Angiotensin II stimulates phosphorylation of 4E-binding protein 1 and p70 S6 kinase in cultured vascular smooth muscle cells

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KEY WORDS angiotensin II; 4E-binding protein 1; p70 S6 kinase; phosphatidylinositol 3-kinase; vascular smooth muscle

ABSTRACT

AIM: To examine the regulatory effects of angiotensin II (Ang II) on the phosphorylation of 4E-binding protein 1 (4E-BP1) and p70 S6 kinase in cultured vascular smooth muscle cells (VSMC), and the contribution of phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) signaling pathway in this process. **METHODS:** VSMC obtained from rat thoracic aortas were cultured. The phosphorylation of 4E-BP1 and p70 S6 kinase was detected by immunoblotting. **RESULTS:** Ang II significantly increased the phosphorylation of 4E-BP1 and p70 S6 kinase, with the peaks occurring at, respectively, 10 min and 30 min, after stimulation with Ang II. The stimulatory effect of Ang II on 4E-BP1 and p70 S6 kinase phosphorylation was abrogated by Ang II type 1 receptor (AT_1 receptor) antagonist losartan, and suppressed by PI3K inhibitor LY294002 in a concentration-dependent manner. **CONCLUSION:** Ang II treatment of VSMC induces the phosphorylation of 4E-BP1 and p70 S6 kinase via AT_1 receptor, and PI3K signaling pathway is involved in this process.

INTRODUCTION

Angiotensin II (Ang II) potently stimulates protein synthesis, the major hallmark of hypertrophy, in vascular smooth muscle cells (VSMC) by acting through the G protein-coupled Ang II type 1 receptor (AT_1 receptor)[1]. However, the molecular basis for this hypertrophic effect remains largely unknown.

Recent studies have established the initiation phase of mRNA translation as a pivotal site of regulation for global rates of protein synthesis[2]. At the heart of the translational control lies 4E-binding protein 1 (4E-BP1) and p70 S6 kinase, and together they coordinate the behavior of both eukaryotic initiation factors (eIFs) and ribosome. In its nonphosphorylated state, 4E-BP1 binds eIF4E, inhibiting cap-dependent translation. Hyperphosphorylation of 4E-BP1 disrupts this binding, activating cap-dependent translation[3]. P70 S6 kinase phosphorylates the 40s subunit ribosomal protein S6 and is involved in an activation of protein synthesis at the level of initiation[4]. The activity of p70 S6 kinase is controlled by its phosphorylation at multiple sites, with the Thr^{389} position shown to correlate most clearly with activity *in vivo*[5]. Upstream signaling pathways responsible for the phosphorylation of the two translational regulators have been investigated in some recent studies. A growing body of evidence suggests phosphatidyl-inositol 3-kinase (PI3K)/protein kinase B (PKB) pathway is a strong candidate[6-9].

Despite the emergence of an increasingly refined

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picture of mRNA translation, there have been relatively few effort made to understand the role of the two translational regulators, especially 4E-BP1, in Ang II-induced pathological VSMC hypertrophy. The purpose of the present study was to examine the effects of Ang II on phosphorylation of two translational regulators, and the contribution of PI3K/PKB signaling pathway in this process.

MATERIALS AND METHODS

Reagents Ang II was obtained from Sigma. AT_1 receptor antagonist losartan was a gift from Merck. LY294002 (the specific inhibitor of PI3K) and all the antibodies were purchased from New England Biolabs. Immunoblotting chemiluminescence kit was purchased from Santa Cruz Biotechnology. All other reagents were of analytic grade.

Cell culture VSMC were isolated from thoracic aortas of 8-week-old Sprague-Dawley rats by explant method and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum 15 %, benzylpenicillin 100 kU/L, streptomycin 100 mg/L. The culture was maintained at 37 ºC in a humidified incubator with CO_2 5 %+air 95 %. The cells showed a spindle-shaped appearance, and confluent VSMC in culture showed the characteristic "hill and valley" growth pattern. The identification of VSMC was confirmed by smooth muscle α-actin staining. Cells were passaged twice a week by harvesting with trypsin 0.25 %. For subsequent experiments, cells between passage levels 4 and 6 were used.

Preparation of cell extracts and immunoblotting VSMC in 6-well plates were made quiescent by incubation with serum-free DMEM for 24 h. VSMC were treated with Ang II 100 nmol/L for the indicated durations at 37 ºC. The cells were washed twice with ice-cold PBS, and lysed with SDS Sample Buffer (Tris-HCl 62.5 mmol/L, SDS 2 %, glycerol 10 %, DTT 50 mmol/L, bromophenol blue 0.01 %, pH 6.8). Solubilized proteins were centrifuged (13 000×*g*, 30 min, 4 ºC) and protein concentrations in the supernatant were determined using the Bradford protein assay.

Equal amounts of protein were then separated by SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific binding was blocked in Tris-buffered saline containing Tween 20 0.1 % (TBST) with fat-free milk powder 5 % at room temperature. The membranes were incubated overnight at 4 ºC with one of the following antibodies at a 1:1000 dilution: anti-phosphospecific 4E-BP1 (Ser^{65}); anti-phosphospecific p70 S6 kinase (Thr^{389}). After they were washed with TBST, the membranes were incubated for another hour with anti-rabbit horseradish peroxidase-conjugated immunoglobulin G at a 1:2000 dilution at room temperature. Membranes were then washed three times with TBST and once with TBS, and the signals were detected using an chemiluminescence kit after exposure to an X-ray film. Signals were quantified by densitometer analysis.

Statistical analysis Values were expressed as mean±SD, and assessed by one-way ANOVA test.

RESULTS

Time course of Ang II-stimulated phosphorylation of 4E-BP1 The basal level of 4E-BP1 phosphorylation was low in quiescent VSMC. Ang II 100 nmol/L induced a rapid, transitory increase of 4E-BP1 phosphorylation, with a peak occurring at 10 min. The phosphorylation of 4E-BP1 was still above baseline at 30 min (Fig 1).

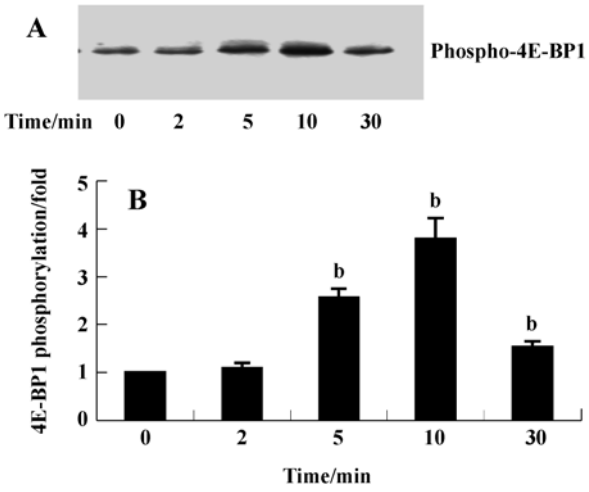

Fig 1. Immunoblotting analysis of time course of 4E-BP1 phosphorylation stimulated by Ang II (100 nmol/L). A) autoradiogram; B) quantitation of immunoreactive bands. *n*=3. Mean±SD. $^bP<0.05$ *vs* control.

Time course of Ang II-stimulated phosphorylation of p70 S6 kinase Though the basal level was low, stimulation with Ang II 100 nmol/L induced a robust and sustained increase of p70 S6 kinase phosphorylation, which peaked at 30 min, and then slowly decreased (Fig 2).

Effect of losartan on Ang II-stimulated phosphorylation of 4E-BP1 and p70 S6 kinase VSMC were pretreated with losartan (AT_1 receptor antagonist) 10 μmol/L for 30 min before exposure to Ang II 100 nmol/L. Phosphorylation of 4E-BP1 and p70 S6 kinase

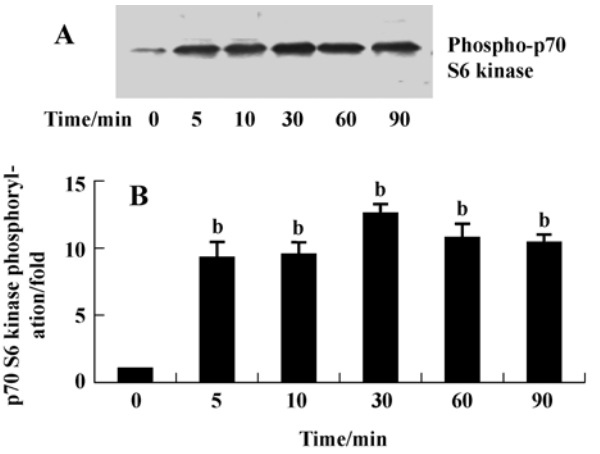

Fig 2. Immunoblotting analysis of time course of p70 S6 kinase phosphorylation stimulated by Ang II (100 nmol/L). A) autoradiogram; B) quantitation of immunoreactive bands. *n*=3. Mean±SD. $^bP < 0.05$ *vs* control.

stimulated by Ang II was abrogated by losartan, indicating that Ang II-induced phosphorylation of the two proteins is mediated by AT_1 receptor (Fig 3).

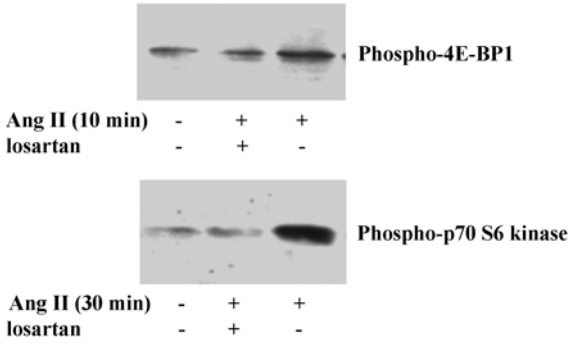

Fig 3. Immunoblotting analysis of effect of losartan on Ang II-stimulated phosphorylation of 4E-BP1 and p70 S6 kinase.

Concentration-dependent effect of PI3K inhibitor on Ang II-stimulated phosphorylation of 4E-BP1 and p70 S6 kinase To assess the role of PI3K in Ang II-stimulated phosphorylation of 4E-BP1 and p70 S6 kinase, VSMC were pretreated with LY294002, a specific inhibitor of PI3K, for 1 h before addition of Ang II 100 nmol/L. Both 4E-BP1 and p70 S6 kinase phosphorylation induced by Ang II was dramatically inhibited by the inhibitor in a concentration (0.1-10 μmol/L)-dependent manner (Fig 4), suggesting that PI3K is an upstream mediator of 4E-BP1 and p70 S6 kinase phosphorylation in VSMC.

DISCUSSION

In this study, we showed that Ang II could rapidly

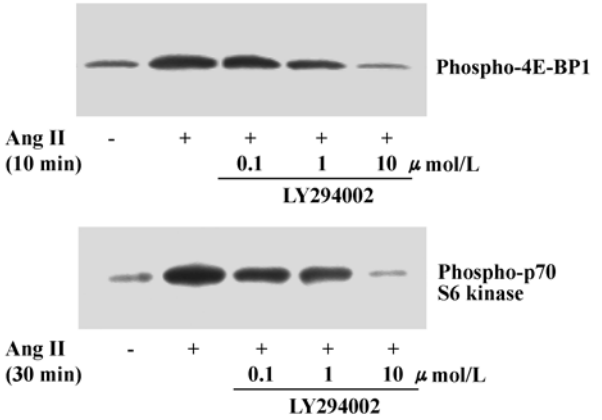

Fig 4. Immunoblotting analysis of effect of LY294002 on Ang II-stimulated phosphorylation of 4E-BP1 and p70 S6 kinase.

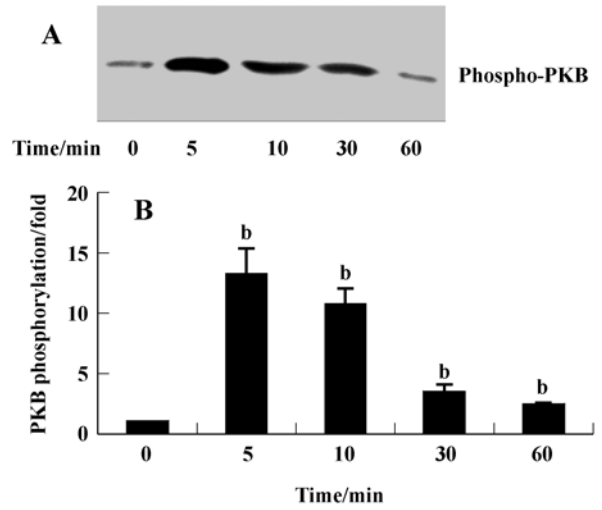

Fig 5. Immunoblotting analysis of time course of PKB phosphorylation stimulated by Ang II (100 nmol/L). A) autoradiogram; B) quantitation of immunoreactive bands. *n*=3. Mean±SD. $^bP<0.05$ *vs* control.

stimulate phosphorylation of 4E-BP1 and p70 S6 kinase through AT_1 receptor mediation. Considering the important role of 4E-BP1 and p70 S6 kinase in translational control, our study substantiates the idea that hypertrophic effect of Ang II is partly mediated through their phosphorylation.

The signal transduction pathways coupling AT_1 receptor activation to the stimulation of 4E-BP1 and p70 S6 kinase phosphorylation remain to be elucidated. There was some evidence to support that PI3K mediated 4E-BP1 and p70 S6 kinase phosphorylation. For example, the phosphorylation of both 4E-BP1 and p70 S6 kinase was strongly diminished by wortmannin, an inhibitor of PI3K[6,7]. In addition, expression of an activated

110-kDa catalytic subunit of PI3K stimulates the phosphorylation of 4E-BP1[8], and a dominant-negative mutant bearing a mutation of the 85-kDa regulatory subunit of PI3K inhibits the phosphorylation and activity of p70 S6 kinase[9]. Because PI3K has been traditionally associated with receptor tyrosine kinase action, the contribution of this enzyme to Ang II-mediated signaling pathways has been relatively little documented. Aim to investigate whether PI3K was involved in Ang II-induced phosphorylation of two translational regulators in VSMC, LY294002, which was considered to exert more highly selective inhibitory effect on PI3K than wortmannin[10], was used. As expected, LY294002 did suppress their phosphorylation in a concentration-dependent manner. These results provide experimental evidence for the involvement of a PI3K-dependent pathway in Ang II-induced phosphorylation of the two translational regulators in VSMC. To our knowledge, this is the first time to determine the role of PI3K in Ang II-induced 4E-BP1 phosphorylation.

Activation of PI3K leads to the formation of phosphatidylinositol-3,4-bisphosphate and phosphatidyl-inositol-3,4,5-trisphosphate resulting in PKB activation. As a key signaling component lying downstream of PI3K, PKB mediates many of the biological functions of PI3K[11,12]. These findings inspire the idea that PKB may act as a potential signal transducer linking PI3K to the translational regulators. To gain some insight into this idea, we compared the kinetics of PKB activation with that of two translational regulators phosphorylation in Ang II-stimulated VSMC. As shown in Fig 5, Ang II caused PKB activation (phosphorylation of PKB on Ser^{473} is required for activation, therefore, phosphorylation at this site was routinely taken as a measure of PKB enzymatic activity[13]). PKB activation was rapid but transitory. The kinetics of this phosphorylation is, to some extent, similar with that of 4E-BP1 phosphorylation, but greatly distinct from that of p70 S6 kinase phosphorylation. P70 S6 kinase reached maximal phosphorylation at 30 min when PKB activity had decreased to very low level. Therefore it seemed impossible for PKB to be a direct regulator of p70 S6 kinase phosphorylation. These findings challenge the previous hypothesis that PKB mediates PI3K-induced phosphorylation of p70 S6 kinase. Support for the hypothesis has largely relied on results obtained with highly active, constitutively membrane-localized alleles of wild-type PKB[14]. However, recent studies found that highly activated alleles of PKB or constitutive membrane localization might not reflect wild-type PKB signaling[15]. Thus, the results presented here together with other findings[15] support a model in which PI3K signaling to p70 S6 kinase is PKB independent, whereas 4E-BP1 phosphorylation is PKB dependent.

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