Cyclosporine Promotes Glomerular Endothelin Binding In Vivo

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ABSTRACT

It has previously been shown that administration of cyclosporine causes a prompt (within 15 min after infusion) increase in circulating level of endothelin and a pattern of glomerular hypoperfusion and hypofiltration which can be ameliorated with anti-endothelin antibody. We now show that 60 min after cyclosporine, serum endothelin level falls to <2.55 ± 0.31 pg/mL (N = 6), a value comparable to that found in normal animals (<2 pg/mL). The study presented here also examines whether sustained cyclosporine-induced glomerular dysfunction is associated with altered endothelin receptor characteristics. Saturation and competitive inhibition binding studies in isolated glomerular membranes showed two binding sites. Of these, the density of the low-affinity site was affected by cyclosporine treatment (851 ± 117 versus 425 ± 61 fmol/mg of protein; P < 0.05; N = 6) without a change in equilibrium dissociation constant, Kd. The high-affinity site was not affected. The receptor characteristics of another vasoconstrictor, angiotensin II, were not affected by cyclosporine. In addition, there was no difference in endothelin binding sites in hepatic tissue between cyclosporine and control rats. These results raise the intriguing possibility that cyclosporine-induced glomerular dysfunction involves upregulation of endothelin binding sites and that altered endothelin receptors appear specific to the kidney.

Key Words: Endothelin receptor, cyclosporine, angiotensin, kidney

The positive therapeutic impact of cyclosporine (Cy) in the management of organ transplantation has been marred by its most frequent side effect, namely nephrotoxicity. Renal dysfunction, particularly in the initial days/weeks of Cy, appears to be a functional and reversible disturbance whose mechanisms remain largely undefined. Of interest in this regard are the recent studies which show that regulation of vasomotor tone involves endothelium-derived substances, including prostaglandins, endothelium-derived relaxation factor, and what is described as the most powerful vasoconstricting agent known, endothelin (Et). Et has already been implicated as having a pivotal role in the pathophysiologic mechanisms underlying Cy nephrotoxicity (1,2). Because Et is rapidly removed from the circulation, yet its vasoconstricting actions persist, we investigated the possibility that the persistent glomerular dysfunction after Cy accompanies alterations in the characteristics of Et binding.

METHOD

Animal Preparation

Experiments were done in male Munich-Wistar rats weighing 200 to 293 g. Under Inactin anesthesia (70 mg/kg body wt; i.p., BYK-Gulden, Konstanz, Germany), tracheotomy was performed and indwelling vascular catheters were inserted to monitor systemic blood pressure and for infusions as previously described (1). Group 1 rats were infused with Cy (20 mg/kg; i.v.; N = 6) or the identical volume of its vehicle, Cremophor (polyoxyethylene castor oil and alcohol) [Sandoz Pharmaceutical Co., East Hanover, NJ] administered over 10 min. The blood pressure was continuously monitored. After 1 h, the kidneys
and liver were harvested and processed for radioligand binding studies as described below.

Group 2 rats (N = 6) were prepared as for the radioligand binding study. Blood (~5 mL) was harvested from the aorta and immediately centrifuged at 4,000 rpm for 10 min at 0°C. Et measurements were performed by radioimmunoassay as described previously by us (1,3).

In group 3 rats, the functional implications of changes in the Et receptor characteristics were examined. After baseline measurement of clearances of p-aminohippuric acid and insulin to determine the renal plasma flow (RPF) and glomerular filtration rate (GFR), the rats were infused with Cy (N = 5) as in group 1 and clearances repeated. Et-1 (50 ng/kg/min; i.v.) was infused, and RPF and GFR were measured. In a separate group of normal rats (n = 4) not given Cy, RPF and GFR were determined before and after infusion of the identical dose of Et.

Group 4 rats were tested for the response to exogenous angiotensin II (All). After baseline clearance measurement, Cy was infused and clearance was repeated (N = 5); after 20 to 30 min of infusion of All (4.5 μg/kg/h; i.v.), clearance was again measured. In four normal rats without Cy treatment, clearance measurements were performed before and after All infusion.

**Tissue Preparation**

Isolation of glomeruli from rats was performed by a method previously reported by us (4). Glomeruli were homogenized in 4 vol of ice-cold 250 mM sucrose with a sonicator at setting 7 with four 15-s bursts separated by 15-s cooling intervals. The volume of the homogenate was adjusted to 12 mL with 250 mM sucrose and centrifuged at 200 × g for 10 min to remove unbroken cells and nuclei. The supernatant was then centrifuged at 40,000 × g for 60 min, and the resulting pellet was resuspended in Hank’s balanced salt solution (HBSS) containing 25 mM HEPES and 1 mM phenylmethylsulphonyl fluoride (PMSF). For the binding studies, the glomerular membrane was suspended in the final assay buffer of HBSS containing 0.2 g/dL of BSA, 5.5 mM glucose, 25 mM HEPES, 1 mM bacitracin, and 1 mM PMSF.

The liver was harvested at the same time as the kidneys and prepared as previously described (5). Liver was minced, homogenized in 4 vol of ice-cold 250 mM sucrose at setting 6 with 15 bursts on ice. The homogenate was centrifuged at 200 × g for 10 min. The supernatant was centrifuged at 40,000 × g for 60 min, and the resultant pellet was resuspended in HBSS with 25 mM HEPES and 1 mM PMSF. Membrane fractions (consisting of hepatocytes) (5) were stored at −70°C until the time of assay.

**Radioligand Binding Studies**

The experiments were performed in pairs with tissues from Cy-treated and identically prepared Cre-mophor-treated animals. The 125I-labeled endothelin ([125I]Et-1) and the homologous unlabeled peptide Et-1 were used for radioligand studies (Peptide International, Inc., Louisville, KY). Et-1 was radioliodinated by the lactoperoxidase method (6).

Ten 12.5 pM [125I]Et (SA, 2,200 Ci/mmol) and appropriate concentrations of unlabeled Et-1 (100 pM to 100 nM) were placed into borosilicate glass tubes in a volume of 200 μL, and the reaction was initiated by the addition of 800 μL of the glomerular or liver membrane suspension (membrane concentration, 20 μg/mL). Preliminary experiments showed that the binding curve is linear with membrane concentration from 12.5 to 45 μg/mL (Figure 1). Time course experiments showed that maximum binding was achieved at 120 min and remained stable up to 180 min at 25°C. Therefore, all of the following incubations were at 25°C for 150 min. Bound radioactivity was separated from the free within 5 s by suction/filtration through glass microfiber filters (Whatman 934AH; Whatman Ltd., Mainstone, England) pretreated with 4% BSA, followed by three washings with ice-cold phosphate-buffered saline containing 0.2 g/dL of BSA. Nonspecific binding was defined as radioactivity bound in the presence of saturating concentrations (0.5 μM) of unlabeled Et-1 and was approximately 3% of total bound ligand. Saturation binding studies were performed by membranes being incu-
treated rats (N = 102 ± 3 to 132 ± 8 mm Hg) as previously reported (1). One hour after administration of Cy, the Et level was, on average, 2.55 ± 0.31 pg/mL. This value, although statistically significantly higher, is numerically only slightly higher than the <2 pg/mL obtained in rats not receiving Cy and is markedly lower than the 41.7 ± 14.7 pg/mL previously observed by us within 20 min after administration of the identical dose of Cy (1).

Both competitive and saturation binding data were best fit with two sites model by using LIGAND analysis (Figures 1 and 2). In vehicle-treated normal rats, the equilibrium dissociation constant, Kd, for the two binding sites are in the same order of magnitude as previously reported in cultured glomerular mesangial cells (0.76 nM and 44.70 nM) (9). As can be seen in Table 1, the Et-1 binding characteristics were dramatically different between the vehicle-treated animals and those treated with Cy. In pilot studies, it was found that Et-1 binding characteristics were identical between normal animals and Cremophor-treated rats. The Et receptor density of the low-affinity site (Et receptor 2 [ER-2]) was approximately two-fold higher in Cy than in the control (CON) (851 ± 117 versus 425 ± 61 fmol/mg of protein; P < 0.05) without changes in K<sub>d</sub> (36.22 ± 5.52 versus 26.62 ± 4.81 nM). In contrast, there was no difference in either the receptor density or K<sub>d</sub> in the high-affinity site (Et receptor 1 [ER-1]). Saturation binding studies also demonstrated an increase in ER-2 density in Cy (857 ± 62 versus 375 ± 65 fmol/mg of protein; P < 0.05; K<sub>d</sub>, 26.8 ± 6.5 versus 27.3 ± 0.3 nM) with no change in ER-1 characteristics (Figure 2). Receptor characteristics of another vasoconstrictor, namely All, showed that there were no differences in either receptor density or K<sub>d</sub> between Cy and CON (receptor density [Ro], 169 ± 31 versus 155 ± 27 fmol/mg of protein; K<sub>d</sub>, 0.90 ± 0.08 versus 0.75 ± 0.04 nM) (Table 2).

Figure 3 shows the competitive inhibition study in the liver between [125I]Et-1 and other ligands, including Et-3, All and atrial natriuretic peptide (ANP), in liver. Binding of [125I]Et-1 was inhibited by increasing concentrations of unlabeled Et-1. The binding was highly specific and was not affected by excess amount of the other ligands, namely ANP or All. On the other hand, an endothelin isoform, Et-3, did displace the binding of [125I]Et-1 to some extent, although the potency was less than for Et-1, an observation previously noted in vascular smooth muscles (11). As in the kidney, the liver has two classes of high-affinity Et-1 binding sites. However, in contrast with the kidney tissue, there was no difference in either high- or low-affinity sites between normal and Cy-treated rats (Table 3).

Baseline RPF decreased from 4.73 ± 0.33 to 3.24 ± 0.04 mL/min after Cy (N = 5) and GFR fell from an average of 1.00 ± 0.08 to 0.78 ± 0.09 mL/min (P < 0.05). Infusion of exogenous Et-1 in Cy-treated rats (having upregulated Et receptors) dramatically decreased RPF to 1.95 ± 0.78 mL/min (P < 0.01) and GFR to 0.46 ± 0.15 (P < 0.01). By comparison, an identical dose of Et-1 infused into normal rats caused only subtle changes: RPF decreased from 4.25 ± 0.64 to 3.74 ± 0.58 mL/min after Et-1 and GFR fell from 0.94 ± 0.10 to 0.86 ± 0.08 mL/min. A different hemodynamic pattern was seen after infusion of All. Whereas Cy decreased GFR from the baseline of 1.05 ± 0.13 to 0.89 ± 0.14 mL/min, there was no further decrease with All (0.95 ± 0.04 mL/min; N = 5). In normal animals, All decreased GFR on average from 0.92 ± 0.04 to 0.76 ± 0.05 mL/min (N = 4).
TABLE 1. Glomerular Et-1 receptor characteristics

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>( R_0 ) (fmol/mg of protein)</th>
<th>( K_0 ) (nM)</th>
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<tbody>
<tr>
<td>Cy ((N = 6))</td>
<td>99 ± 9</td>
<td>0.096 ± 0.010</td>
</tr>
<tr>
<td>Con ((N = 6))</td>
<td>92 ± 23</td>
<td>0.108 ± 0.018</td>
</tr>
<tr>
<td>Cy ((N = 4))</td>
<td>169 ± 31</td>
<td>0.90 ± 0.08</td>
</tr>
<tr>
<td>Con ((N = 4))</td>
<td>155 ± 27</td>
<td>0.75 ± 0.04</td>
</tr>
</tbody>
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\( a \) Values are mean ± SE. Cy, rats treated with cyclosporine A; CON, control rats treated with Cremophor.

\( b \) \( P < 0.05 \) compared with CON.

TABLE 2. Glomerular All receptor characteristics

<table>
<thead>
<tr>
<th>Experimental Groups</th>
<th>( R_0 ) (fmol/mg of protein)</th>
<th>( K_0 ) (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cy ((N = 6))</td>
<td>851 ± 117( ^b )</td>
<td>36.22 ± 5.52</td>
</tr>
<tr>
<td>Con ((N = 6))</td>
<td>425 ± 61</td>
<td>26.62 ± 4.81</td>
</tr>
</tbody>
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\( ^a \) Values are mean ± SE. Abbreviations are as defined in footnote to Table 1.

DISCUSSION

We recently showed that i.v. administration of Cy causes a prompt increase (occurring within 20 min) in the circulating level of Et-1 (1). The study presented here shows that the elevation in circulating Et-1 after Cy administration is only a transient phenomenon. Sixty minutes after Cy, Et-1 level was, on average, <2.55 pg/mL, which is remarkably similar to the value obtained in normal animals (<2 pg/mL) yet strikingly lower than the 41.7 pg/mL observed within 15 to 20 min after Cy administration (1). The low circulating level observed was somewhat surprising in view of our previous findings that 1 h after Cy administration (dose identical to that used in the present study), the glomerular plasma flow rate was 48% and single nephron GFR (42%) was below normal control values. That the circulating Et-1 level of 2.55 pg/mL is physiologically important seems unlikely, because in two Cy-treated animals whose levels were <2 pg/mL, substantial hypoperfusion and hypofiltration were also observed. Moreover, that this hypoperfusion/hypofiltration is related to Et-1 is supported by our previous observation that anti-Et serum substantially improved the dysfunction (1), a finding recently repeated by Perico et al. (2).

The dissociation between the Et-dependent glomerular dysfunction after Cy and the circulating level of Et prompted evaluation as to whether the receptor characteristics for Et-1 are altered by Cy. Our glomerular preparations indicate two binding sites for Et-1. Although these may reflect binding to different cell types, a previous study in cultured mesangial cells also showed two binding sites (9). Also, although the possibility of one receptor with negative cooperativity can not be ruled out, previous studies in mesangial cells by using a cross-linking technique favor the existence of two distinct binding sites (10). Characteristics of Et-1 binding sites in isolated glomeruli and mesangial cells were similar. In view of the in vivo effect of Et-1, it is conceivable that glomerular Et-1 binding represents mostly that of mesangial cells. In previous studies with cultured mesangial cells, only high concentrations of Et-1 (5 to 100 nM) caused increases in intracellular calcium, inositoltrisphosphate, cell contraction, and intracellular alkalinization, thymidine incorporation, and mitogenesis (11–13). A lower dose of Et-1 (0.1 to 10 pM) caused slow and sustained increase in calcium with no demonstrable biological effects (12). Therefore, it is plausible that the lower-affinity binding site is linked to the functional receptor, whereas the high-affinity site may be related to some other yet unknown function.

Of these, the low-affinity binding site was found to have strikingly different characteristics in kidneys from rats treated with Cy when compared with vehicle-treated animals. Thus, the density for the low-
affinity site had almost doubled without a change in $K_0$ values. We examined the functional implications of this Cy-induced upregulation of renal Et-1 binding by assessing the renal hemodynamic response to exogenous Et-1 in normal and Cy-treated rats (having increased Et-1 receptors). In the Cy-treated rat, Et-1 caused RPF to fall precipitously, on average, by 39% and GFR by 37% when compared with only a 5% fall in RPF and an 8% fall in GFR in the normal rats given the identical infusion dose of Et-1.

To assess whether Cy affected receptors for other vasoconstrictory substances, receptor characteristics of AII were also examined in these animals. There were no appreciable differences in the receptor binding for AII between Cy- and vehicle-treated kidneys, pointing to a selective nature of Cy-induced upregulation of glomerular Et binding. In agreement with the binding data, exogenous infusion of AII in Cy-treated animals did not further decrease GFR. In addition, examination of hepatic tissue revealed that, in striking contrast to the kidney, there was no difference in the Et-1 binding characteristics in livers between Cy- and vehicle treated rats.

The long-lasting pressor/vasoconstrictive effects of Et-1 have been recognized since the original description of the purification and sequencing of Et by Yanagisawa et al. (14). It is now also recognized that although the biological actions of Et-1 are remarkably potent and long lasting, the estimated half-life for circulating Et is <1 min and 60% of exogenous Et injected in vivo is removed on first passage through the lungs (15,16). It appears, then, that persistent elevation in the circulating level of this substance is not necessary to effects its compelling actions; instead, the long-lasting effect of Et has been ascribed to a persistent activation of the vascular smooth muscle cells (17). In this regard, the observation presented here may have important implications in Cy-induced nephrotoxicity. The following possibilities remain to be clarified. First, the Et-1 binding sites may be associated with nonplasma membrane fractions which may differ between Cy and control. Of interest, recent reports show that changes in the Et receptor characteristics reflect receptor internalization/externalization (18). Thus, upregulation in Et-1 receptors in ischemic rat hearts was shown to reflect externalization of Et-1 binding sites (18), which was postulated to involve unmasking of already existing receptors which have been described in several cellular compartments (19). These studies with ischemia are of particular interest because renal hypoperfusion is a feature of Cy toxicity and may, therefore, be a factor in the pathophysiologic mechanisms. It is also possible that degradation of the ligand differs between Cy and control rats. In view of its versatile cellular effects (20), it is possible that Cy affects certain proteolytic enzymes. Cy is also known to increase membrane fluidity (20), which has a significant effect on the binding of biological substances. Finally, prior binding site occupancy may be different between Cy and controls. However, if this were the case, decreased binding sites rather than upregulation are expected in Cy because Et-1 production is increased, at least initially. Although much remains to be clarified on the mechanisms and functions of Et binding sites, this report demonstrates the upregulation of glomerular Et-1 binding site by Cy, which may play a role in glomerular dysfunction in Cy nephrotoxicity.

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