Involvement of Tyrosine Hydroxylase Upregulation in Cyclosporine-Induced Hypertension

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ABSTRACT—To identify the mechanism of cyclosporine-induced hypertension, we studied the effect of cyclosporine on the catecholamine synthetic pathway in rats. We administered cyclosporine (10 mg/kg per day, s.c.) for 3 days to 10-week-old male Wistar rats. Systolic blood pressure increased significantly in the cyclosporine-treated group in comparison to that in the control group. Norepinephrine and epinephrine levels in the adrenal medulla and plasma of cyclosporine-treated rats were also significantly higher than levels in the control rats. Moreover, tyrosine hydroxylase (TH) activity and TH mRNA expression in the adrenal medulla of cyclosporine-treated rats were significantly elevated. Administration of the TH inhibitor G61-methyl-p-tyrosine (200 mg/kg, b.i.d., s.c.) for 3 days significantly suppressed cyclosporine-induced increases in systolic blood pressure. Phosphorylation of cyclic AMP responsive element-binding protein (CREB) and its binding activity to DNA in the nuclear fraction from the adrenal medulla of cyclosporine-treated rats were much higher than that of the control rats. Calcineurin protein expression of cyclosporine-treated rats was less than that of the control rats. These results suggest that cyclosporine increased blood pressure via activation of the catecholamine synthetic pathway due to the activation of transcription factor CREB.

Keywords: Cyclosporine, Hypertension, Tyrosine hydroxylase, Catecholamine, Transcription factor

Cyclosporine is one of the most effective drugs administered for immunosuppression in patients who have undergone organ transplantation. However, since cyclosporine often causes hypertension, it is not always possible to continue cyclosporine therapy. Schom et al. (1) reported that high blood pressure was detected much more frequently in kidney transplant patients treated with cyclosporine than in such patients treated with azathioprine and prednisolone. Ponticelli et al. (2) reported that 81.6% of patients treated with cyclosporine after kidney transplant develop hypertension. Moreover, cyclosporine-induced hypertension occurs in over 70% of liver transplant patients, and it approaches 100% in cardiac transplant recipients (3, 4). Several mechanisms have been proposed for cyclosporine-induced hypertension such as activation of the renin-angiotensin system and suppression of the nitric oxide system (5, 6). These mechanisms have not been explained sufficiently. The sympathetic nervous system is important in the regulation of blood pressure. Scherrer et al. (7) reported cyclosporine-induced hypertension to be accompanied by increased sympathetic nerve firing in the peroneal nerve, and Morgan et al. (8) demonstrated that cyclosporine increases renal and lumbar sympathetic nerve activity, but the mechanisms of cyclosporine-induced hypertension have not yet been clarified in detail. Iida et al. (9) reported that injection of cyclosporine increased binding activities of transcription factors to the cAMP response element (CRE) in the striatum and nucleus accumbens. Thus, we investigated the relationships between cyclosporine-induced hypertension, catecholamines derived from the sympathetic nervous system, and the transcription factors of the tyrosine hydroxylase gene such as phosphorylated CRE-binding protein (CREB).

MATERIALS AND METHODS

Animals

Male Wistar rats (10 weeks of age) were obtained from SLC (Japan Shizuoka Laboratory Animal Center, Hamamatsu). All studies were performed according to the “Guiding Principles for the Care and Use of Laboratory Animals” of The Japanese Pharmacological Society. The rats were housed in a room where temperature (23 ± 1°C),
humidity (55 ± 5%), and lighting (light from 6 AM to 6 PM) were controlled. We sacrificed animals and collected blood under the pentobarbital anesthesia (35 mg/kg, i.p.).

**Drugs**

Cyclosporine (10 mg/kg per day, s.c.; Sigma, St. Louis, MO, USA) was administered for 3 days. Corn oil was administered to control rats as a vehicle. α-Methyl-p-tyrosine (α-MT) (200 mg/kg, b.i.d., s.c.; Aldrich Chemical Co., Milwaukee, WI, USA) was also administered for 3 days alone or in combination with cyclosporine.

**Blood pressure measurement**

Systolic blood pressure was measured by the tail-cuff method (PS-100; Riken Kaibatsu Co., Tokyo) in conscious rats placed on a hot plate (37°C). Six to seven blood pressure readings were obtained from each rat and averaged.

**Epinephrine and norepinephrine analysis**

The method of epinephrine and norepinephrine analysis was as described previously (10). The tissues were homogenized in a glass tissue grinder in ice-cold 0.05 M perchloric acid with dihydroxy benzylamine (1 µg/2 ml) as an internal standard. The homogenate was centrifuged at 15,000 × g for 20 min at 4°C and the supernatant was used for assay of epinephrine and norepinephrine. The supernatant was mixed with 10 µg aluminum oxide and 100 µl of 2 M Tris-EDTA (pH 8.7) for 15 min. The sedimented aluminum oxide was washed with 1 ml of 16.5 mM Tris-EDTA (pH 8.1). The sedimented aluminum oxide was then dried and mixed with 200 µl of solvent medium (100% acetic acid : 10% sodium metabisulfite : 5% EDTA : water = 0.1 : 0.05 : 0.05 : 9.8) for 15 min, followed by centrifugation at 1,800 × g for 1 min. The supernatant was passed through a 0.22-µm filter, and an aliquot (10 µl) was injected to a high performance liquid chromatography (HPLC) (510 pump; Waters, Milford, MA, USA) with an electrochemical detector (460 detector, Waters) and a column (Cosmosil SC8-AR packed column, 4.6 × 150 mm; Nacalai Tesque, Kyoto). The mobile phase consisted of the following components: 50 mM sodium acetate, 20 mM citric acid, 3.75 mM sodium octyl sulphate, 1 mM di-n-butylamine, 0.134 mM EDTA and 5% (V/V) methanol. All separations were performed isocratically at a flow rate of 0.9 ml/min at 35°C. The detector potential was maintained at +0.65 V. Recovery of alumina absorption was more than 95%.

**Northern blot and dot blot analysis**

RNA isolation was performed by the Chomczynski and Sacchi method (12) and as described previously (10). The total RNA was extracted with guanidine thiocyanate and purified by phenol chloroform and ethanol. TH mRNA levels were determined by Northern blot and dot blot analysis. TH cDNA was produced by RT-PCR (Retro script; Ambion, Austin, TX, USA). The sequences of the primers were as follows: 5'-TCGCCACAGCCCCAAGGCTTCAGA-3' (sense) and 5'-CACAGGCTGTAGTTTGGAT-3' (antisense) (13). The first 100 bases of the RT-PCR product from the 5' region were confirmed. Human β-actin cDNA (0.4 kb; Wako Junyaku Co., Osaka) was used as the control. The TH cDNA and β-actin cDNA probe were labeled with [α-32P]dCTP (Dai-ichi Kagaku Yakuhin Co., Tokyo). Total RNA (20 µg for Northern blot and 3 µg for dot blot) were incubated with glyoxal and hybridized with each radio-labeled probe overnight. The membranes were washed and exposed to scientific imaging with an intensifying screen at −80°C for 1 day.

**Electrophoretic mobility-shift assay**

Adrenal medulla was homogenized in homogenizing buffer (10 mM HEPES-NaOH buffer (pH 7.6), 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 1 mM dithiothretiol, 1 mM phenyl-methyl sulfonyl fluoride, 2 M sucrose and 10% glycerol) and then centrifuged 100,000 × g at 4°C, 30 min. Nuclear fractions were resuspended in nuclear extraction buffer (20 mM HEPES-
NaOH buffer (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol and 1 mM phenyl-methyl sulfonyl fluoride (PMSF). Electrophoretic mobility-shift assay was performed as described by Ishige et al. (14) with slight modifications. Briefly, the binding reactions were carried out by incubating 10 μg of nuclear extract with 1 μg of poly (dl-dC) in the reaction mixture (20 mM HEPES-NaOH (pH 7.9), 1 mM dithiothreitol, 0.3 mM EDTA, 0.2 mM EGTA, 80 mM NaCl, 10% glycerol and 0.2 mM PMSF) for 15 min on ice. Approximately 10,000 cpm of 32P-labeled CRE (sense sequence 5’-GATTGGCTGACGT CAGAGAGCT-3’) oligonucleotide were added and the incubation was continued for a further 30 min at room temperature. DNA-protein complexes were applied on a 5.0% polyacrylamide gel in a buffer containing 25 mM Tris-HCl (pH 8.2) and 0.5 mM EDTA. Electrophoresis was carried out at 4°C. The gel was dried and exposed to scientific imaging film (Eastman Kodak Co., Rochester, NY, USA) with an intensifying screen at ~80°C.

Immunoblot analysis

TH, phosphorylated CREB and calcineurin immunoreactive protein content were examined. Aliquots of samples (TH: cytosolic fraction, phosphorylated CREB and calcineurin: nuclear extract) containing 2.5 μg protein each were subjected to SDS-PAGE using a 10.0% acrylamide gel, and then the resolved proteins were transferred to nitrocellulose membranes. Subsequently, the membranes were reacted with the polyclonal antibody to each of the primary antibodies and developed by the ECL method (Amersham, Arlington Heights, IL, USA). The intensities of the bands corresponding to the proteins on membrane of TH protein were measured with a densitometer (AE-6900-M; ATTO, Tokyo). Antibody for TH was obtained from Chemicon International Inc. (Temecula, CA, USA); antibody for phosphorylated CREB was obtained from Calbiochem (La Jolla, CA, USA); and antibody for calcineurin was obtained from Santa Cruz Biotec. Inc. (Santa Cruz, CA, USA).

Statistical analyses

Data are presented as the mean ± S.E.M. Statistical differences between mean values were analyzed by Dunnett’s multiple range test. A probability value of less than 0.05 was considered to be statistically significant.

RESULTS

Effects of cyclosporine on systolic blood pressure

Systolic blood pressure before and after treatment with cyclosporine is shown in Fig. 1. Control rats showed no change in systolic blood pressure, but cyclosporine-treated rats showed a significant increase (P<0.05).

Effects of cyclosporine on plasma catecholamine levels

Norepinephrine and epinephrine levels in plasma of control rats were 18.0 ± 2.4 and 55.4 ± 8.9 ng/ml, respectively; levels in cyclosporin-treated rats showed significant increases to 27.0 ± 4.1 and 80.1 ± 5.1 ng/ml, respectively (P<0.05).

Effects of cyclosporine on catecholamine levels in the adrenal medulla

Mean norepinephrine and epinephrine levels in the adrenal medulla of control rats were 0.320 ± 0.016 and 1.54 ± 0.082 mg/g tissue, respectively; in cyclosporine-treated rats, they increased to 0.409 ± 0.040 and 2.18 ± 0.29 mg/g tissue, respectively (P<0.05).

Effects of cyclosporine on TH activity in the adrenal medulla

Mean TH activity in the cytosolic fraction from cells of the adrenal medulla of control rats and cyclosporine-treated rats were 12.8 ± 0.50 and 16.3 ± 1.18 μg/g tissue per hour, respectively. Activity was significantly higher in the cyclosporine-treated rats (P<0.05).

Effects of cyclosporine on TH protein expression in the adrenal medulla

TH protein expression in the adrenal medulla of cyclosporine-treated rats was significantly higher than that of control rats (P<0.01), as shown in Fig. 2.
Effects of cyclosporine on TH mRNA level (ratio of TH mRNA to \(\beta\)-actin mRNA) in the adrenal medulla

TH mRNA level in the adrenal medulla of cyclosporine-treated rats was significantly higher than that of control rats \((P<0.05)\), as shown in Fig. 3.

Effects of cyclosporine and \(\alpha\)-MT on systolic blood pressure

The effects of cyclosporine and \(\alpha\)-MT on systolic blood pressure are shown in Fig. 4. There was no obvious difference in systolic blood pressure between the control rats and the \(\alpha\)-MT-treated rats. \(\alpha\)-MT significantly depressed cyclosporine-induced increases in systolic blood pressure \((P<0.05)\).

Effects of cyclosporine on CREB phosphorylation and its binding activity to CRE

The effects of cyclosporine on CREB phosphorylation and its binding activity to CRE are shown in Fig. 5. CREB phosphorylation was much higher in cyclosporine-treated rats than in control rats. CRE-binding activity of CREB in the nuclear fraction of cells from the adrenal medulla of cyclosporine-treated rats was much higher than that of the control rats. We used adrenal medulla of 10 rats as one sample of nuclear extract for one examination.

Effects of cyclosporine on calcineurin protein expression

The effect of cyclosporine on calcineurin protein expression in the nuclear fraction of cells from the adrenal medulla of rats is shown in Fig. 6. Calcineurin levels were lower in cyclosporine-treated rats than in control rats. We used adrenal medulla of 10 rats as one sample of nuclear extract for one examination.

Fig. 2. The effect of cyclosporine treatment on tyrosine hydroxylase (TH) protein expression or level in the cytosolic fraction of adrenal medulla cells. Upper panel shows Western Blot analysis. Lower panel shows data of densitometric analysis. Values are the mean \(\pm\) S.E.M. Cont: control group, Cyclo: cyclosporine-treated group.

Fig. 3. The effect of cyclosporine treatment on tyrosine hydroxylase (TH) mRNA to \(\beta\)-actin mRNA in the adrenal medulla of rats. Upper panel shows Northern Blot analysis. Lower bar graph shows dot blot analysis. Values are the mean \(\pm\) S.E.M. Cont: control group, Cyclo: cyclosporine-treated group.
DISCUSSION

Our results confirmed that cyclosporine significantly increases systolic blood pressure in rats. We also observed elevation of plasma norepinephrine and epinephrine levels with administration of cyclosporine. Blood pressure is controlled positively by circulating catecholamines.

Scherrer et al. (7) reported that cyclosporine increases sympathetic nerve firing in the peroneal nerve and induces hypertension; Grobecker et al. (15) showed that prazosin, an α₁-blocker, inhibited increases in blood pressure in cyclosporine-treated patients. Thus, the elevated plasma norepinephrine and epinephrine levels in this study are likely related to the mechanism of cyclosporine-induced hypertension.

Plasma catecholamines are derived mainly from the adrenal medulla. Circulating norepinephrine originates from the adrenal medulla and postganglionic sympathetic nerve endings, and circulating epinephrine originates almost exclusively from the adrenal medulla (16). We investigated the effects of cyclosporine on the catecholamine synthetic pathway in the adrenal medulla based on indices of systemic sympathetic nervous activity. Cyclosporine increased norepinephrine and epinephrine levels in the adrenal medulla in association with increased plasma levels. We speculate that cyclosporine increased the synthesis of norepinephrine and epinephrine in the peripheral sympathetic nervous system (i.e., in the adrenal medulla), which

![Fig. 4. Bar graph shows the effect of α-methyl-p-tyrosine, a tyrosine hydroxylase inhibitor, on changes in systolic blood pressure after treatment with cyclosporine. Values are the mean ± S.E.M. Cont: control group, Cyclo: cyclosporine-treated group, α-MT: α-methyl-p-tyrosine-treated group, Cyclo + α-MT: cyclosporine and α-methyl-p-tyrosine-treated group. Cyclosporine (10 mg/kg per day, s.c.) and/or α-MT (200 mg/kg, b.i.d., s.c.) were administered for 3 days. The number of rats is shown in parentheses.](image)

![Fig. 5. The effect of cyclosporine on cyclic AMP responsive element-binding protein (CREB) phosphorylation (pCREB) and its DNA binding activity to cyclic AMP responsive element (CRE) in the adrenal medulla of rats. Left panel shows Western blot analysis. Right panel shows electrophoresis mobility shift assay. Cont: control group, Cyclo: cyclosporine-treated group.](image)

![Fig. 6. The effect of cyclosporine treatment on calcineurin protein expression in the nuclear fraction of adrenal medulla. Cont: control group, Cyclo: cyclosporine-treated group.](image)
were then secreted to plasma.

TH is a rate-limiting enzyme of the catecholamine synthetic pathway. In our study, cyclosporine increased TH activity and TH protein expression in the adrenal medulla, suggesting that elevations in TH protein, TH activity and catecholamine levels, which are followed by an increase in TH mRNA expression, are associated with cyclosporine-induced hypertension.

To evaluate further whether TH contributes to cyclosporine-induced hypertension, we investigated the effect of TH inhibition on cyclosporine-induced increases in systolic blood pressure. α-MT, a reversible and competitive inhibitor of TH, significantly inhibited cyclosporine-induced increases in blood pressure, suggesting strongly that TH plays an important role in the mechanism of cyclosporine-induced hypertension.

Furthermore, we investigated in detail the mechanism involved in elevation of TH upregulation by cyclosporine treatment. There are several transcription factor binding domains in the promoter region that regulate transcription of the TH gene in the adrenal medulla (17, 18). Cyclosporine inhibits calcineurin activity after complexing with cyclophilin. Calcineurin is a Ca²⁺/calmodulin-dependent phosphatase that has been shown to play an important role in the regulation of gene expression (19). We found that cyclosporine increased CREB-binding activity in the nuclear fraction of the adrenal medulla and that calcineurin immunoreactivity in the nuclear fraction decreased with cyclosporine treatment. Cyclosporine is known to inhibit the formation of the calcineurin-cyclophilin complex, and calcineurin has been shown to enhance phosphorylated-CREB dephosphorylation by nuclear protein phosphatase 1 when complexed with cyclophilin (20). Phosphorylated CREB has DNA binding activity, and phosphorylation of CREB is usually controlled negatively by the calcineurin-cyclophilin complex. We found that cyclosporine increased phosphorylated CREB immunoreactivity and that the calcineurin protein level decreased in the nuclear fraction with cyclosporine treatment. Thus, it was assumed that inhibition of the calcineurin-cyclophilin complex by cyclosporine suppresses dephosphorylation of CREB. It is reasonable to consider that as a result of inactivation of calcineurin by cyclosporine, dephosphorylation of CREB by calcineurin is inhibited and that increased CREB phosphorylation enhances the transcription activity of TH mRNA.

In conclusion, we showed that cyclosporine increases catecholamine levels in plasma and the adrenal medulla. We also showed that cyclosporine increases TH activity and its mRNA expression. Results suggest that cyclosporine increases blood pressure via activating the catecholamine synthetic pathway. Furthermore, changes in the activation of transcription factors that may be related to activation of the sympathetic nervous system are proposed for cyclosporine-induced hypertension. Further studies are needed to clarify the interactions of these factors.

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