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Phosphatidylinositol 3-kinase in angiotensin II-induced hypertrophy of vascular smooth muscle cells

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Abstract

Activation of 4E-binding protein 1 (4E-BP1) by growth factors regulates protein synthesis in vascular smooth muscle cells. The interaction between G protein-coupled receptors and activated 4E-BP1 is unclear. We examined phosphadityl inositol (PI) 3-kinase in angiotensin II-induced 4E-BP1 phosphorylation in cultured rat vascular smooth muscle cells. Angiotensin II time and dose dependently stimulated phosphorylation of 4E-BP1 through the angiotensin AT_1 receptor. Pretreatment with wortmannin or 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), a PI 3-kinase inhibitor, suppressed angiotensin II-induced phosphorylation, but a mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases (ERK) kinase-1 (MEK-1) inhibitor, 2′-Amino-3′-methoxyflavone (PD98059), and a p38 MAPK inhibitor, 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580), had no effect. With regard to the involvement of mammalian target of rapamycin (mTOR) and p70 S6 kinase, angiotensin II-induced phosphorylation was abolished by pretreatment with rapamycin, but not by tosylphenylalanine chloromethyl ketone or tosyllysine chloromethyl ketone. Ca^{2+} was involved, since intracellular Ca^{2+} chelation inhibited angiotensin II-induced phosphorylation while a Ca^{2+} ionophore, A23187, stimulated phosphorylation. Thus, angiotensin II induces the phosphorylation of 4E-BP1 via the PI 3-kinase/mTOR pathway, but not via ERK or p70 S6 kinase.

Keywords: Angiotensin II; 4E-BP1 (4E-binding protein 1); Phosphatidylinositol 3-kinase; Angiotensin AT_1 receptor; mTOR (mammalian target of rapamycin)

1. Introduction

The arteries of hypertensive patients (Aalkjaer et al., 1989) have been shown to be thickened. Previous studies have reported that medial thickening, at least of large conduit vessels, reflects increased vascular smooth muscle cell content or mass, which occurs primarily by enlargement or hypertrophy of preexisting vascular smooth muscle cells, with little to no change in vascular smooth muscle cell number (Lichtenstein et al., 1986; Owens and Schwartz, 1982, 1983). Therefore, there has been considerable interest in identifying the cellular mechanisms that mediate hypertrophic growth of vascular smooth muscle. Angiotensin II, a major effecter peptide of the renin–angiotensin system, now is believed to play a critical role in cardiovascular remodeling associated with hypertension, heart failure, and atherosclerosis (Goodfriend et al., 1996). Our group and others (Sasaki et al., 1991; Murphy et al., 1991) previously cloned the angiotensin AT_1 receptor, which not only mediates diverse hemodynamic effects of angiotensin II (Timmermans et al., 1993), but also promotes hypertrophy and/or hyperplasia of vascular smooth muscle cells (Geisterfer et al., 1988; Gibbons et al., 1992). The angiotensin AT_1 receptor belongs to the superfamily of heterotrimeric G protein-coupled receptors (Sasaki et al., 1991; Murphy et al., 1991). In cultured vascular smooth muscle cells, this receptor activates phospholipase C, which initiates the generation of inositol triphosphate and diacylglycerol to bring about intracelluar calcium mobilization and protein kinase C activation, respectively (Griendling, 1997; Griendling et al., 1986). In addition, angiotensin II induces

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several signaling events commonly evoked by growth factor receptors, such as activation of mitogen-activated protein kinase (MAPK) also known as extracellular signal-regulated kinase (ERK) (Duff et al., 1992; Tsuda et al., 1992) and the ribosomal S6 kinase (Giasson and Meloche, 1995). Considerable interest has been focused on identifying the mechanisms and cellular signaling pathways by which angiotensin II stimulates vascular smooth muscle cell hypertrophy.

The initiation phase of mRNA translation, which generally is rate-limiting for protein synthesis, is mediated in part by the eIF-4F complex. The complex consists of three subunits, eIF-4g, eIF-4A, and eIF-4E (Proud, 1992). Being the least abundant of the eIF-4F subunits, eIF-4E is considered to be rate-limiting for translation initiation. The availability of eIF-4E is regulated by 4E-binding protein 1 (4E-BP1), the eIF-4E binding protein (Pause et al., 1994; Lin et al., 1994). The nonphosphorylated form of this 4E-BP1 binds tightly to eIF-4E, preventing its binding to eIF-4g. When phosphorylated at the appropriate sites, 4E-BP1 dissociates from eIF-4E, allowing the factor to participate in translation initiation. Recently, angiotensin II has been reported to stimulate phosphorylation of 4E-BP1 through angiotensin AT_1 receptor (Fleurent et al., 1997). The tyrosine kinases of growth factor receptors and oncogene products are specifically associated with phosphadityl inositol (PI) 3-kinase (Cantley and Cantley, 1995). Several growth factors, such as platelet-derived growth factor and epidermal growth factor, activate PI 3-kinase through an association with phosphotyrosines located at the C-terminus of their receptors. Although the angiotensin AT_1 receptor lacks intrinsic tyrosine kinase activity, it is reported to stimulate PI 3-kinase activity, suggesting cross-talk between the angiotensin AT_1 receptor and a tyrosine kinase. We previously demonstrated that angiotensin II-induced p70 S6 kinase phosphorylation was mediated through the ERK1/2 cascade and PI 3-kinase/Akt/mammalian target of rapamycin (mTOR) cascade (Eguchi et al., 1999). To understand the cellular mechanisms involved in the hypertrophic action of angiotensin II, we examined the involvement of PI 3-kinase, p70 S6 kinase, and mTOR in 4E-BP1 function stimulated by angiotensin II in vascular smooth muscle cells.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle medium (DMEM), fetal calf serum, penicillin, and streptomycin were obtained from Life Technologies (Rockville, MD, USA). 2′-Amino-3′-methoxyflavone (PD98059), MAPK/ERK kinase (MEK)1/2 inhibitor, polyclonal antibodies to Thr^{202} and Tyr^{204}-phosphorylated ERK1/2, phospho-p38 MAPK, phospho-p70 S6 kinase, and phospho-4E-BP1 were purchased from New England Biolabs (Beverly, MA, USA). 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002), 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580), 2-[1-(3-Dimethylaminopropyl)-1*H*-indol-3-yl]-3-(1*H*-indol-3-yl)-maleimide (GF-109203X), 2-[*N*-(2-hydroxyethyl)]-*N*-(4-methoxybenzenesulfonyl)]amino-*N*-(4-chlorocinnamyl)-*N*-methylbenzylamine) (KN93), losartan, 12-tetradecanylphorbol-13-acetate (TPA), bis (2-amino phenoxy) ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethylester (BAPTA-AM), A23187, and rapamycin were obtained from Calbiochem (Darmstadt, Germany). ((*S*-[+]-1-[(4-[dimethylamino]-3-methylphenyl)-methyl]-5-[diphenylacetyl]-4,5,6,7-tetrahydro-1*H*-imidazo[4,5-*c*]pyridine-6-carboxylic acid), ditrifluoroacetate) (PD123319) was purchased from Research Biochemicals International (Natick, MA, USA). Polyclonal antibodies to 4E-BP1 and p70 S6 kinase were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibodies were purchased from Amersham Biosciences (Piscataway, NJ, USA).

2.2. Cell culture

Vascular smooth muscle cells were prepared from the thoracic aorta of 12-week-old Sprague–Dawley rats (Charles River Breeding Laboratories, Wilmington, MA, USA) by the explant method as previously described (Eguchi et al., 1996). Subcultured vascular smooth muscle cells from passages 3–15, used in the experiments, showed >99% positive immunostaining against smooth muscle α-actin antibodies and were negative for mycoplasma infection. For subsequent experiments, cells at ~80% confluence in culture wells were used 1 day after serum depletion.

2.3. Preparation of cell extracts and Western blotting

Vascular smooth muscle cells were stimulated with agonists for specified times. After treatment, cells were washed with ice-cold PBS. Cells were lysed with ice-cold lysis buffer, pH 7.4, containing 500 mM HEPES, 5 mM EDTA, 50 mM M NaCl, 1% Triton X-100, a mixture of protease inhibitors, and 1 mM sodium orthovanadate. Solubilized proteins were centrifuged at 14,000 × *g* for 30 min, and supernatants were stored at −80 °C. Proteins (25 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Western blot analysis with the indicated antibodies, using the enhanced chemiluminescent (ECL) detection system (Amersham).

2.4. [^{3}H] leucine incorporation

After serum starvation for 48 h, cells in 24-well plates were stimulated with angiotensin II for 24 h. [^{3}H] leucine (2 μCi/ml) was added for the last 24 h. Cells were washed twice with PBS, followed by treatment with 5% trichloroacetic acid (TCA), added for 30 min at 4 °C to precipitate

proteins. TCA-precipitable radioactivity was counted in a scintillation counter.

3. Results

3.1. 4E-BP1 phosphorylation by angiotensin II

4E-BP1 generally appears as three migrating bands when separated by SDS-PAGE and analyzed by immunoblotting. These bands are designated as α, β, and γ (Lin et al., 1995; Beretta et al., 1996). The nonphosphorylated α-form migrates most rapidly. Increases in phosphorylation of the intermediate β-form to the most highly phosphorylated γ-form decrease the electrophoretic migration of 4E-BP1 in a proportional manner. Under most conditions, the nonphosphorylated, α-form of 4E-BP1 is barely detectable in vascular smooth muscle cells. Fig. 1A shows a rapid increase in the phosphorylation of 4E-BP1 following addition of 0.1 μM angiotensin II to quiescent cultured rat vascular smooth muscle cells, where maximal phosphorylation occurred 10 to 20 min after angiotensin II addition and then gradually decreased to basal levels. The 10-min response to angiotensin II was concentration dependent, with peak activity occurring at angiotensin II concentrations between 0.01 and 0.1 μM, although there was a clear increase even at concentrations below 1 nM (Fig. 1B). Fig. 1C demonstrates that the response to angiotensin II was mediated through the AT_1 receptor but not through the angiotensin AT_2 receptor, since pretreatment with losartan (an AT_1 receptor antagonist), but not with PD123319 (an angiotensin AT_2 receptor antagonist), caused nearly complete blockade of phosphorylation. To exclude the possibility that this induction was caused by autocrine or paracrine factors released by angiotensin II, we examined the effect of conditioned medium, in which vascular smooth muscle cells had been incubated previously with 0.1 μM angiotensin II for 1 h, on fresh cells. In the presence of losartan plus angiotensin II, the conditioned medium did not increase 4E-BP1 mRNA (data not shown).

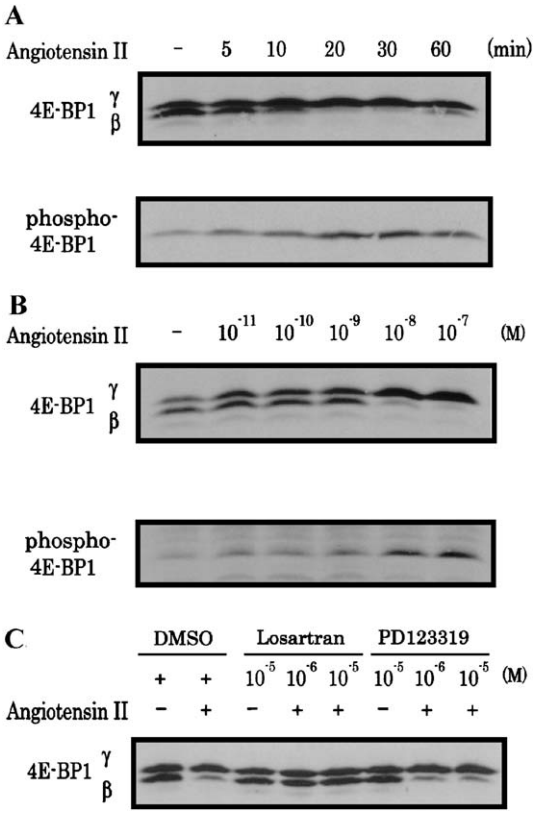

Fig. 1. Phosphorylation of 4E-BP1 induced by angiotensin II. (A) Cells were stimulated with angiotensin II (0.1 μM) for indicated times. (B) Cells were stimulated with angiotensin II for 20 min with indicated concentrations. (C) Cells were pretreated with losartan or PD123319 in the indicated concentrations and stimulated with angiotensin II for 20 min. Whole-cell lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Results shown are representative of three separate experiments.

3.2. Angiotensin II induction of 4E-BP1 phosphorylation is sensitive to PI 3-kinase inhibitors

To examine the possible involvement of PI 3-kinase in 4E-BP1 phosphorylation evoked by angiotensin II, we tested the effect of the selective PI 3-kinase inhibitors, wortmannin and LY294002, on angiotensin II-induced phosphorylation of 4E-BP1. Pretreatment with wortmannin or LY294002 completely inhibited angiotensin II-induced 4E-BP1 phosphorylation (Fig. 2A). The inhibitory effect of LY294002 on angiotensin II-induced phosphorylation of 4E-BP1 was concentration dependent (Fig. 2B).

3.3. ERK are not involved in 4E-BP1 phosphorylation

G protein-coupled receptor-induced ERK activation is in part mediated by PI 3-kinase (Nishioka et al., 1995). In addition, p90 S6 kinase activation is mediated by ERK1/2. To assess the involvement of ERK1/2 on 4E-BP1 phosphorylation, we examined the effect of PI 3-kinase inhibitors on ERK1/2 phosphorylation/activation. As shown in Fig. 2C, pretreatment with wortmannin or LY294002 had no effect on angiotensin II-induced ERK1/2 phosphorylation and activation. Although a MEK-1 inhibitor, PD98059, prevented the angiotensin II-induced phosphorylation and activation of ERKs, angiotensin II-induced 4E-BP1 phosphorylation was not suppressed by pretreatment with PD98059 (Fig. 2D). SB203580, a specific inhibitor of p38MAPK, also had no effect on 4E-BP1 phosphorylation, suggesting that 4E-BP1 phosphorylation is not mediated through these pathways.

3.4. Role of mammalian target of rapamycin (mTOR) in angiotensin II-induced phosphorylation of 4E-BP1

Insulin-like growth factor-1 (Graves et al., 1995) and platelet-derived growth factor in smooth muscle cells, as

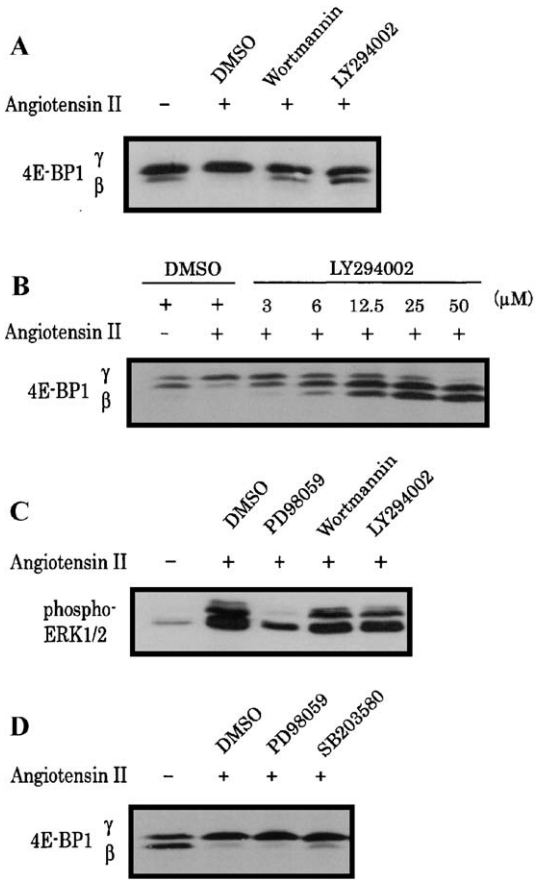

Fig. 2. Effects of PI 3-kinase inhibitors on 4E-BP1 phosphorylation. Cells were pretreated with or without Wortmannin 500 nM, LY294002 50 μM (A) or indicated concentrations (B) for 30 min, or PD98059 25 μM, SB203580 5 μM (D) and stimulated with angiotensin II (0.1 μM) for 20 min. Cells were pretreated with or without PD98059 25 μM, wortmannin 500 nM or LY294002 50 μM and stimulated with angiotensin II (0.1 μM) for 7 min (C). Whole-cell lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Results shown are representative of three separate experiments.

well as insulin in 3T3 L1 adipocytes (Lin et al., 1995), increase 4E-BP1 phosphorylation by a rapamycin-sensitive pathway. To investigate the involvement of mTOR in angiotensin II-induced 4E-BP1 phosphorylation, the effect of the specific mTOR inhibitor, rapamycin, was tested. Pretreatment with rapamycin almost completely abolished angiotensin II-induced 4E-BP1 phosphorylation (Fig. 3A). This effect of rapamycin was concentration dependent (Fig. 3B), whereas angiotensin II-induced ERK phosphorylation was not reduced by rapamycin (Fig. 3C).

3.5. Involvement of p70 S6 kinase

To investigate the involvement of p70 S6 kinase in angiotensin II-induced 4E-BP1 phosphorylation in vascular smooth muscle cells, we examined the effect of angiotensin II on p70 S6 kinase phosphorylation. Fig. 4A shows a rapid increase in the phosphorylation of p70 S6 kinase following the addition of 0.1 μM angiotensin II to vascular smooth

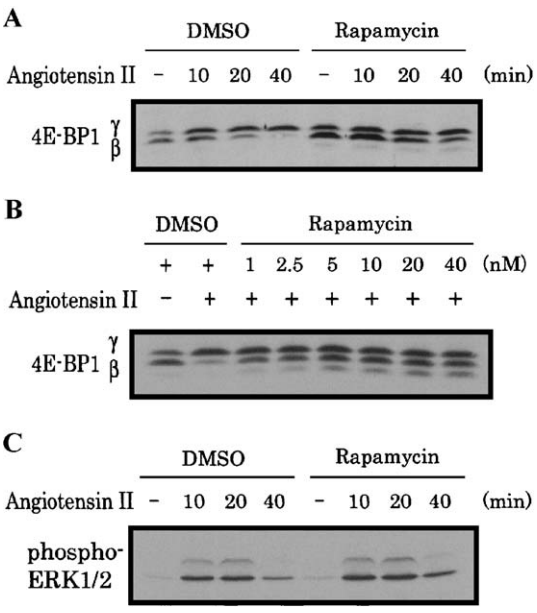

Fig. 3. Effects of Rapamycin on 4E-BP1 phosphorylation. Cells were pretreated with or without rapamycin 20 nM for the indicated times (A, C) or in the indicated concentrations (B) for 30 min and stimulated with angiotensin II (0.1 μM) for 20 (A, B) or 7 min (C). Whole cell lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Results shown are representative of three separate experiments.

muscle cells, with maximal phosphorylation occurring 20 min after angiotensin II addition and then gradually decreasing to basal levels. Pretreatment with LY294002 completely inhibited angiotensin II-induced p70 S6 kinase phosphorylation (Fig. 4B), suggesting the possible involvement of PI 3-kinase in angiotensin II-induced p70 S6 kinase activation.

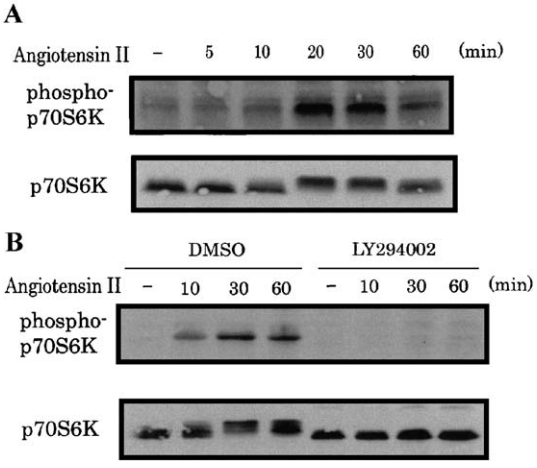

Fig. 4. Effect of LY294002 on p70 S6 kinase phosphorylation. (A) Cells were stimulated with angiotensin II (0.1 μM) for the indicated times. (B) Cells were pretreated with or without LY294002 50 μM and stimulated with angiotensin II (0.1 μM) for the indicated times. Whole-cell lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Results shown are representative of three separate experiments.

3.6. Effects of tosylphenylalanine chloromethyl ketone and tosyllysine chloromethyl ketone

Serine protease inhibitors, tosylphenylalanine chloromethyl ketone (TPCK) and tosyllysine chloromethyl ketone (TLCK), are reported to inhibit p70 S6 kinase (Grammer and Blenis, 1996). To elucidate whether p70 S6 kinase influences 4E-BP1 activation, we examined the effect of both inhibitors on angiotensin II-induced 4E-BP1 phosphorylation. As shown in Fig. 5, pretreatment with TPCK or TLCK had no effect on angiotensin II-induced 4E-BP1 phosphorylation even though p70 S6 kinase phosphorylation was inhibited by both agents.

3.7. Involvement of Ca^{2+} in 4E-BP1 phosphorylation

We next explored the action of Ca^{2+} in angiotensin II-induced 4E-BP1 phosphorylation. Previously, we found that intracellular Ca^{2+} was involved in angiotensin II-induced p70 S6 kinase phosphorylation (Eguchi et al., 1999). To confirm the role of intracellular Ca^{2+} in 4E-BP1 phosphorylation, we examined the effect of an intracellular Ca^{2+} chelator, BAPTA-AM, on angiotensin II-induced 4E-BP1 phosphorylation. BAPTA-AM completely inhibited angiotensin II-induced 4E-BP1 phosphorylation. We also examined the effect of a Ca^{2+} ionophore, A23187, on 4E-BP1 phosphorylation. As shown in Fig. 6B, the Ca^{2+} ionophore stimulated 4E-BP1 phosphorylation. These data suggest that intracellular Ca^{2+} is involved in angiotensin II-induced 4E-BP1 phosphorylation. We examined the effect of KN93, a calmodulin inhibitor, on angiotensin II-induced phosphorylation of 4E-BP1. As shown in Fig. 6, pretreatment with KN93 suppressed angiotensin II-induced 4E-BP1 phosphorylation.

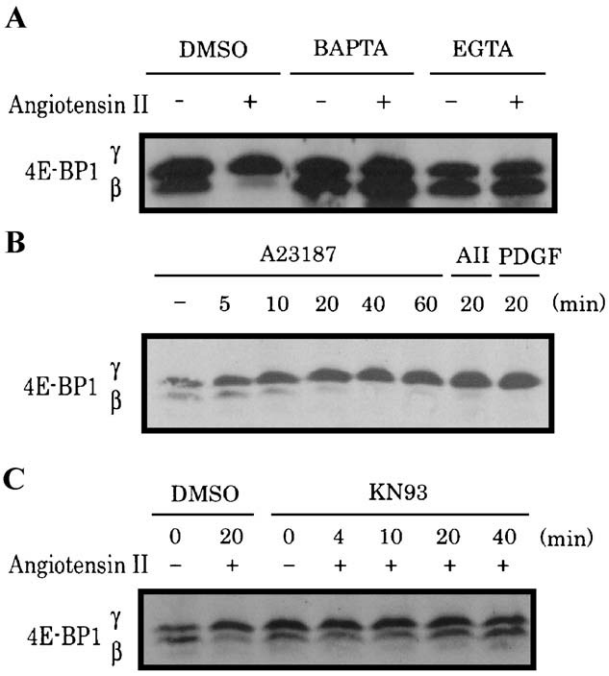

Fig. 6. The role of intracellular Ca^{2+} on 4E-BP1 phosphorylation. (A, C) Cells were pretreated with or without BAPTA-AM 10 μM or EGTA 5 mM and stimulated with angiotensin II (0.1 μM) for 20 min. (B) Cells were stimulated with A23187 10 μM, angiotensin II, or PDGF-BB for the indicated times. Whole-cell lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Results shown are representative of three separate experiments.

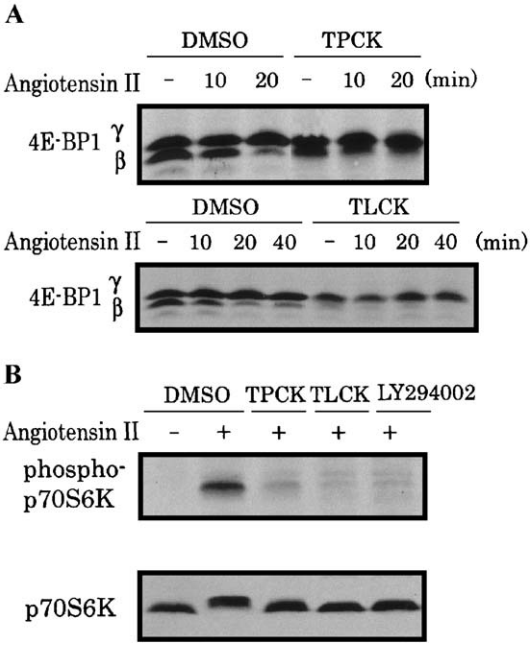

Fig. 5. Effects of inhibitors of p70S6 kinase on 4E-BP1 phosphorylation. (A, B) Cells were pretreated with or without TPCK 40 μM, TLCK 160 μM for 45 min, or LY294002 50 μM for 30 min and stimulated with angiotensin II (0.1 μM) for 20 min. Whole-cell lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. Results shown are representative of three separate experiments.

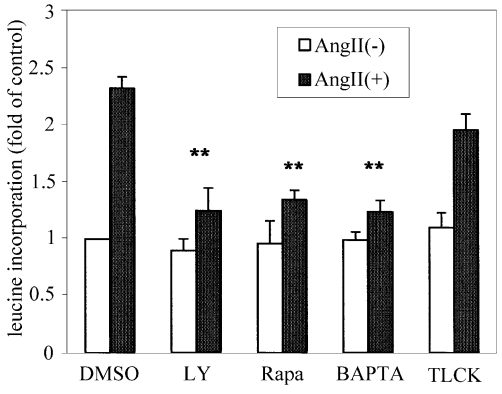

Fig. 7. Effects of pharmacological treatments on leucine incorporation by angiotensin II. Shown are the results of leucine incorporation analyzed in vascular smooth muscle cells subjected to various treatments as indicated: LY294002 (LY, 50 μM for 30 min), rapamycin (20 nM for 10 min), BAPTA (20 μM for 30 min), TLCK (160 μM for 45 min), or DMSO. Treated cells were exposed to angiotensin II for 24 h. $^{**}P < 0.01$ vs. DMSO treated control.

3.8. [^{3}H] leucine incorporation

We next characterized the effects of different pharmacologic interventions on angiotensin II-induced leucine incorporation. As shown in Fig. 7, pretreatment with LY294002, rapamycin, or BAPTA-AM, but not TLCK, inhibited leucine incorporation stimulated by angiotensin II in vascular smooth muscle cells.

4. Discussion

Angiotensin II-induced hypertrophy of vascular smooth muscle cells could involve two parallel pathways: One involves the ERK cascade and the other involves PI 3-kinase/mTOR/4E-BP1 phosphorylation. The role of angiotensin II in the activation of PI 3-kinase and p70 S6 kinase and the influence of rapamycin and PI 3-kinase inhibitors on their activation and 4E-BP1 phosphorylation have not been investigated in vascular smooth muscle cells. In addition, we examined the involvement of Ca^{2+} in these pathways. Our data provide new insights into the signaling mechanisms by which angiotensin II contributes to vascular hypertrophy.

Several growth factors have been shown to activate PI 3-kinase. Insulin stimulation leads to tyrosine phosphorylation of insulin receptor substrates 1 and 2 and to association of PI 3-kinase with SH2 binding sites contained within these proteins. Platelet-derived growth factor and epidermal growth factor activate PI 3-kinase through association with phosphotyrosines located in the C-terminal regions of their receptors. PI 3-kinase also can be activated by various agonists of G protein-coupled receptors, including the angiotensin AT_1 receptor (Saward and Zahradka, 1997; Murga et al., 1998). Insulin-induced 4E-BP1 phosphorylation is mediated through PI 3-kinase (Mendez et al., 1996). We, therefore, specifically examined the relative contribution of the PI 3-kinase to angiotensin II-induced 4E-BP1 phosphorylation. The inhibitors wortmannin or LY294002, both of which are known to selectively or specifically inhibit PI 3-kinase, blocked 4E-BP1 phosphorylation by angiotensin II. Thus, we believe that angiotensin II-induced phosphorylation of 4E-BP1 in vascular smooth muscle cells occurs via activation of PI 3-kinase.

Increasing number of reports using a variety of physiological stimuli indicate a role for the ERK pathway in the phosphorylation of 4E-BP1 (Rao et al., 1999; Bhandari et al., 2001; Herbert et al., 2002) However, insulin is thought to regulate the phosphorylation of 4E-BP1 through the PI 3-kinase, independently of ERK (Von Manteuffel et al., 1996). To elucidate whether angiotensin II phosphorylates 4E-BP1 via ERK, we examined the effect of a specific inhibitor of MEK-1, PD98059, on angiotensin II-induced 4E-BP1 phosphorylation. Neither PD98059 nor SB203580 affected 4E-BP1 phosphorylation, suggesting that angiotensin II stimulates the phosphorylation of 4E-BP1 by an ERK-independent mechanism, an observation compatible with a previous report (Fleurent et al., 1997).

Recent studies of 4E-BP1 have implicated a signaling pathway involving the mTOR. Rapamycin blocks growth factor-induced phosphorylation of 4E-BP1 (Beretta et al., 1996; Lin et al., 1995; Diggle et al., 1996; Von Manteuffel et al., 1996; Brunn et al., 1997). For example, insulin-like growth factor-1 (Graves et al., 1995) and platelet-derived growth factor in smooth muscle cells, as well as insulin in 3T3 L1 adipocytes (Lin et al., 1995), increase 4E-BP1 and p70 S6 kinase phosphorylation by a rapamycin-sensitive pathway. However, both rapamycin sensitive and insensitive pathways have been implicated in the phosphorylation of 4E-BP1 in response to insulin in rat epididymal adipocytes (Diggle et al., 1996). Whether 4E-BP1 phosphorylation is rapamycin sensitive or not in relation to G-protein-coupled receptors also is controversial. Phosphorylation of 4E-BP1 by the μ-opioid receptor, a Gi-coupled receptor, is mediated through the PI 3-kinase/mTOR pathway (Polakiewicz et al., 1998). Whether angiotensin II-induced 4E-BP1 phosphorylation is rapamycin sensitive or not is unclear. As this study showed, pretreatment with rapamycin completely blocked angiotensin II-induced 4E-BP1 phosphorylation, suggesting that angiotensin II-induced phosphorylation occurs through activation of mTOR.

Previously, we have reported that angiotensin II activated p70 S6 kinase in vascular smooth muscle cells (Eguchi et al., 1999). Whether G-protein-coupled receptor agonists induce 4E-BP1 via p70 S6 kinase remains unknown. Insulin-induced 4E-BP1 phosphorylation is mediated by the mTOR-p70 S6 kinase pathway (Von Manteuffel et al., 1996). P70 S6 kinase also was activated by mTOR upon insulin stimulation. However, p70 S6 kinase itself does not phosphorylate either the free or the eIF4E-bound form of 4E-BP1 in vitro, and thus it might not be involved in intact cells (Diggle et al., 1996). In addition, phosphorylation of 4E-BP1 was detected in p70 S6 kinase −/− cells to the same extent as in parental cells (Kawasome et al., 1998). Thus, whether 4E-BP1 phosphorylation is mediated through the p70 S6 kinase may depend upon the agonist or cell type involved. In the present study, inhibitors of p70 S6 kinase had no effect on angiotensin II-induced 4E-BP1 phosphorylation, suggesting that angiotensin II-induced 4E-BP1 phosphorylation is independent of p70 S6 kinase.

Incubation of a variety of cell types in medium containing EGTA to deplete intracellular Ca^{2+} stores leads to a sharp, rapid decrease in the rate of protein synthesis (Palfrey and Nairn, 1995). Stimulation of the β-adrenergic receptor in cardiac myocytes opens L-type voltage-sensitive Ca^{2+} channels, and the resulting Ca^{2+} signal has been reported to mediate wortmannin-sensitive activation of protein kinase B (Morisco et al., 2000). A Gq-coupled receptor, the $\alpha_{(1A)}$ adrenoreceptor, induces 4E-BP1 phosphorylation via a Ca^{2+}-dependent pathway (Rybkin et al., 2000). We, therefore, investigated the effect of Ca^{2+} on AT_1 receptor-mediated protein synthesis in vascular smooth muscle cells.

Treatment of the cells with EGTA led to a loss of angiotensin II-induced 4E-BP1 phosphorylation. In addition, the use of BAPTA-AM to chelate intracellular Ca^{2+} reduced angiotensin II-induced 4E-BP1 phosphorylation. Accordingly, Ca^{2+} plays a role in angiotensin II-induced 4E-BP1 phosphorylation.

The Ca^{2+} dependence of 4E-BP1 phosphorylation and p70 S6 kinase activation does not appear to be mediated by protein kinase C (PKC). Down-regulation of Ca^{2+}-dependent PKC by long-term treatment with TPA or pretreatment with GF-109203X, a PKC inhibitor, had little effect on the angiotensin II-induced phosphorylation of 4E-BP1 (data not shown). Instead, these Ca^{2+}-dependent events apparently require calmodulin or a closely related protein. We tested the effect of KN93 on angiotensin II-induced phosphorylation of 4E-BP1. Pretreatment with KN93 suppressed angiotensin II-induced 4E-BP1 phosphorylation. Thus, the Ca^{2+}/calmodulin pathway is involved in 4E-BP1 phosphorylation by angiotensin II, although the mechanisms of Ca^{2+}/calmodulin in this pathway require further elucidation.

In conclusion, we demonstrated that angiotensin II induces phosphorylation of 4E-BP1 through the PI 3-kinase/mTOR pathway, but not via ERKs or p70 S6 kinase. Further elucidation of these mechanisms should unravel the details of the action of angiotensin II in vascular remodeling in pathological states, such as hypertension, atherosclerosis, and restenosis after angioplasty.

Acknowledgements

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