of the autoinduction of preproET-1 mRNA and peptide by Et-1 (34). Thus, the initial stimulation of Et-1 may, through this receptor subtype, amplify its own message, result in a sustained increase in the local Et-1 level and thereby mediate and perpetuate renal vasoconstriction (35,36). The augmented arteriopathy in salt-depleted animals receiving only the ETA receptor antagonist suggests that the initial stimulation of Et-1 may modulate this vascular lesion. Thus, the failure of ETA antagonism to fully abrogate the renal hypoperfusion/hypofiltration seen with CsA may relate to these actions of the ETB receptor.

The improved function in CsA + BQ123-treated rats is even more striking when one considers that this occurred in spite of worsening fibrosis (Table 1). Of note, renal dysfunction occurs in the absence of systemic hypertension in this model, and the amelioration of injury is therefore not dependent on any antihypertensive effects of BQ123. Although both groups showed tubular dilation/vacuolization and arteriolopathy, remarkably, animals treated with CsA + BQ123 had worse tubulointerstitial fibrosis. These observations suggest that the histologic damage with chronic CsA is not fully dependent on the hemodynamic perturbations. Because the ETA receptor mediates cell proliferation and matrix deposition, antagonism of this receptor would predict the blockade of these functions. However, Et-1 can potentiate the actions of other factors that stimulate cell proliferation or fibrosis. Our recent study in the chronic CsA model showed that a combined ETA/ETB antagonist improved function without ameliorating the structural lesion (37). In those studies, treatment with angiotensin-converting enzyme inhibitors showed beneficial effects on the structural injury, although renal hypoperfusion actually worsened, as also seen by Bennett (38) and likely related to the angiotensin-converting enzyme inhibitor effect via bradykinin to decrease efferent arteriolar tone (39). The potential importance of the renin-angiotensin system (RAS) in the fibrosis process is underscored by the finding of mild, albeit significant, tubulointerstitial fibrosis in salt-depleted animals, which are characterized by a stimulated RAS. These findings confirm previous reports that the RAS is pivotal in the development of renal fibrosis (15). The possibility remains, as has also been suggested by others, that the arteriolar lesion, at least in part, reflects the accumulation of renin and not smooth muscle cell necrosis (18). These studies also touch on another interesting interaction of vasoconstricting and fibrogenic processes, suggested by the work of Correa-Rotter et al. in the remnant kidney (40). Those authors observed increased RAS activation, specifically localized in the border zone adjacent to the infarcted tissue. Indirect interactions of Et and angiotensin II may thus occur, because it is possible that ETA antagonism could increase perfusion into such a border zone and provide a setting in which RAS and/or other cytokines linked to fibrosis could be increased.

In summary, these findings indicate an important role for Et-1 through the ETA receptor in mediating glomerular dysfunction associated with chronic CsA treatment. The study further indicates that, although the ETA receptor mediates vasoconstriction, factors other than ETA are responsible for the structural damage. These studies suggest that the optimization of renal function during chronic CsA treatment must encompass a multifaceted approach targeting the distinct processes of vasoconstriction and fibrosis.

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