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Short communication

Cyclosporin A inhibits angiotensin II-induced c-*Jun* NH_2-terminal kinase activation but not protein synthesis in vascular smooth muscle cells

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Abstract

Angiotensin II activates three major mitogen-activated protein kinases (MAPK) in vascular smooth muscle cells. Although other angiotensin II-induced MAPKs activation require transactivation of a growth factor receptor, the detailed mechanism by which angiotensin II activates c-*Jun* NH_2-terminal kinase (JNK) remains unclear. Here, an immunosuppressant, cyclosporin A but not FK506, selectively inhibited angiotensin II-induced JNK activation in vascular smooth muscle cells. However, cyclosporin A had no inhibitory effect on angiotensin II-induced protein synthesis. Thus, angiotensin II-induced JNK activation but not protein synthesis is mediated by a mechanism sensitive to cyclosporin A, which is independent from calcineurin in vascular smooth muscle cells.

Keywords: Angiotensin II; Smooth muscle cell, vascular; Cyclosporin A; c-*Jun* NH_2-terminal kinase

1. Introduction

Angiotensin II not only controls cardiovascular homeostasis but also contributes to various cardiovascular diseases such as hypertension, atherosclerosis and cardiac hypertrophy. Angiotensin II promotes hypertrophy and/or hyperplasia of vascular smooth muscle cells, cardiac myocytes and cardiac fibroblasts through the angiotensin II type-1 receptor (Kim and Iwao, 2000; Touyz and Schiffrin, 2000). The angiotensin II type-1 receptor activates phosphlipase C, causing intracellular Ca^{2+} mobilization and protein kinase C activation. This receptor also mediates activation of three major mitogen-activated protein kinases (MAPKs) by angiotensin II in vascular smooth muscle cells. These MAPKs may play critical roles in mediating the growth promoting action of angiotensin II (Berk, 1999; Griendling et al., 1997; Schmitz et al., 1998; Ushio et al., 1998). Although angiotensin II-induced epidermal growth factor (EGF) receptor transactivation is required for activation of extracellular signal-regulated kinase (ERK) and p38MAPK by angiotensin II (Eguchi et al., 1998, 2001), the detailed mechanism by which angiotensin II activates c-*Jun* NH_2-terminal kinase (JNK) remains unclear.

Cyclosporin A, an immunosuppressant, interacts with cyclophilins and inhibits a Ca^{2+}/calmodulin-dependent protein phosphatase, calcineurin (Crabtree, 2001). Recently, cyclosporin A was shown to attenuate cardiac hypertrophy by inhibiting calcineurin-dependent activation of nuclear factor of activated T lymphocytes 3 (Molkentin et al., 1998). However, whether the antihypertrophic action of cyclosporin A can solely be explained through its action on calcineurin and whether cyclosporin A inhibits vascular hypertrophy remain unanswered. Here, we have examined the effect of cyclosporin A on MAPK family activation and protein synthesis induced by AngII in vascular smooth muscle cells.

2. Material and methods

2.1. Materials

Angiotensin II was obtained from Sigma. Cyclosporin A, W7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide,

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HCl], FK506 and anisomycin were purchased from Calbiochem. Antibodies toward phosphorylated-ERK, ERK2 and JNK2 were purchased from Santa Cruz Biochemistry. Antibody toward phosphorylated JNK was purchased from Promega. Antibody toward phosphorylated-p38MAPK was purchased from Cell Signaling.

2.2. Cell culture

Vascular smooth muscle cells were prepared from thoracic aorta of Sprague–Dawley rats as previously described (Eguchi et al., 1996). For subsequent experiments, subcultured cells from passage 3–12 at 80–90% confluency were used after serum depletion for 3 days.

2.3. Immunoblotting

After stimulation, cells were lysed with Laemmli sample buffer, subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and immunoblotted as previously described (Eguchi et al., 1996).

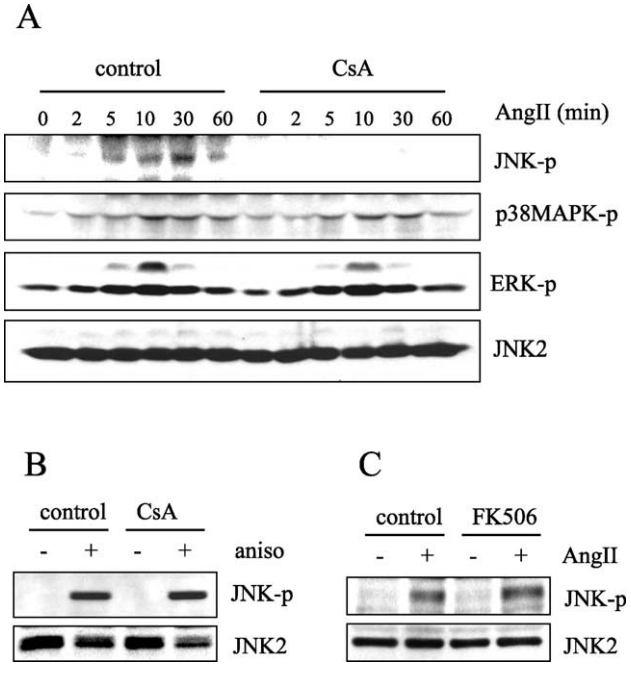

Fig. 1. Effects of cyclosporin A on angiotensin II-induced activation of MAPKs. (A) Cells were pretreated with cyclosporin A (100 ng/ml) for 30 min and stimulated with angiotensin II (100 nM) for the indicated time periods. (B) Cells were pretreated with cyclosporin A (100 ng/ml) for 30 min and stimulated with anisomycin (20 μg/ml) for 30 min. (C) Cells were pretreated with FK506 (1 μg/ml) for 30 min and stimulated with angiotensin II (100 nM) for 30 min. Cell lysates were immunoblotted with phospho-specific anti-JNK (JNK-p), anti-ERK (ERK-p) and anti-p38MAPK antibodies (p38MAPK-p) or anti-JNK2 antibidy as indicated. Results are representative of two independent experiments giving similar results.

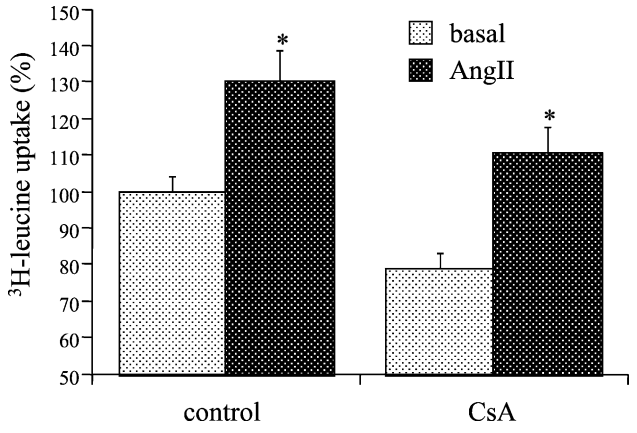

Fig. 2. Effect of cyclosporin A on angiotensin II-induced protein synthesis. Cells were pretreated with cyclosporin A (100 ng/ml) for 30 min and incubated with angiotensin II (100 nM) in the presence of [^{3}H]leucine. Incorporation of [^{3}H]leucine was determined after 24 h. Data are presented as percent change relative to basal incorporation ($n = 3$, mean ± S.D.). * $P < 0.05$ compared to basal.

2.4. Protein synthesis

Protein synthesis was assessed by incorporation of [^{3}H]leucine into the cells as previously described (Eguchi et al., 1999). In brief, cells were incubated with angiotensin II and 1 μCi [^{3}H]leucine for 24 h. Trichloroacetic acid-insoluble radioactivity was measured in a liquid scintillation counter.

3. Results

Fig. 1A shows the effect of cyclosporin A pretreatment on the time-dependent phosphorylation of MAPKs induced by angiotensin II. We and others have shown that these MAPK phosphorylations are well-correlated with their kinase activity (Eguchi et al., 2001). Consistent with our previous observation (Eguchi et al., 2001), angiotensin II stimulated p54 JNK phosphorylation maximally at 30 min. JNK phosphorylation by angiotensin II was completely inhibited by cyclosporin A through out the time course of its activation. In contrast, cyclosporin A had no inhibitory effect on angiotensin II-induced ERK or p38MAPK phosphorylation. Also, cyclosporin A did not affect JNK phosphorylation in response to a stress inducer, anisomycin (Fig. 1B). Since cyclosporin A inhibits Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, we further examined the effect of a calmodulin inhibitor, W7, and an alternative calcineurin inhibitor, FK506. Pretreatment of 1 μM W7 for 30 min also markedly inhibited angiotensin II-induced JNK phosphorylation but not ERK phosphorylation (data not shown). However, FK506 up to 1 μg/ml had no effect on angiotensin II-induced JNK phosphorylation (Fig. 1C). These data suggest that angiotensin II-induced JNK activation is mediated through a step sensitive to cyclosporin A and to W7, which is independent from calcineurin.

To determine whether a cyclosporin A-sensitive pathway mediates angiotensin II-induced protein synthesis in vascular smooth muscle cells, we examined the effect of cyclosporin A on angiotensin II-induced [^{3}H]leucine incorporation. Angiotensin II increased [^{3}H]leucine incorporation by 1.3-fold over basal control (Fig. 2). Although cyclosporin A reduced both basal and angiotensin II-induced [^{3}H]leucine incorporation, comparable enhancement of incorporation by angiotensin II was still noticeable in the presence of cyclosporin A. These data suggest that the cyclosporin A-sensitive pathway is not required for growth of vascular smooth muscle cells induced by angiotensin II.

4. Discussion

In this study, we found that cyclosporin A inhibited angiotensin II-induced JNK activation but not ERK or p38MAPK activation in vascular smooth muscle cells. In cardiac myocytes, the activation of JNK induced by angiotensin II was strongly suppressed by down regulation of protein kinase C as well as chelation of intracellular Ca^{2+} (Kudoh et al., 1997). In contrast, angiotensin II activates JNK mainly through a Ca^{2+}-dependent mechanism in vascular smooth muscle cells (Schmitz et al., 1998), cardiac fibroblasts (Murasawa et al., 2000) and liver epithelial cells (Zohn et al., 1995), thus indicating the dominant role of Ca^{2+} in JNK activation. Although intracellular Ca^{2+} elevation seems to be indispensable for MAPK family activation by angiotensin II in vascular smooth muscle cells, ERK and p38MAPK but not JNK are downstream of EGF receptor transactivation (Eguchi et al., 2001). Our present results are in agreement with these previous findings and further suggest that a Ca^{2+}/calmodulin and a step sensitive to CsA are specifically involved in angiotensin II-induced JNK activation. To support this notion, it has been reported that cyclosporin A inhibits Ca^{2+} ionophore-stimulated JNK activation but not ERK activation in T lymphocytes (Matsuda et al., 1998; Werlen et al., 1998).

It has been recently reported that JNK is activated in the heart of calcineurin transgenic mice (De Windt et al., 2000). However, the lack of JNK inhibition by FK506 suggest that angiotensin II activates JNK through a pathway independent from calcineurin and is not due to lack of FK-binding proteins in our cells. This is supported by the observation of FK506-sensitive antagonism of the rapamycin effect (Cao et al., 1995) and inhibition of nitric oxide synthase induction by cyclosporin A but not by FK506 (Marumo et al., 1995) in vascular smooth muscle cells. Therefore, peptidyl prolyl-*cis*/*trans*-isomerase activity of cyclophilins (the selective targets of cyclosporin A) could be a required step of JNK activation by angiotensin II. Regarding the route of JNK activation by angiotensin II, we and others have reported that a Ca^{2+}-dependent tyrosine kinase, proline-rich tyrosine kinase-2 (PYK2) also termed cell adhesion kinase-β is involved in angiotensin II-induced JNK activation (Frank et al., 2001; Murasawa et al., 2000) possibly through Nck, Rac and p21-activated kinase (Schmitz et al., 2001). Although our data indicate that cyclosporin A dose not directly inhibits JNK, whether cyclosporin A inhibits the above molecules function directly or indirectly through intermediate step(s) are under current investigation.

Cyclosporin A has been shown to inhibit cardiac hypertrophy (Molkentin et al., 1998). Also, cyclosporin A inhibited angiotensin II-induced splenic lymphocyte proliferation (Nataraj et al., 1999). However, in this study, angiotensin II-induced protein synthesis was unaltered by pretreatment with cyclosporin A in vascular smooth muscle cells. Taken together, our results rather suggest a cell type specific role of the cyclosporin A-sensitive pathway for MAPK family activation and cell growth induced by angiotensin II, providing a unique insight of a cyclosporin A-sensitive pathway in mediating cardiovascular remodeling.

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