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Acute Cyclosporin A-Treatment Impairs the Cytosolic Guanylate Cyclase-Mediated Vasodilatation in Rat Thoracic Aorta

Hyun Kook

Department of Pharmacology, Chonnam University Medical School, 5 Hak-dong, Dong-ku, Kwangju 501—190, Korea

Cyclosporin A (CsA), a widely used immunosuppressant, is well known to cause nephrotoxicity and hypertension as major side effects. The present study was aimed at investigating the effects of CsA-pretreatment on the activities of cytosolic guanylate cyclase (cGC) in relation to the alteration of relaxant responses in the rat thoracic aorta. CsA (10 μM)-preincubation for 90 min significantly attenuated the vasodilatation induced by sodium nitroprusside (SNP), a cytosolic guanylate cyclase activator, shifting the dose-response curve to the right. The increase in cGMP contents induced by SNP was markedly attenuated by CsA. SNP (1 μM~1 mM) increased the cGC activity dose-dependently, and the increase was completely abolished by CsA. CsA attenuated the SNP-induced cGC activation dose-dependently. The abolishing effect of CsA-pretreatment on the SNP-induced cGC activation was not affected by washing the preparation, suggesting that the inhibition is irreversible. When CsA was added simultaneously with SNP, cGC activation was not attenuated. 1-(5-isoquinolinylsulfonyl)-2-methyl piperazine (H-7), a protein kinase C (PKC) inhibitor, decreased SNP-induced cGC activation and blocked the CsA-attenuation of cGC activation. These results suggest that CsA directly inhibits cGC participating in the CsA-induced impairment of vasodilatation, and that PKC is involved in the inhibitory action of CsA on cGC.

Key Words: Cyclosporin A, Cytosolic guanylate cyclase, cGMP, Vasodilatation, Rat thoracic aorta

INTRODUCTION

Cyclosporin A (CsA) is one of the indispensable agents widely used in immunosuppressive treatment, particularly in organ transplantation. However, it is endowed with adverse effects, such as hypertension and nephrotoxicity, which are related to CsA-induced vasoconstriction, and restrain the unscrupulous use of the agent (Schulman et al, 1981; Bellet et al, 1985). Both renal dysfunction and hypertension are reproducible in laboratory animals (Whiting et al, 1982; Siegl et al, 1983) and are being extensively investigated. Although the mechanism involved in the vasoconstriction is not fully clarified yet, the increase in resistance of renal blood vessel and subsequent decrease in renal blood flow (Murray et al, 1985) was suggested to result from the abnormalities, either in the renin-angiotensin system (Jao et al, 1986) or in the vascular tone-regulating prostaglandins (Kawaguchi et al, 1985), or from the increased sympathetic tone (Murray et al, 1985).

Acute CsA-treatment is reported to produce transient vasoconstriction (Xue et al, 1987) and to increase the responsiveness to vasoconstrictors (Lamb & Webb, 1987). Besides the increased vasoconstriction, however, impaired vasodilatation has also been suggested as an alternative cause of CsA-induced hypertension, since that chronic administration of CsA exerts direct cytotoxic effect on endothelium (Zoja et al, 1986), resulting in the impairment of endothelium-dependent vasodilatation (Diederich et al, 1992; Gallego et al, 1994). However, no clear consensus has been reached as for the endothelium-independent vasodilatation. Rego et al (1990) and Choi et al (1992) suggested that the function of vascular smooth muscle was also affected

Corresponding to: Hyun Kook, Department of Pharmacology, Chonnam University Medical School, 5 Hak-dong, Dong-ku, Kwangju 501-190, Korea

by CsA, since sodium nitroprusside (SNP)-induced vasodilatation was impaired. In contrast, other investigators asserted that CsA impaired the functions of vascular endothelium rather than those of smooth muscle (Diederich et al, 1994; Gallego et al, 1994).

To prove the CsA-induced impairment of vasodilatation, it is important to assess the direct effects of CsA on the vascular smooth muscle. However, since the long-term administration of CsA deteriorates general conditions of test animals as confirmed histologically (Ryffel et al, 1985; Mihatsch et al, 1988), and subsequent emaciation may also affect blood vessels, the chronic administration does not seem to be suitable for the evaluation of the CsA effects. In addition, because castor oil, a vehicle used in intramuscular preparations, was reported to affect the vascular responses to agonists (Amorena et al, 1990; Yaris & Tuncer, 1995a), it is necessary to obviate the vehicular effect. Therefore, in the present study, to clarify the mechanism of CsA-induced impairment of endothelium-independent vasodilatation, we investigated, employing isolated strips of rat thoracic aorta, the effects of acute CsA-pretreatment on the activities of cytosolic guanylate cyclase (cGC) in relation to the alteration of relaxant responses.

METHODS

Materials

Norepinephrine (NE), sodium nitroprusside (SNP), ethylenediaminetetraacetic acid (EDTA), creatine phosphate, phenylmethylsulfonyl flouride (PMSF), creatine phosphokinase, 3-isobutyl-1-methylxanthine (IBMX), guanosine triphosphate (GTP), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7), 2-amino-2-(hydromethyl)-1, 3-propanediol (Tris), sodium acetate, ascobic acid, bovine serum albumin (BSA), and magnesium chloride ($MgCl_2$) were obtained from Sigma (St. Louis, MO); biocinchonic acid (BCA), and Bradford kit for measuring protein concentration from BioRad (Hercules, CA); and cGMP radioimmunoassay kit from DuPont (NEN; Boston, MA). Cyclosporin A (CsA) in pure form was obtained from Sandoz (Basel, Switzerland) to avoid the possible effects of the vehicle. SNP and CsA were freshly prepared before each experiment. CsA was dissolved in ethanol to make 1 or 2 mM stock solution and diluted further with distilled water. The 0.5% ethanol, used for diluting CsA, decreased cGC activity slightly, but not significantly. Therefore, 0.5% ethanol was added in control experiment.

Tension experiments and cGMP contents

The effects of CsA on the tension of rat thoracic aorta were examined with the methods of Kook et al (1996). Briefly, Sprague-Dawley rats were sacrificed by decapitation, their thoracic aortas excised, and then cut into 5 mm rings. The ring segments were mounted in muscle bath containing 4 ml physiological salts solution (in mM: 122 NaCl, 4.7 KCl, 1.6 $CaCl_2$, 1.2 KH_2PO_4, 1.18 $MgSO_4$, 15 $NaHCO_3$, 11.5 dextrose, 0.026 EDTA and 0.12 ascorbic acid) and connected to an isometric transducer.

Cyclic GMP contents were measured as described previously (Kook et al, 1996) using [^{125}I] radioimmunoassay kit with acetylated protocol. The pellet was used for protein assay with BCA kit against BSA standard.

cGC activity

Aortic preparations were pooled and homogenized in ice-cold homogenization buffer (50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA, 0.2 mM PMSF, and 250 mM sucrose). The homogenates were centrifuged at 1,000×g and the supernatants were fractionated further by centrifuging at 100,000×g for 1 h at 4°C. The resulting supernatant was collected and its protein concentration was measured by Bradford kit and stored for further use as cytosolic fraction.

Cytosolic GC activity was measured by the method of Rapoport & Murad (1988) with slight modification. Ninety μl of working solution (50 mM Tris-HCl, pH 7.6, containing 4 mM $MgCl_2$, 1 mM GTP, 15 mM phosphocreatine, and 20 μg/ml creatine phosphokinase, and 2 mM IBMX) was warmed to 37°C. The test agents (CsA or H-7) were added to pre-warmed cytosolic fraction containing 3~6 μg of protein and the mixture was incubated further for 90 min. The reaction was started by adding the cytosolic fraction mixture to working solution containing SNP. After 5 min, the reaction was stopped by cold stopping solution containing 50 mM sodium acetate, pH 4.0, and centrifuged at 1,000×g for 10 min at 4°C. The supernatant was collected and the cGMP contents were assayed as described above.

The Km value against SNP in cGC activity was

9.6×10^{-5} M, and the contamination of membrane-bound guanylate cyclase in the cytosolic fraction was ruled out by the absence of response to ANP (data not shown).

In co-incubation experiments CsA was simultaneously added with SNP (CsA + SNP Co I). In order to examine the reversibility of CsA-induced effects, the aortic preparations were washed three-times with fresh physiological salts solution after CsA-incubation and their cytosolic fraction was obtained (CsA-Washed).

Statistics

Inhibitory concentration fifty (IC_{50}) of dose-response was calculated by using ALLFIT program (Erithacus). Statistical significance was evaluated with Student's unpaired t-test and two-way ANOVA with repeated measure. Significance was accepted at the 0.05 level of probability.

RESULTS

Effects of CsA-pretreatment on SNP-induced vasodilatation

CsA itself contracted the rat thoracic aorta slightly; however, it did not affect the NE-induced vasoconstriction. SNP reduced the NE-induced tension dose-dependently in the concentration range of $10^{-10} \sim 10^{-7}$ M in both Control and CsA-pretreated preparations. Pretreatment with CsA significantly attenuated the SNP-induced vasodilatation, shifting the dose-response curve to the right (Fig. 1). The IC_{50} obtained from CsA-pretreated rings (14.2 ± 3.8 nM) was significantly greater than that of the SNP alone (Control, 3.8 ± 0.6 nM, $p < 0.05$).

Effects of CsA-pretreatment on SNP-induced increase in cGMP contents

Treatment with CsA did not affect the basal cGMP level. SNP significantly increased cGMP contents in both Control and CsA-pretreated groups. The SNP-induced increase in cGMP was markedly attenuated by pretreatment with CsA (Fig. 2).

Effects of CsA-pretreatment on SNP-induced increase in cGC activity

SNP increased the cGC activity in a dose-dependent fashion in concentration ranges from 10^{-6} to 10^{-3} M; however, this increase in cGC activity was

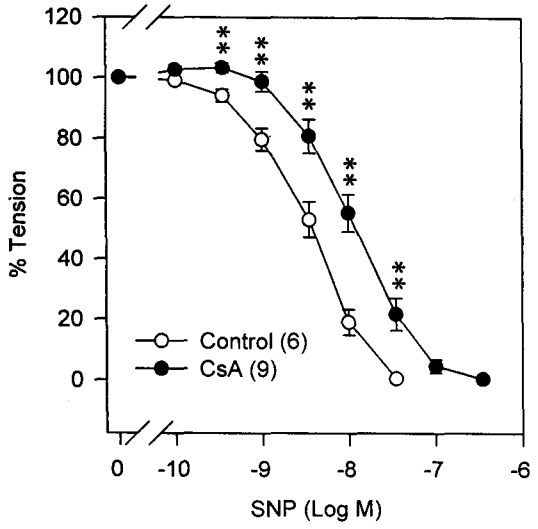

Fig. 1. Effects of pretreatment with 10 μM cyclosporin A (CsA) on the sodium nitroprusside (SNP)-induced vasodilatation. Each dot represents mean ± SEM. Asterisks indicate significant differences from the control (*$p < 0.05$, **$p < 0.01$). Numerals in parentheses are the numbers of experiments.

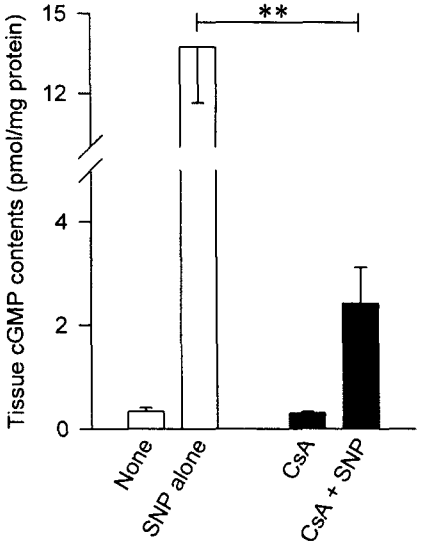

Fig. 2. Effects of pretreatment with 10 mM CsA on the increase in cGMP contents induced by 10^{-7} M SNP. Each bar represents mean ± SEM from 5 experiments. Asterisk with bracket indicates the significant differences between two groups ($p < 0.01$).

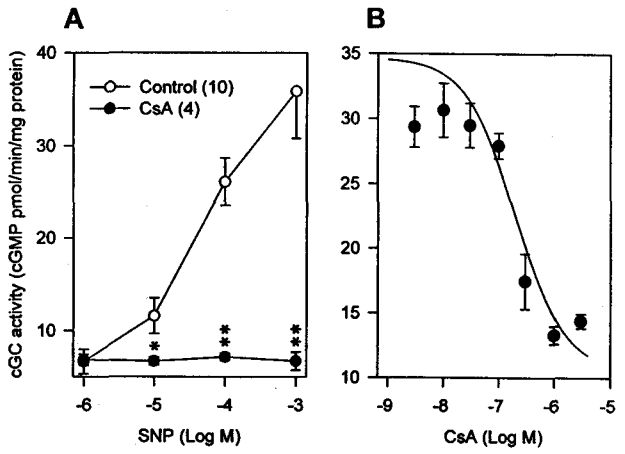

Fig. 3. A. Ten μM CsA-induced abolishment of the increase in cytosolic guanylate cyclase (cGC) activity induced by varying doses of SNP. B. Dose-dependent CsA-induced attenuation of 0.35 mM SNP-induced increase in cGC activity (n=5). Other legends are as in Fig. 1.

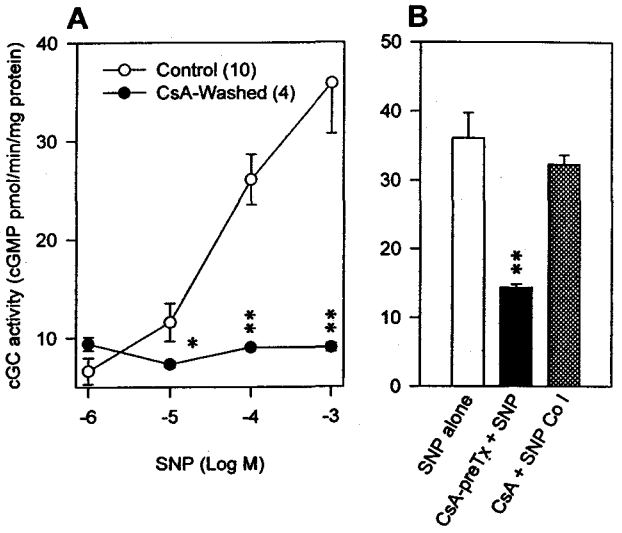

Fig. 4. A. SNP-induced cGC activation in the 'CsA-Washed' preparations, which had been pretreated with 10 μM CsA 90 min before SNP. B. Effects of simultaneous incubation of CsA with SNP (CsA+SNP Co I) in comparison with those of pretreatment of CsA for 90 min before adding SNP (CsA-preTx+SNP; n=6). See methods for details.

completely abolished by pretreatment with CsA (Fig. 3A). To ascertain the dose-response relation of the CsA-attenuation of cGC activity, CsA in various amounts was added to the preparations pretreated with 0.35 mM SNP, which produce 80% of the maximal response. CsA attenuated cGC activity dose-dependently, with IC_{50} calculated as 232.6 ± 53.9 nM (Fig. 3B).

To test whether the CsA-attenuation of SNP-induced cGC activation is reversible, SNP was added to the 'CsA-Washed' preparation. As shown in Fig. 4A, SNP failed to increase cGC activity at all, suggesting the CsA-action is essentially irreversible. When CsA was added with SNP simultaneously (CsA+SNP Co I) instead of the 90 min preincubation before SNP addition, the increase in cGC was not affected; thus, the magnitude of increase in the CsA-SNP-coincubated preparation was not different from that of SNP alone (Fig. 4B).

Involvement of protein kinase C (PKC) in CsA-induced attenuation of cGC activity

Fig. 5A shows the effects of H-7, a non-specific PKC inhibitor (Hidaka et al, 1984), on the SNP-induced increase in cGC activity in CsA-untreated (Control) and CsA-pretreated (CsA) preparations. SNP-induced increase in cGC activity was halved by H-7. However, in CsA-pretreated preparations, H-7

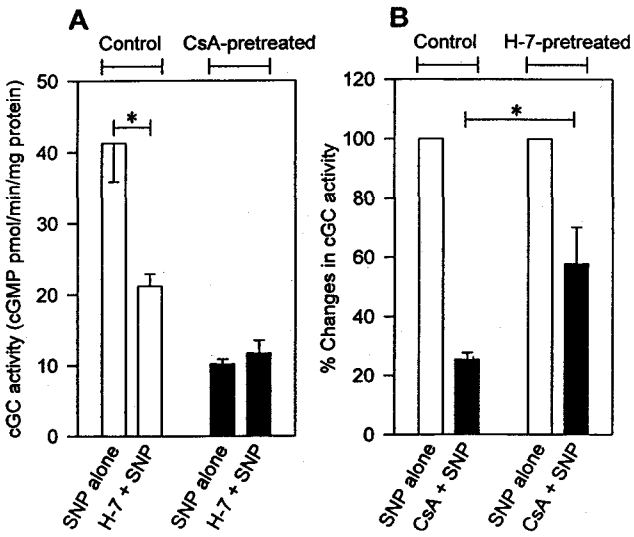

Fig. 5. A. Effects of H-7 on SNP-induced increase in cGC activity in the preparations with or without CsA-pretreatment. Left two columns show the H-7 (10 μ M)-induced attenuation of cGC activation induced by 0.35 mM SNP (Control) in CsA-untreated preparations, while right two columns represent the CsA-pretreated ones. B. Percent changes of cGC activity in relation to H-7-pretreatment comparing the groups of CsA+SNP and CsA alone. See methods and results for details.

failed to attenuate cGC activity further.

In Fig. 5B, relative effect of CsA on cGC activity was compared in the H-7-pretreated and H-7-untreated preparations. The CsA-untreated cGC activity of each preparation was regarded as 100%. In H-7-untreated preparations, CsA reduced the SNP-induced increase in cGC activity by approximately 75%. In the H-7-pretreated, CsA-induced reduction of the activity was about 40%, significantly less than that of H-7-untreated control, indicating that the cGC activation was restored.

DISCUSSION

In this study we observed that the relaxation of rat aortic strip in response to SNP is attenuated by CsA, indicating that the endothelium-independent vasodilatation is impaired by CsA-pretreatment. This observation agrees with the data reported by such investigators as Rego et al (1990) and Choi et al (1992). At the same time, it differs from those of Diederich et al (1994) and Gallego et al (1994), who claimed that the endothelium-independent vasodilatation was not affected by CsA. Balligand & Godfraind (1991) also found that acute CsA-treatment did not affect the endothelium-independent vasodilatation and suggested that chronic administration, which deteriorates the entire vascular structure, might have caused attenuation in the endothelium-independent vasodilatation in the studies of Rego et al (1990) and Choi et al (1992). However, this suggestion may be refuted, since we found in the present study that the acute treatment with CsA inhibited endothelium-independent vasodilatation induced by SNP.

Interestingly, Amorena et al (1990) reported that the vehicle of commercially available CsA preparation besides CsA itself can inhibit the vasodilatation induced by acetylcholine or carbamylcholine and that the substance responsible appeared to be cremophor EL, a polyoxyethylated derivative of castor oil. Yaris & Tuncer (1995a; 1995b) found further that cremophor EL affects the vascular tone, observing that it can inhibit endothelium-dependent vasodilatation and potentiate the contractile response of the vascular smooth muscle to various agonists. Besides cremophor EL, other vehicles of CsA preparations, such as ricinoleic acid and labrafil, were also reported to affect the vascular tone. Ricinoleic acid, a main fatty acid component of castor oil, constricts blood vessel directly by affecting the thromboxane/prostaglandin pathway (Lodge, 1994). Also labrafil, a polyoxyethylated derivative of the vehicle of oral CsA preparation directly inhibits endothelium-dependent vasodilatation (Amorena et al, 1990). However, in this study, these effects of CsA vehicle were excluded by using least amount of ethanol, which did not affect the vascular tone significantly. Lodge (1994) also reported that CsA dissolved in ethanol produced negligible vasoconstriction in comparison with CsA in castor oil.

We found that the cGMP contents were increased by SNP in agreement with others (Murad, 1994), and observed further that CsA prevented the cGMP increase. However, in contrast to other reports by Gallego et al (1994) who found that CsA prevented the increase in cGMP contents induced only by acetylcholine, but not by SNP, the SNP-induced increase in cGMP contents was abolished by pretreatment with CsA for 90 min. This result indicates that the failure of endothelium-independent increase in cGMP contents in response to nitric oxide as well as endothelium-dependent increase is involved in the impairment of vasodilatory mechanism.

We observed that SNP increased cGC activity and that CsA dose-dependently attenuated the SNP-induced increase in cGC activity. Hence, it can be deduced that CsA-action is brought about by the decrease in cGMP formation rather than by the increase in destruction. If an increased destruction of cGMP had dominated, the basal cGMP contents without SNP-treatment would have been reduced by the pretreatment with CsA. However, no significant difference in basal cGMP contents between CsA-treated and Control groups was observed, suggesting that cGMP formation in vascular smooth muscle by SNP might be impaired in CsA-pretreated group. Furthermore, the present study has clearly shown that cGC in vascular smooth muscle serves as an important target for CsA-induced impairment of vasodilatation.

The CsA effect of attenuating the SNP-induced cGC activation was not removed when the preparation was washed repeatedly, indicating that the inhibition of CsA is irreversible. In addition, the SNP-binding site in cGC molecule does not seem to be involved in the CsA-induced inhibition of the enzyme, since the simultaneous incubation of CsA with SNP failed to block the SNP-induced increase in cGC activity. If CsA binds to SNP-binding site, simultaneous administration of CsA with SNP should

also reduce the SNP-induced increase in cGC activity by competing with SNP. Rather, CsA seems to block cGC by interfering the catalytic action of the cGC.

H-7, a PKC inhibitor, markedly attenuated SNP-induced increase in cGC activity, suggesting the involvement of PKC in the activation of cGC. It has been reported that phosphorylation by PKC activates cGC in PC12 cells (Louis et al, 1993) and that chelerythrine, a specific inhibitor of PKC, decreased SNP-induced cGMP accumulation in rat pinealocytes (Spessert et al, 1995). It was found, in this study, that H-7 failed to attenuate cGC activity further in the CsA-treated preparations. This finding may be interpreted as either CsA sharing with H-7 a common binding site or CsA blocking some part of the H-7 mechanism. Indeed, a mechanism related to protein kinase/phosphatase has been suggested to be involved in the CsA-induced impairment of vascular function. Oriji & Keiser (1997) suggested that PKC mediates CsA-induced vasoconstriction, associated with the influx of extracellular calcium. Yang et al (1982) also reported that calcineurin, a target of CsA, serves as a protein phosphatase. However, since calcineurin is calcium-dependent (Tonks & Cohen, 1983; Stewart et al, 1983), and we found changes of cGC activity in the absence of calcium, this calcium system does not seem to be involved. One remaining possibility that demands further studies is superoxide free radicals, which might be produced by CsA (Diederich et al, 1994), and their involvement should also be elucidated.

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