Cyclosporin A Protects Against Angiotensin II–Induced End-Organ Damage in Double Transgenic Rats Harboring Human Renin and Angiotensinogen Genes

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Abstract—Leukocyte infiltration and adhesion molecule activation play a central role in the pathogenesis of angiotensin II (Ang II)–induced end-organ damage in double transgenic rats (dTGR) harboring human renin and angiotensinogen genes. We tested the hypothesis that the immunosuppressive agent cyclosporine (CsA) protects against the Ang II–induced myocardial and renal damage in dTGR. Furthermore, we investigated the influence of CsA on interleukin-6 (IL-6) and inducible nitric oxide synthase (iNOS) expression and the DNA binding activity of transcription factor nuclear factor of activated T cells (NF-κB). The 4-week-old rats were divided into 4 groups: (1) control dTGR (n = 20), (2) dTGR plus CsA (5 mg/kg SC for 3 weeks, n = 15), (3) normotensive Sprague-Dawley (SD) rats (n = 10), and (4) SD rats plus CsA (n = 8). In dTGR, CsA completely prevented cardiovascular death (0 of 15 versus 9 of 20), decreased 24-hour albuminuria by 90% and systolic blood pressure by 35 mm Hg, and protected against the development of cardiac hypertrophy. Whole blood CsA concentrations 24 hours after the last drug treatment were 850 ± 15 ng/mL. Semiquantitative ED-1 and Ki-67 (a nuclear cell proliferation–associated antigen) scoring showed that CsA prevented perivascular monocyte/macrophage infiltration and prevented cell proliferation in the kidneys and hearts of dTGR, respectively. The beneficial effects of CsA were, at least in part, mediated by the suppression of IL-6 and iNOS expression. Electrophoretic mobility shift assay revealed that CsA regulated inflammatory response in part through the NF-κB transcriptional pathway. In contrast to dTGR, CsA increased blood pressure in normotensive SD rats by 10 mm Hg and had no effect on cardiac mass or 24-hour urinary albumin excretion. Perivascular monocyte/macrophage infiltration, IL-6, and iNOS expression or cell proliferation were not affected by CsA in SD rats. Our findings indicate that CsA protects against Ang II–induced end-organ damage and underscores the central role of vascular inflammatory response in the pathogenesis of myocardial and renal damage in dTGR. The beneficial effects of CsA in the kidney and heart are mediated, at least in part, by suppression of IL-6 and iNOS expression via NF-κB transcriptional pathway. (Hypertension. 2000;35[part 2]:360-366.)

Key Words: angiotensin II ● cyclosporin A ● albuminuria ● monocytes ● interleukins ● nitric oxide

Cyclosporine (CsA) has a selective inhibitory effect on T lymphocytes, suppressing the early cellular response to antigenic and regulatory stimuli.1,2 CsA and its binding protein cyclophilin associate with calcineurin at the endoplasmic reticulum of T cells and inhibit calcineurin catalytic activity. As a consequence, the nuclear translocation of nuclear factor of activated T cells (NFAT) and the induction of several cytokine genes are not initiated.1–3 Interestingly, a calcineurin-dependent transcriptional pathway has also been described in myocytes.4 CsA abolishes the hypertrophic response to angiotensin II (Ang II) in primary rat cardiomyocytes and prevents cardiac hypertrophy in transgenic mice overexpressing activated forms of calcineurin in the heart.4,5 These findings support the notion that calcineurin inhibition might be useful in the treatment of cardiac hypertrophy and congestive heart failure.6 However, studies on conventional animal models of in vivo acquired or genetic rodent hypertrophy have failed to demonstrate any beneficial cardioprotective effects by CsA,7–10 and therefore the potential role of calcineurin inhibition in the prevention of cardiac hypertrophy is still unclear.11,12 We showed previously that leukocyte infiltration and adhesion molecule activation play a central role in the pathogenesis of Ang II–induced myocardial and renal damage in double transgenic rats (dTGR) harboring human renin and angiotensinogen genes.13,14 Ang II concentrations in the plasma and kidney tissue are constantly 4- to
5-fold higher in dTGR compared with normotensive Sprague-Dawley (SD) rats. Furthermore, perivascular inflammation and cell proliferation occur via an Ang II–dependent and blood pressure–independent mechanism. We have also provided evidence that transcription factors nuclear factor-κB (NF-κB) and activator protein-1 are involved with the initiation of chemokine and cytokine overexpression in dTGR. We now test whether CsA prevents perivascular inflammation in the kidney and heart and thereby ameliorates the development of Ang II–induced end-organ damage. We also investigated the influence of CsA on interleukin-6 (IL-6) and inducible nitric oxide synthase (iNOS) expression and the DNA binding activity of transcription factor NF-κB.

Methods

Animals

Experiments were conducted in 35 male dTGR (4-week-old, body weight 60±3 g) and in 18 normotensive SD (63±2 g). The dTGR line and characteristics are described in detail elsewhere. The rats were purchased from Biological Research Laboratories Ltd and were allowed free access to standard 0.3% sodium rat chow (SSNIFF Spezialitäten GmbH) and drinking water. The procedures and protocols were approved by the local council on animal care, whose standards correspond to those of the American Physiological Society. dTGR and normotensive SD control rats were divided into 4 groups: (1) dTGR control group (n=20), (2) dTGR plus CsA group (n=15), (3) SD control group (n=10), and (4) SD plus CsA group (n=8). CsA (50 mg/mL Sandimmune infusion concentrate; Sandoz Ltd) was diluted in a 0.9% NaCl to produce a 2.5 mg/mL solution, which was administered once a day subcutaneously at a daily dose of 5 mg/kg for 3 weeks. This CsA dosage produced whole blood CsA concentrations of 700 to 1000 ng/mL in our previous study. Control dTGR and SD rats received the same volume of vehicle. Systolic blood pressure was measured weekly according to the tail-cuff method under light ether anesthesia 24 hours after the last drug dose starting at the age of 5 weeks. Urine samples were collected over a 24-hour period by metabolic cages at 5, 6, and 7 weeks. Rats were sacrificed while under thiopental anesthesia (150 mg/kg IP) at the age of 7 weeks. Blood samples for CsA determination were drawn via aortic puncture into prechilled tubes containing EDTA (6.25 mmol/L) as an anticoagulant. Tissue samples for an electrophoretic mobility shift assay (EMSA) of NF-κB were snap-frozen in liquid nitrogen, and samples for immunohistochemistry were snap-frozen in isopentane (−35°C). All samples were stored at −80°C until assayed.

Immunohistochemistry

Frozen kidneys and hearts were cryosectioned at 6 μm and air dried as described previously. The sections were fixed with cold acetone, air dried, and washed with Tris-buffered saline (0.05 mol/L Tris buffer and 0.15 mol/L NaCl, pH 7.6). The sections were incubated for 60 minutes in a humid chamber at room temperature with primary monoclonal antibodies against rat monocytes/macrophages (ED1; Serotec); Ki-67, a nuclear cell proliferation–associated antigen ex-
pressed in all active stages of the cell cycle (MIB-5; Dianova); and IL-6 (R and D Systems Europe) and with a polyclonal antibody against iNOS (Affinity BioReagents Inc). Semiquantitative scoring of ED-1– and MIB-5–positive cells in the heart and kidney was performed with the use of a computerized cell count program (KS 300 3.0; Zeiss). Fifteen separate areas of each heart and kidney samples (n = 5 in both groups) were analyzed. The kidney samples were examined without knowledge of the group of the rat.

**EMSA**

Tissue extraction and EMSA for the transcription factor NF-κB were performed as described in detail elsewhere.19,20 For EMSA, total kidney and heart homogenates (50 μg) were incubated in binding reaction medium [2 μg poly(dI/dC), 1 μg BSA, 1 mmol/L DTT, 20 mmol/L HEPES, pH 8.4, 60 mmol/L KCl, and 8% Ficoll] with 0.5 ng 32P-dATP end-labeled oligonucleotide containing the NF-κB binding site from the MHC enhancer (H2K; 5′- gatcCAGGGCTGGGGATTCCCACTTCCACAGC) at 30°C for 30 minutes. The DNA/protein complexes were analyzed in a 5% polyacrylamide gel/0.5% Tris buffer, dried, and autoradiographed. In competition assays, 50 or 100 ng unlabeled H2K oligonucleotides was used.

**Concentration Assays**

Whole blood CsA concentration was determined with the use of fluorescence polarization immunoassay (Abbott TDx cyclosporine monclonal whole blood method; Abbott Laboratories) with a monoclonal antibody specific for the parent molecule according to the manufacturer’s instructions. Urinary rat albumin was measured with a commercially available ELISA with rat albumin as the standard (Celltrend).

**Statistical Analysis**

Data are presented as mean±SEM. Statistically significant differences in mean values were tested with ANOVA and Tukey’s multiple range test. A value of P<0.05 was considered statistically significant. The data were analyzed with the use of SYSTAT statistical software (SYSTAT Inc).

**Results**

The dTGR had hypertension, severe renal damage, cardiac hypertrophy with focal necrosis, and a 50% mortality rate at 7 weeks. However, small vessels showed increased intimal and medial thickness, as well as hyaline deposits; tubules were frequently swollen and filled with proteinaceous material (Figure 1). Long-term treatment with CsA prevented vascular injury in small renal vessels and extracellular matrix formation (Figure 1). CsA completely prevented cardiovascular death in dTGR (0 of 15 versus 9 of 20, P=0.005). CsA decreased 24-hour urinary albumin excretion by 90% (Figure 2A). The systolic blood pressure of dTGR significantly increased from week 4 to 7; however, CsA treatment ameliorated the increase in blood pressure after 2 weeks of treatment. Nevertheless, untreated dTGR and CsA-treated dTGR showed significantly elevated blood pressure levels from week 5 on compared with SD and CsA-treated SD rats. In addition, CsA ameliorated cardiac hypertrophy (Figure 2C). In SD rats, CsA increased blood pressure by 10 mm Hg and had no effect on cardiac mass or 24-hour urinary albumin excretion (Figure 2). The whole blood CsA concentration was 850±15 ng/mL at 24 hours after the last drug treatment.

The perivascular monocyte/macrophage infiltration in the kidney (Figure 3A) and heart of untreated dTGR (not shown) was severe. The number of Ki-67–positive cells in the kidney (Figure 3D) and heart (not shown) vascular wall was also significantly higher in untreated dTGR than in SD rats. The use of CsA prevented local monocyte/macrophage infiltration and vascular cell proliferation (Figures 3B and 3E). In SD rats, CsA did not influence the number of ED-1– or Ki-67–positive cells. Semiquantitative scoring of ED-1– and Ki-67–positive cells is shown in Figures 3C and 3F, respectively. IL-6 expression in the vessel wall of renal artery was higher compared with SD rats (Figures 4A to 4C). Interestingly, the IL-6–positive cells colocalized with infiltrating neutrophils. iNOS expression in the glomeruli and renal arterial wall was also significantly higher compared with SD rats (Figures 4D

Figure 2. Effects of CsA on 24-hour urinary albumin excretion (A), blood pressure (B), and cardiac hypertrophy (C) in dTGR and normotensive SD rats. dTGR indicates dTGR control animals (n = 20); dTGR plus CsA, CsA-treated dTGR (n = 15); SD, SD control animals (n = 10); and SD plus CsA, CsA-treated SD rats (n = 8). CsA reduced albuminuria by 90%, decreased blood pressure, and ameliorated development of cardiac hypertrophy in dTGR. *P<0.01, dTGR vs dTGR plus CsA. #P<0.01, dTGR and dTGR plus CsA vs SD and SD plus CsA. Mean±SEM values are given.
to 4F). CsA suppressed the IL-6 and iNOS expression in the kidney (Figures 4B and 4E). EMSA for the detection of NF-κB showed greater DNA binding activity in the kidney (Figure 5) and heart of untreated dTGR compared with SD rats. CsA significantly reduced NF-κB DNA binding activity.

Discussion

Human renin and human angiotensinogen gene–harboring dTGR develop hypertension and severe hypertension-related end-organ damage due to local Ang II formation in the heart, kidney, and vasculature. We have shown recently that leukocyte infiltration and adhesion molecule activation play a central role in the pathogenesis of Ang II–induced end-organ damage. We currently tested the hypothesis that at therapeutic concentrations, the immunosuppressive agent CsA protects against Ang II–induced end-organ damage. Furthermore, we investigated the influence of CsA on IL-6 and iNOS expression and the DNA binding activity of transcription factor NF-κB in the kidney and heart. The most important finding in our study was that CsA completely prevented cardiovascular death, decreased albuminuria by 90%, and markedly attenuated the development of hypertension and cardiac hypertrophy in dTGR. The beneficial effects of CsA were closely related to suppression of monocyte/macrophage infiltration and cell proliferation in the kidneys and heart. We also provide evidence that CsA suppresses IL-6 and iNOS expression through the NF-κB transcriptional pathway. Our findings underscore the central role of vascular inflammation in the pathogenesis of myocardial and renal damage in dTGR and strongly suggest that immunomodulatory drugs might be useful as adjunctive therapy in the prevention of Ang II–induced cardiovascular complications.

Hypertension is a major risk factor for left ventricular hypertrophy and end-stage renal failure. In the present study, CsA slightly but significantly decreased blood pressure in dTGR. Conceivably, the beneficial effects of CsA were mediated in part by lowered blood pressure. However, in our previous study, even the complete normalization of blood pressure with non–renin-angiotensin system–dependent, triple-drug therapy (ie, hydralazine, reserpine, and hydrochlorothiazide) had no major effects on cardiac hypertrophy or albuminuria. Therefore, it is unlikely that the beneficial effects of CsA were mediated by blood pressure–dependent mechanisms.

CsA very effectively suppressed perivascular monocyte/macrophage infiltration and vascular smooth muscle cell proliferation in the kidneys and heart. The immunosuppressive effect of CsA is usually explained on the basis of calcineurin inhibition in the T cells. CsA inhibits the transcription of several inflammatory mediators, including IL-2,
granulocyte macrophage colony–stimulating factor, tumor necrosis factor-α, and interferon-α. Recently, Khanna and Hosenpud\textsuperscript{21} provided evidence that the antiproliferative effect of CsA occurs through the induction of the cell cycle inhibitor p21 and that this induction is dependent on transforming growth factor-β1. We found in the present study that CsA suppresses iNOS expression in the kidneys and heart. With the use of EMSA, we were able to demonstrate that the CsA-induced suppression of iNOS was associated with decreased DNA binding activity of NF-κB. Thus, our finding is in good agreement with a previous report by McCaffrey et al\textsuperscript{22} demonstrating the CsA sensitivity of the NF-κB site of the IL-2Rα promoter in untransformed murine T cells. Whether CsA interferes directly with NF-κB or indirectly by influencing the cross-talk between transcription factors NFAT and NF-κB\textsuperscript{23} remains to be determined.

IL-6 is a multifunctional proinflammatory cytokine that has several biological activities, including the induction of B

Figure 4. Representative immunohistochemical photomicrographs of IL-6 (A to C) and iNOS (D to F) expression in kidneys of untreated dTGR and CsA-treated dTGR and SD control rats. CsA reduced IL-6 and iNOS expression.
cell differentiation, T cell activation, the induction of acute-phase proteins in the liver, and the production of platelets. IL-6 is secreted from macrophages, T cells, endothelial cells, mesangial cells, and vascular smooth muscle cells. Previous studies have demonstrated that vascular smooth muscle cells inducibly secrete IL-6 in response to Ang II. Several regulatory DNA cis-elements, such as activator protein-1, cAMP response element, NF-IL-6, and NF-κB, have been found in the promoter region of the IL-6 gene. Recently, Han et al. showed that Ang II induces IL-6 transcription in vascular smooth muscle cells through the NF-κB transcription pathway. The present study provides the first evidence that Ang II also regulates IL-6 secretion in vivo. Because IL-6 has been shown to have chemotactic properties, it is tempting to speculate that within the vascular wall, IL-6 may play an important role in monocyte/macrophage recruitment.

CsA inhibits calcineurin, which is a calcium-dependent phosphatase that activates the NF-AT transcription factors. Transgenic mice expressing activated forms of calcineurin or NF-AT3 in the heart develop cardiac hypertrophy and heart failure. The hypertrophy was blocked with CsA administration. However, inhibition of cardiac hypertrophy was not achieved with the use of CsA in rats with constricted abdominal aortas. Aortic banding activates the renin-angiotensin system; however, the hypertrophy is principally related to pressure overload. Our model is primarily initiated by the

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**Figure 5.** Activation of DNA binding nuclear factor by Ang II. EMSA for detection of NF-κB shows a higher binding activity of dTGR kidney extracts compared with SD rats. CsA treatment reduced NF-κB level in kidney. EMSA was performed 3 times independently, with similar results.
local effects of Ang II. We showed that NF-κB is activated in this model and that possibly NF-AT transcription factors are also involved in cardiac hypertrophy. Signaling in cardiac hypertrophy involves both extracellular and intracellular events. Although controversial, the role of calcineurin-related pathways in cardiac hypertrophy deserves additional scrutiny.

In conclusion, our findings indicate that CsA protects against Ang II–induced end-organ damage and underscores the central role of vascular inflammatory response in the pathogenesis of myocardial and renal damage in TGR. The beneficial effects of CsA in the kidney and heart are mediated, at least in part, through the suppression of IL-6 and iNOS expression via the NF-κB transcriptional pathway.

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