Role of Angiotensin II and Reactive Oxygen Species in Cyclosporine A–Dependent Hypertension

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Abstract—Treatment with cyclosporine A (CysA), a potent immunosuppressive agent, is associated with systemic and renal vasoconstriction, leading to hypertension. The present study was conducted to elucidate the contribution of angiotensin II (Ang II) to CysA-induced hypertension and reactive oxygen species (ROS) generation. CysA (30 mg/kg per day SC), given for 3 weeks in rats, increased systolic blood pressure (SBP) from 119±2 to 145±3 mm Hg (n=7). Plasma and kidney Ang II levels were significantly higher in CysA-treated rats (136±10 fmol/mL and 516±70 fmol/g) than in vehicle-treated (1 mL olive oil) rats (76±10 fmol/mL and 222±21 fmol/g, n=7). CysA treatment increased AT1 receptor protein expression in the aorta (by 251±35%), whereas it was reduced in the kidney (by −32±4%). Superoxide anion production in aortic segments and kidney thiobarbituric acid–reactive substance (TBARS) contents were higher in CysA-treated rats (26±2 counts/min per milligram and 37±3 nmol/g) than in vehicle-treated rats (17±1 counts/min per milligram and 24±3 nmol/g). Concurrent administration of an AT1 receptor antagonist, valsartan (30 mg/kg per day, in drinking water), to CysA-treated rats (n=7) significantly decreased SBP (113±4 mm Hg) and prevented increases in vascular superoxide (16±2 counts/min per milligram) and kidney TBARS contents (21±3 nmol/g). Similarly, treatment with a superoxide dismutase mimetic, 4-hydroxy-2,2,6,6,-tetramethylpiperidine-N-oxyl (Tempol; 3 mmol/L in drinking water, n=7), prevented CysA-induced increases in SBP (115±3 mm Hg), vascular superoxide (16±1 counts/min per milligram), and kidney TBARS contents (19±2 nmol/g). These data suggest that ROS generation induced by augmented Ang II levels contributes to the development of CysA-induced hypertension. (Hypertension. 2003;42[part 2]:754-760.)

Key Words: angiotensin antagonist ■ antioxidants ■ receptors, angiotensin II ■ renin

Cyclosporine A (CysA) is a potent immunosuppressive agent and is widely used after organ transplantation and in the treatment of several autoimmune diseases.1 However, its clinical use is frequently complicated by systemic and renal vasoconstriction, leading to arterial hypertension.2 Although numerous factors have been implicated,2 several lines of evidence suggest an involvement of the renin-angiotensin system in the development of CysA-induced hypertension.3–10 Increased plasma renin activity and renin content in the development of CysA-induced hyperten-

Received May 12, 2003; first decision June 2, 2003; revision accepted June 25, 2003.

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DOI: 10.1161/01.HYP.0000085195.38870.44
incubation with CysA. In addition, impaired acetylcholine-induced relaxation in arteries isolated from CysA-treated rats was normalized by pretreatment with a superoxide dismutase (SOD). These observations suggest that an increased ROS production participates in CysA-induced endothelial dysfunction that might be responsible for the development of hypertension.

The aim of the present study was to elucidate the contribution of Ang II to CysA-induced hypertension and ROS generation. Accordingly, studies were performed to determine if the renin-angiotensin system is actually activated and ROS production is increased in CysA-induced hypertensive rats. We also investigated whether AT1 receptor blockade or treatment with an antioxidant prevents CysA-induced hypertension and ROS generation. In this study, we used an AT1 receptor antagonist, valsartan, and a membrane-permeable, metal-independent SOD mimetic, 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (Tempol). Tempol has been shown to be specific for superoxide anion and to reduce blood pressure in hypertensive animals.

### Methods

#### Animal Preparation

All experimental procedures were performed under the guidelines for the care and use of animals as established by the Kagawa Medical University and Tulane University Health Sciences Center. Male Sprague-Dawley (SD) rats (Clea Japan) weighing 162 to 184 g at the beginning of the experiments were selected at random to receive daily subcutaneous injection of CysA (Novartis Pharma) at a dose of 30 mg/kg per day (n = 7) or vehicle (1 mL of olive oil, n = 7) for a period of 3 weeks. In a separate experimental series, CysA-treated rats were treated with valsartan (Novartis Pharma, 30 mg/kg body weight per day in the drinking water) or Tempol (Sigma Chemical Co, 3 mmol/L in the drinking water) (n = 7, respectively). The doses of CysA, valsartan, and Tempol were chosen on the basis of results from previous studies in rats. Superoxide anion production in aortic segments was determined through the use of lucigenin chemiluminescence, as described previously. In brief, the aortic ring was placed in bicarbonate buffer with the following composition (in mmol/L): 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 KH2PO4, 1.2 MgSO4, 25.0 NaHCO3, 5.5 glucose, and 0.026 EDTA, which was bubbled continuously with 95% O2–5% CO2 to maintain the pH at 7.4 and allowed to equilibrate for 30 minutes at 37°C. After equilibration, the ring was rinsed with prewarmed (37°C) modified Krebs-HEPES buffer with the following composition (in mmol/L): 119 NaCl, 20 HEPES, 4.6 KCl, 1.0 MgSO4, 0.15 Na2HPO4, 0.4 KH2PO4, 25 NaHCO3, 1.2 CaCl2, and 5.5 glucose (pH 7.4). The ring was placed in 1 mL of Krebs-HEPES buffer containing lucigenin (Sigma Chemical Co; 20 μmol/L) and equilibrated in the dark for 10 minutes at 37°C. Recent studies indicate that lucigenin itself could act as a source of superoxide anion through auto-oxidation of the lucigenin cation radical. However, it was shown that auto-oxidation of lucigenin did not occur at <100 μmol/L in rat renal cortical tissues. Our preliminary data showed that vascular superoxide anion levels could be detected with the use of 20 μmol/L of lucigenin. Furthermore, these levels were significantly decreased by treatment with Tiron, which is often used as an alternative for native superoxide dismutase. On the basis of these preliminary data, we decided to use 20 μmol/L of lucigenin in the present study. The chemiluminescence was then recorded every 15 seconds for 5 minutes, with the use of a luminescence reader (BLR-301, Aloka). The lucigenin chemiluminescence was expressed as counts per minute per milligram if dry tissue weight.

#### Analysis of Kidney Samples for Angiotensigen and Ang II Receptors

Protein levels of angiotensigen as well as AT1 and AT2 receptors in the aorta and kidney tissues were analyzed by Western blot, as previously described in detail. Membranes were reprobed with an antibody against β-actin. All values were normalized by arbitrarily setting the densitometry of vehicle-treated rats to 1.0. Protein levels of angiotensigen in plasma were also measured, as previously described.

#### Analytical Procedures

Urinary protein excretion was determined with the use of a protein assay kit (microTP-test, Wako). We determined the degree of lipid peroxidation by using biochemical assays of LOOH and TBARS levels in plasma as well as TBARS contents in kidney tissues, as described previously in detail. Plasma renin activity, kidney renin content, and Ang II levels were measured by radioimmunoassay, as previously reported.

#### Statistical Analysis

The values are presented as mean±SEM. Statistical comparisons of the differences were performed with the use of 1-way or 2-way ANOVA combined with the Newman-Keuls post hoc test. A value of P<0.05 was considered statistically significant.

### Results

#### Blood Pressure, Plasma Creatinine Concentration, and Urinary Protein Excretion

SBP was identical among 4 groups at the beginning of the protocol. As shown in Figure 1, daily administration of CysA for 3 weeks at a dose of 30 mg/kg per day significantly increased SBP from 119±2 to 145±3 mm Hg (P<0.05). Concurrent administration of valsartan or Tempol prevented the development of CysA-induced hypertension (113±4 and
115 ± 3 mm Hg at week 3, respectively). At week 3, plasma creatinine levels in CysA-treated rats (0.55 ± 0.03 mg/dL) were higher than those in vehicle-treated rats (0.37 ± 0.03 mg/dL). Concurrent administration of valsartan or Tempol significantly decreased plasma creatinine levels in CysA-treated animals (0.37 ± 0.02 and 0.36 ± 0.01 mg/dL, respectively). Urinary protein excretion of vehicle-treated rats was 4.8 ± 0.5 mg/d. Treatment with CysA for 3 weeks did not alter urinary protein excretion (5.0 ± 0.8 mg/d). Furthermore, valsartan or Tempol did not alter urinary protein excretion in CysA-treated rats (3.5 ± 0.4 and 4.7 ± 0.6 mg/d, respectively).

**Renin-Angiotensin System in CysA–Induced Hypertensive Rats**

Analyses of integrated densitometric values (IDV) showed that CysA treatment did not alter plasma angiotensinogen levels (densitometric value/average control value ratios of 0.97 ± 0.06 versus 1.00 ± 0.06). Similarly, kidney angiotensinogen levels were similar between CysA-treated and vehicle-treated rats (densitometric value/average control value ratios of 0.95 ± 0.07 versus 1.00 ± 0.07). CysA-treated rats showed higher plasma renin activity (8.7 ± 1.0 ng Ang I/mL per hour) and kidney renin content (5.2 ± 0.9 × 10^3 ng Ang I/g per hour) compared with vehicle-treated rats (3.2 ± 0.8 ng Ang I/mL per hour and 3.4 ± 0.7 × 10^3 ng Ang I/g per hour, respectively, Figures 2A and 2B). Plasma Ang II levels were significantly higher in CysA-treated rats (136 ± 10 fmol/mL) than in vehicle-treated rats (76 ± 10 fmol/mL, Figure 2C). Similarly, CysA-treated rats showed higher Ang II contents in the kidney (516 ± 70 fmol/g) compared with vehicle-treated rats (222 ± 21 fmol/g, Figure 2D). Concurrent administration of valsartan markedly increased plasma Ang II levels in CysA-treated rats (411 ± 11 fmol/mL, Figure 2C). In contrast, kidney Ang II contents were significantly decreased by treatment with valsartan (146 ± 14 fmol/g, Figure 2D). On the other hand, Tempol did not alter plasma Ang II levels in CysA-treated rats (91 ± 16 fmol/mL, Figure 2C) but significantly decreased kidney Ang II contents in CysA-treated rats (292 ± 53 fmol/g, Figure 2D). CysA-treated rats showed augmented AT_1 receptor protein expression in the aorta, whereas AT_1 receptor expression in the kidney was reduced (Figure 3). Analyses of IDV showed that the ratios of aorta samples for AT_1 receptors were 2.5 ± 0.4-fold higher in CysA-treated rats compared with vehicle-treated rats, whereas those of kidney samples were significantly lower in CysA-treated rats (Figure 3). On the other hand, both aorta and kidney AT_2 receptor expressions were increased by long-term treatment with CysA (Figure 4). Analyses of IDV showed that the ratios of both aorta and kidney samples for AT_2 receptors were increased in CysA-treated rats by 2.8 ± 0.3- and 3.9 ± 0.3-fold, respectively (Figure 4). As a control study to check the equal loading, membranes were reprobed with an antibody against β-actin. The results showed that IDV were unaltered between both groups.

![Figure 1. Temporal profile of SBP. SBP was identical among 4 groups at week 0. Daily administration of CysA (30 mg/kg per day SC) significantly increased SBP. Concurrent administration of valsartan or Tempol prevented the development of CysA-induced hypertension. *P<0.05 vs baseline.](http://hyper.ahajournals.org/figures/02/00001.jpg)

**Figure 2.** Plasma renin activity (A), kidney renin contents (B), plasma angiotensin II (Ang II) concentrations (C), and kidney Ang II contents (D). CysA-treated rats showed significantly higher plasma renin activity and kidney renin contents compared with vehicle-treated rats (A and B, respectively). Similarly, plasma Ang II concentration and kidney Ang II contents were higher in CysA-treated rats than in vehicle-treated rats (C and D, respectively). AT_1 receptor blockade with valsartan markedly increased plasma Ang II levels in CysA-treated rats (C). In contrast, kidney Ang II contents were significantly decreased by treatment with valsartan (D). Tempol did not alter plasma Ang II levels (C) but significantly decreased kidney Ang II contents in CysA-treated rats (D). *P<0.05 vs vehicle-treated rats.
Effects of Valsartan and Tempol on ROS Levels

Plasma LOOH and TBARS levels in CysA-treated rats averaged 7.6±0.4 and 25.0±2.1 μmol/L, respectively. These levels were significantly higher than those of vehicle-treated rats (4.2±0.5 and 10.7±1.4 μmol/L, respectively). Concurrent administration of valsartan to CysA-treated rats significantly reduced plasma LOOH and TBARS levels (5.2±0.5 and 12.4±1.4 μmol/L, respectively). On the basis of group comparisons, these levels were not significantly different from those of vehicle-treated rats. Administration of Tempol resulted in similar decreases in plasma LOOH and TBARS levels (5.1±0.5 and 13.1±0.9 μmol/L, respectively). The lucigenin chemiluminescence from aortic segments of vehicle-treated rats averaged 17±1 counts/min per milligram dry tissue wt. In CysA-treated rats, the lucigenin chemiluminescence was significantly higher than in vehicle-treated rats (26±2 counts/min per milligram dry tissue wt, Figure 5A). In CysA-treated rats, valsartan or Tempol significantly decreased the lucigenin chemiluminescence to levels that were the same as those of vehicle-treated rats (16±2 and 16±1 counts/min per milligram dry tissue wt, respectively, Figure 5A). Kidney TBARS contents in CysA-treated rats averaged 37±3 nmol/g, which was significantly higher than those in vehicle-treated rats (24±3 nmol/g). As shown in Figure 5B, administration of valsartan or Tempol significantly reduced kidney TBARS contents in CysA-treated rats (21±3 and 19±2 nmol/g, respectively).

Discussion

In this study, we have demonstrated that circulating and intrarenal Ang II levels are increased in CysA-induced hypertensive rats. The present study also demonstrated that in CysA-induced hypertensive rats, increases in ROS production are associated with elevated Ang II levels. Furthermore, AT1 receptor blockade prevents both increases in ROS levels and the development of hypertension induced by CysA. In addition, treatment with a SOD mimic, Tempol, has a similar antihypertensive effect in these animals. These data suggest that ROS production induced by elevated Ang II levels contributes to the development of CysA-induced hypertension.

The present study provides evidence that Ang II levels are increased in both plasma and kidney tissues of CysA-induced hypertensive rats. However, the mechanisms by which CysA induces Ang II production remain to be elucidated. In vitro studies have shown that CysA stimulates renin release from cortical slices31 or isolated juxtaglomerular cells.32 In agreement with previous studies,9 we observed that circulating and

Figure 3. Western blot analysis of aorta (A) and kidney (B) AT1 receptor protein expression. Densitometric analysis showed that CysA treatment significantly increased AT1 receptor protein levels in the aorta by ~2.5-fold (A). In contrast, CysA-treated rats showed significantly reduced kidney AT1 receptor protein levels. Data are expressed as a relative difference in CysA-treated rats compared with vehicle-treated rats after normalization to the expression of β-actin, as described in the Methods section. *P<0.05 vs vehicle-treated rats.

Figure 4. Western blot analysis of aorta (A) and kidney (B) AT2 receptor protein expression. Densitometric analysis showed that CysA treatment significantly increased AT2 receptor protein levels in the aorta (A). CysA-treated rats also showed markedly increased kidney AT2 receptor protein levels (B). Data are expressed as relative difference in CysA-treated rats compared with vehicle-treated rats after normalization to expression of β-actin, as described in the Methods section. *P<0.05 vs vehicle-treated rats.

Figure 5. A, Vascular superoxide anion (O2−) production assessed by lucigenin chemiluminescence in aortic segments. B, Kidney TBARS contents. CysA treatment significantly increased vascular O2− (A) and kidney TBARS contents (B). Concurrent administration of valsartan or Tempol to CysA-treated rats reduced vascular O2− (A) and kidney TBARS contents (B). *P<0.05 vs vehicle-treated rats.
kidney renin activity was significantly increased in CysA-induced hypertensive rats. We also found that neither plasma nor kidney angiotensinogen levels were affected by treatment with CysA. These data indicate that increases in renin activity rather than angiotensinogen expression are responsible for the elevated Ang II levels in CysA-induced hypertensive rats. As expected, AT$_1$ receptor blockade with valsartan markedly increased plasma Ang II levels in CysA-treated rats. In contrast, kidney Ang II contents were significantly decreased by treatment with valsartan. These observations were consistent with previous studies showing that the progressive increases in intrarenal Ang II levels were prevented by AT$_1$ receptor blockade in Ang II–dependent hypertension. Thus, it is possible that CysA-induced augmentation of intrarenal Ang II was also prevented by AT$_1$ receptor blockade with valsartan. Interestingly, we also observed that although Tempol did not alter plasma Ang II levels in CysA-treated rats, kidney Ang II contents were significantly reduced by Tempol. At present, we have no satisfactory explanation why kidney Ang II contents were reduced by Tempol in these animals. Several studies indicate that CysA activates sympathetic nerve activity. Since renal nerve stimulation increases intrarenal Ang II formation, it is possible that CysA-induced activation of sympathetic nerve activity is involved in the augmentation of Ang II production. We recently reported that Tempol markedly reduced renal sympathetic nerve activity in spontaneously hypertensive rats. Therefore, it can be speculated that Tempol decreases intrarenal Ang II levels through its sympathoinhibitory effects. Clearly, further studies are needed to determine the effects of Tempol on kidney angiotensinogen levels and renin activity/contents as well as sympathetic nerve activity in CysA-induced hypertensive rats.

Avdonin et al. showed that pretreatment with CysA for 24 hours increased $[^{125}\text{I}]$Ang II binding in vascular smooth muscle cells without changing its affinity, suggesting an enhancement of Ang II receptor expression by acute CysA treatment. In the present study, we observed that long-term treatment with CysA increased protein expression of AT$_1$ receptors in the aorta. These observations are in accordance with previous studies showing that mRNA expression of AT$_1$ receptors in aortic vascular smooth muscle cells and endothelial cells were significantly increased in CysA-induced hypertensive rats. Thus, it is possible that the combination of elevated circulating Ang II levels and increased vascular AT$_1$ receptor expression contributes to the CysA-induced vascular dysfunction, leading to the development of hypertension. In contrast to the results obtained in the aorta, protein expression of AT$_1$ receptors in renal tissue was significantly reduced in CysA-induced hypertensive rats. These data are consistent with previous observations that AT$_1$ receptor mRNA expression is downregulated in the kidney after chronic CysA treatment in the rat. In the present study, we also found that AT$_1$ receptor expression was markedly increased in renal tissues (≈4-fold). Although functional roles of AT$_2$ receptors in the kidney are not clear, it can be speculated that the regulation of both AT$_1$ and AT$_2$ receptor expression in the kidney may represent an adaptive mechanism to attenuate CysA-induced renal dysfunction.

In agreement with previous studies, we observed that plasma creatinine levels were significantly increased in CysA-induced hypertensive rats, suggesting CysA-induced glomerular dysfunction. We also observed that treatment with valsartan or Tempol significantly decreased plasma creatinine levels in CysA-treated animals. These results indicate that CysA-induced glomerular dysfunction was ameliorated by treatment with valsartan or Tempol. On the other hand, the present study showed that urinary protein excretion of protein was not altered in CysA-induced hypertensive rats, as has been previously reported. It is possible that the duration of CysA treatment to the rats conducted in this study is relatively short compared with the duration of CysA treatment in humans. Therefore, a histologically distinguishable lesion resembling the CysA nephropathy identified in sections of human renal biopsies was not seen in the present experimental settings (data not shown). Nevertheless, recent studies showed that this lesion is produced by CysA treatment in SHR fed a high salt diet and salt-depleted normotensive rats. Further investigations in these animals are required to determine the role of intrarenal Ang II in the progression of CysA nephropathy.

Ang II stimulates NAD(P)H oxidase–dependent superoxide production in different types of cells or isolated vessels. The superoxide produced on Ang II stimulation is rapidly converted to other ROS, including peroxynitrite and H$_2$O$_2$. In Ang II–dependent hypertensive animals, the production of vascular superoxide and the levels of other markers of ROS were elevated. Furthermore, peroxynitrite, which is the chemical combination of superoxide with NO, oxidizes arachidonic acid and thus may stimulate the formation of a potent vasoconstrictor isoprostane. Recent studies also indicate that ROS can exert vasoconstrictor effects independent of NO mechanism. In the present study, we demonstrated that CysA-induced hypertension is associated with elevated Ang II and ROS formation in rats. In addition, AT$_1$ receptor blockade prevented increases in ROS levels and development of hypertension induced by long-term treatment with CysA, suggesting that at least part of increased ROS generation is a consequence of the elevated Ang II levels. Since CysA-induced hypertension was also prevented by treatment with Tempol, it is possible that elevated ROS formation plays a role in the pathogenesis of CysA-induced hypertension.

Although our data suggest a potential participation of Ang II in ROS production in CysA-induced hypertensive rats, other mechanisms may also be involved in CysA-induced ROS production. In vitro studies showed that CysA increased ROS levels in a variety of cells. Furthermore, it was shown that CysA induced lipid peroxidation in isolated hepatic and renal microsomes. These data suggest that CysA or its metabolites directly stimulate ROS production. Furthermore, CysA inhibits mitochondrial respiration, which could also lead to ROS production. Zhong et al. showed that CysA-induced sympathetic nerve activation and ROS production in the kidney were prevented by renal denervation. Therefore, it is possible that CysA increases ROS production.
production by increasing renal sympathetic nerve activity, resulting in vasconstriction. Several studies also showed that CysA-induced hypertension was attenuated by endothelin receptor blockade. Furthermore, it was recently shown that CysA-induced superoxide formation in rat aorta was blocked by an endothelin receptor antagonist, suggesting a role of endothelin in CysA-induced ROS production. It is well known that Ang II stimulates endothelin production. Therefore, the possibility exists that the CysA-induced augmentation of Ang II levels leads to increases endothelin formation, which may also participate in CysA-induced ROS production. Collectively, it seems likely that ROS production is augmented via multiple mechanisms in CysA-induced hypertension.

Perspectives
The involvement of the renin-angiotensin system and ROS in the development of CysA-induced hypertension has been supported by several studies. The present study provides direct evidence that Ang II and ROS formations are actually augmented in CysA-induced hypertensive rats. We have also demonstrated that antihypertensive effects of AT1 receptor blockade and Tempol are associated with reductions in ROS levels in these animals, indicating the contribution of Ang II and ROS formations to CysA-induced hypertension. Recent clinical studies indicate that ROS levels are elevated in CysA-treated hypertensive patients. Therefore, the antioxidative actions of AT1 receptor blockade could explain some of beneficial effects of AT1 receptor antagonists in recently reported clinical studies.

Acknowledgments
This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan (A.N., Y.A.), the Research Foundation for Pharmaceutical Sciences (A.N.), Japan Research Foundation for Clinical Pharmacology (T.F., A.N.), the Uehara Memorial Foundation (A.N. and H.K.), the National Kidney Foundation (H.K.) and Health Excellence Found from the Uehara Memorial Foundation (A.N. and H.K.), the National Institute of Health Sciences (A.N.), the Research Foundation for Pharmaceutical Sciences (A.N.), from the Ministry of Education, Science, and Culture of Japan (A.N., Y.A.), the Research Foundation for Pharmaceutical Sciences (A.N.), and the Ministry of Education, Science, and Culture of Japan (A.N., Y.A.).

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Hypertension. 2003;42:754-760; originally published online July 21, 2003; doi: 10.1161/01.HYP.0000085195.38870.44

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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