Activation of p70 S6 protein kinase is necessary for angiotensin II-induced hypertrophy in neonatal rat cardiac myocytes

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Abstract Although many lines of evidence have suggested that angiotensin II (Ang II) plays an important role in development of cardiac hypertrophy, the mechanism by which Ang II increases protein synthesis in cardiac myocytes remains unclear. It has been reported that the phosphorylation of S6 protein in 40 S ribosome is correlated to the efficiency of protein synthesis. In the present study, we have examined whether Ang II activates p70 S6 kinase ($p70^{S6K}$), which has been reported to phosphorylate S6 protein. Ang II activated $p70^{S6K}$ through AT1 receptor. An immunosuppressant agent, rapamycin, inhibited Ang II-induced $p70^{S6K}$ activation but not the activation of MAP kinases or the induction of *c-fos* gene expression. Rapamycin also abolished Ang II-induced increase in protein synthesis. These results suggest that Ang II induces cardiac hypertrophy by activating $p70^{S6K}$.

Key words: Angiotensin II; p70 S6 protein kinase; Rat cardiomyocyte; Cardiac hypertrophy; MAP kinase; *c-fos*

1. Introduction

Many lines of evidence have suggested that angiotensin II (Ang II) plays a critical role in inducing cardiac hypertrophy [1–7]. All components of the renin–angiotensin system such as renin, angiotensinogen and angiotensin-converting enzyme (ACE) have been identified in the heart at both mRNA and protein levels [5,6]. It has been reported that angiotensinogen and ACE mRNAs are increased in experimental left ventricular hypertrophy induced by hemodynamic overload [4]. ACE inhibitors with subpressor doses induced significant regression of cardiac hypertrophy with no change in systemic systolic blood pressure [1]. Our and other laboratories have reported that Ang II activates phosphorylation cascades of protein kinases such as protein kinase C (PKC), mitogen-activated protein (MAP) kinases and 90 kDa S6 kinase ($p90^{rsk}$), and induces the expression of both immediate early response genes and fetal type genes, followed by the increase in protein synthesis in cultured cardiac myocytes [7–9].

In general, cardiac hypertrophy is formed by the increase in protein synthesis and/or the decrease in protein degradation. The efficiency of protein synthesis is well-correlated to phosphorylated levels of S6 protein in 40 S ribosome [10–12]. Two distinct families of S6 kinases that phosphorylate S6 protein in vitro have been identified [10]. One of them is the 90 kDa S6 kinase ($p90^{rsk}$) and the other is the 70 kDa S6 kinase ($p70^{S6K}$). The $p90^{rsk}$ is activated by MAP kinases through Ras-Raf pathway and mainly mediates the control of transcription. Although the signaling pathway that leads to activation of $p70^{S6K}$ is still unclear, many reports have suggested that $p70^{S6K}$ is responsible for phosphorylation of S6 protein in vivo and mediates the control of translation [10–12]. In the present study, to elucidate the mechanism of cardiac hypertrophy, we examined the role of $p70^{S6K}$ in cardiac hypertrophy induced by Ang II.

2. Materials and methods

2.1. Culture of cardiomyocytes

Primary cultures of cardiomyocytes were prepared from ventricles of 1-day-old Wistar rats, as described previously [8], basically according to the method of Simpson and Savion [13]. Cardiomyocytes were plated at a field density of 1×10^3 cells/mm^2 on 35-mm or 100-mm culture dishes. After starvation for 48 h, cardiomyocytes were stimulated by Ang II (Sigma).

2.2. Kinase assay of $p70^{S6K}$

Cardiac myocytes cultured in 35-mm culture dishes were stimulated with Ang II for the indicated periods of time at 37°C. After stimulation, the cells were washed twice with ice-cold PBS and lysed with 0.15 ml of Triton X-100 lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 50 mM NaF, 5 mM EDTA, 40 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10^{-4} M phenylmethylsulfonyl fluoride, 10^{-6} M leupeptin, 10^{-6} M pepstatin A, 1% Triton X-100) for 25 min on ice. Cell lysates (100 μg) were incubated for 12 h at 4°C with anti-$p70^{S6K}$ polyclonal antibody (Santa Cruz Biotechnology) preabsorbed to protein A-Sepharose beads. The immune complexes were washed 3 × with lysis buffer and once with kinase buffer (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 10 mM β-glycerophosphate). The immunoprecipitates were resuspended in 40 μl of kinase buffer containing 0.2 mM S6 peptide (RRRLSSLRA, Santa Cruz Biotechnology), 20 μM ATP, and 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) and incubated at 30°C for 20 min. After incubation, the reactions were stopped by spotting the mixture on P81 paper (Whatman). The filters were washed 3 × for 10 min each in 1% phosphoric acid, once in acetone and dried. The ^{32}P uptake was measured by Cerenkov counting method.

2.3. MAP kinase assays in myelin basic protein (MBP)-containing gels

MAP kinase assays were performed using MBP-containing gels as described previously [9].

2.4. Northern blot analysis

After starvation for 48 h, cardiac myocytes in 100-mm dishes were stimulated with Ang II (10^{-6} M) for 30 min at 37°C. Northern blot analysis was performed using ^{32}P-labeled human *c-fos* cDNA as described previously [14].

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Abbreviations: Ang II, angiotensin II; PKC, protein kinase C; MAP, mitogen-activated protein; $p90^{rsk}$, 90 kDa S6 kinase; ACE, angiotensin-converting enzyme; $p70^{S6K}$, 70 kDa S6 kinase; MBP, myelin basic protein; FKBP, FK506-binding protein

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2.5. Amino acid uptake into cardiomyocytes

After starvation for 48 h, cardiac myocytes were stimulated with 10^{-6} M Ang II for 24 h. The relative amount of protein synthesis was determined by assessing the incorporation of the radioactivity into a trichloroacetic acid-insoluble fraction as previously reported [8,9].

3. Results

3.1. Ang II stimulates p70^{S6K} in cardiac myocytes

To examine whether Ang II stimulates p70^{S6K} in cultured cardiac myocytes of neonatal rats, we measured the activity of p70^{S6K} using a specific antibody raised against a carboxyl-terminal peptide from the rat p70^{S6K}. This antibody specifically recognizes p70^{S6K} but not p90^{rsk} in mouse, rat and human cells. At 48 h after serum deprivation, cardiac myocytes were stimulated with Ang II (10^{-6} M). The p70^{S6K} activity started to increase by 5 min after the stimulation, and peaked at 15 min

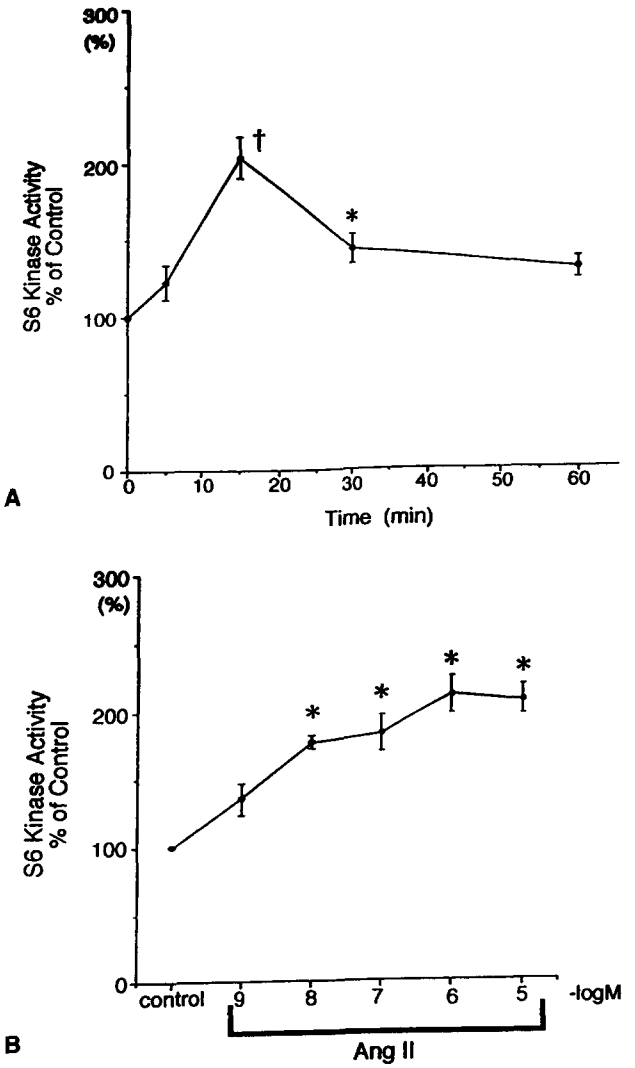

Fig. 1. Time course and dose dependence of Ang II-induced p70^{S6K} activation. (A, above) Cardiac myocytes of neonatal rats were stimulated with 10^{-6} M Ang II for various periods of time. (B, below) Cardiac myocytes were stimulated with various concentrations of Ang II for 15 min. The activity of p70^{S6K} was measured as described in section 2. Data represent the average percentage of control (= 100%, vehicle) from four independent experiments (mean ± S.E.M.). Statistical analysis was performed using the paired-sample *t*-test with *P* values corrected by the Bonferroni method (*$P < 0.05$ and †$P < 0.01$ vs. control).

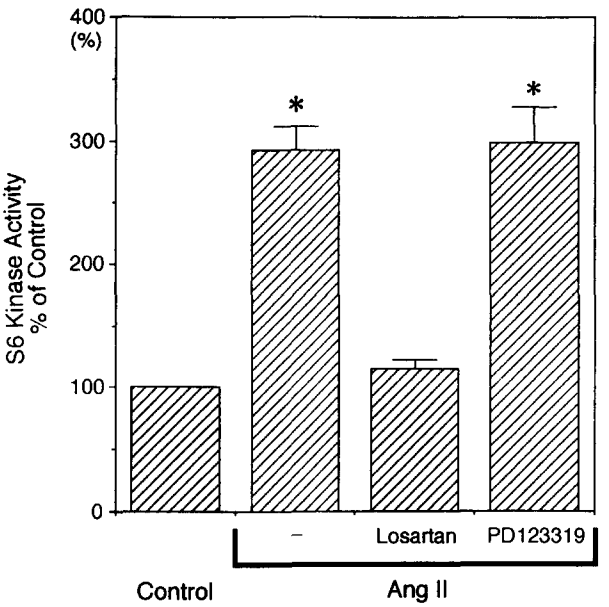

Fig. 2. Ang II activates p70^{S6K} through AT1 receptor. Cardiac myocytes were pretreated with 10^{-5} M losartan or 10^{-5} M PD123319 for 30 min and stimulated with 10^{-6} M Ang II for 15 min. p70^{S6K} activity was measured as described. Data represent the average percentage of control (= 100%, vehicle) from three independent experiments (mean ± S.E.M.) (*$P < 0.05$ vs. control).

(Fig. 1A). The activity decreased gradually thereafter and returned to the control level at 4 h after stimulation (data not shown). Cardiac myocytes were next stimulated with various concentrations (10^{-9} to 10^{-5} M) of Ang II for 15 min (Fig. 1B). A slight increase in p70^{S6K} activity was observed at 10^{-9} M, and the maximal activation of p70^{S6K} was obtained at 10^{-6} M of Ang II. The activity of p70^{S6K} was increased by 110% with 10^{-6} M of Ang II as compared with that of control.

3.2. Ang II stimulates p70^{S6K} through AT1 receptor

Two kinds of Ang II receptors, AT1 and AT2, have been reported to exist in cardiac myocytes [15–19]. To examine which subtype of Ang II receptors is involved in the activation of p70^{S6K}, cardiac myocytes were preincubated with AT1 receptor-specific antagonist, losartan (10^{-5} M) or AT2 receptor-specific antagonist, PD123319 (10^{-5} M) for 30 min and then stimulated with Ang II (10^{-6} M) for 15 min. Losartan completely inhibited the Ang II-induced p70^{S6K} activation, whereas PD123319 had no effect (Fig. 2). These results suggest that p70^{S6K} is activated by Ang II through AT1 receptor.

3.3. Rapamycin but not FK506 inhibits p70^{S6K} activation by Ang II

Although the immunosuppressant agents rapamycin and FK506 are structurally related macrolides, it has been reported that subnanomolar concentrations of rapamycin but not FK506 specifically block serum-induced p70^{S6K} activation in Swiss 3T3 cells [2024]. To examine whether rapamycin inhibits the activation of p70^{S6K} by Ang II, cardiac myocytes were pretreated with rapamycin (0.01–10 ng/ml) or FK506 (30 ng/ml) for 30 min prior to stimulation with Ang II (10^{-6} M). The activity of p70^{S6K} enhanced by Ang II decreased to the control levels by the treatment with 0.1 ng/ml rapamycin. One ng/ml

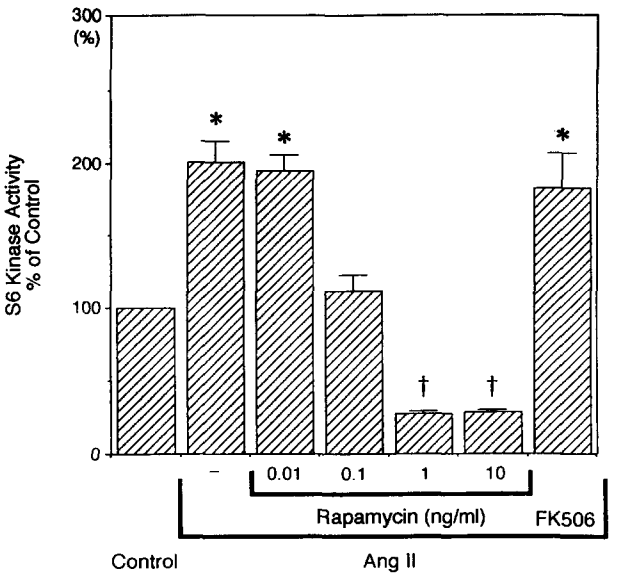

Fig. 3. Rapamycin inhibits $p70^{S6K}$ activated by Ang II. Cardiac myocytes were pretreated with various concentrations of rapamycin or 30 ng/ml of FK506 for 30 min and stimulated with 10^{-6} M Ang II for 15 min. $p70^{S6K}$ activity was measured as described. Data represent the average percentage of control (= 100%, vehicle) from three independent experiments (mean ± S.E.M.) ($^{*}P < 0.05$ and $^{\dagger}P < 0.01$ vs. control).

rapamycin reduced the $p70^{S6K}$ activity to 25% of unstimulated basal levels whereas even high concentration of FK506 had no effect on the $p70^{S6K}$ activity (Fig. 3).

3.4. $p70^{S6K}$ activation is necessary for Ang II-induced cardiac hypertrophy

To examine the role of $p70^{S6K}$ in Ang II-induced cardiac hypertrophy, we measured the relative protein synthesis using [^{3}H]phenylalanine in the absence or presence of rapamycin. As described in the previous papers [9], we found that Ang II (10^{-6} M) increased phenylalanine incorporation into cardiac myocytes by approximately 1.4-fold as compared with control (Fig. 4). Rapamycin (0.5 ng/ml) completely blocked the Ang II-induced increase in amino acid incorporation, suggesting that $p70^{S6K}$ may play a critical role in Ang II-induced cardiac hypertrophy.

3.5. $p70^{S6K}$ activation is independent of MAP kinase activation and c-fos gene expression

Our and other laboratories have recently reported that Ang II increases the activity of Raf-1 and MAP kinases, and mRNA levels of *c-fos* through the AT1 receptor in cardiac myocytes [7,8]. To elucidate the relation between signaling pathways that induce the activation of $p70^{S6K}$ and MAP kinases, we measured the activity of MAP kinases after Ang II stimulation in the presence or absence of rapamycin. Ang II activated MAP kinases as previously reported (Fig. 5A, lane 2) [8,9]. Rapamycin as well as FK506 did not inhibit Ang II-induced the activation of MAP kinases (Fig. 5A, lane 3 and 4). We next examined whether $p70^{S6K}$ is involved in the Ang II-induced *c-fos* gene expression in cardiac myocytes. Ang II induced *c-fos* gene expression as reported before [7–9] and rapamycin or FK506 did not inhibit the Ang II-induced *c-fos* gene expression (Fig. 5B). These data suggest that the pathway of $p70^{S6K}$ activation is different from that of MAP kinase activation and of *c-fos* gene expression in cardiac myocytes.

4. Discussion

A growing body of data has suggested that renin–angiotensin system plays an important role in generating cardiac hypertrophy [1–7]. In the present study, to elucidate the mechanism of the Ang II-induced cardiac hypertrophy, we examined the role of $p70^{S6K}$ in cultured cardiac myocytes of neonatal rats. Ang II increased the activity of $p70^{S6K}$, and rapamycin but not FK506 completely inhibited the activation. Rapamycin also inhibited the Ang II-induced increase in protein synthesis, but had no effects on the Ang II-induced MAP kinase activation and *c-fos* gene expression.

It has been reported that S6 protein exists at the tRNA-mRNA-binding site of 40 S ribosome and that phosphorylation of five serine residues at the carboxyl-terminus of S6 protein is well-correlated to the efficiency of protein synthesis [11,12]. Although the signaling pathway that leads to activation of $p70^{S6K}$ is still unclear, many reports have suggested that $p70^{S6K}$ is responsible for phosphorylation of S6 protein in vivo and regulates the efficiency of translation. In the present study, Ang II activated $p70^{S6K}$ from as early as 5 min in cardiac myocytes. The peak of $p70^{S6K}$ activation was observed at 15 min and the increased activity above basal levels continued for 4 h after Ang II addition (Fig. 1). The maximum activation was obtained by 10^{-6} M Ang II and half maximum activation by $\sim 5 \times 10^{-9}$ M Ang II. Since losartan completely blocked the Ang II-induced activation of $p70^{S6K}$, Ang II activates $p70^{S6K}$ through AT1 receptor. The Ang II concentrations and the receptor type which

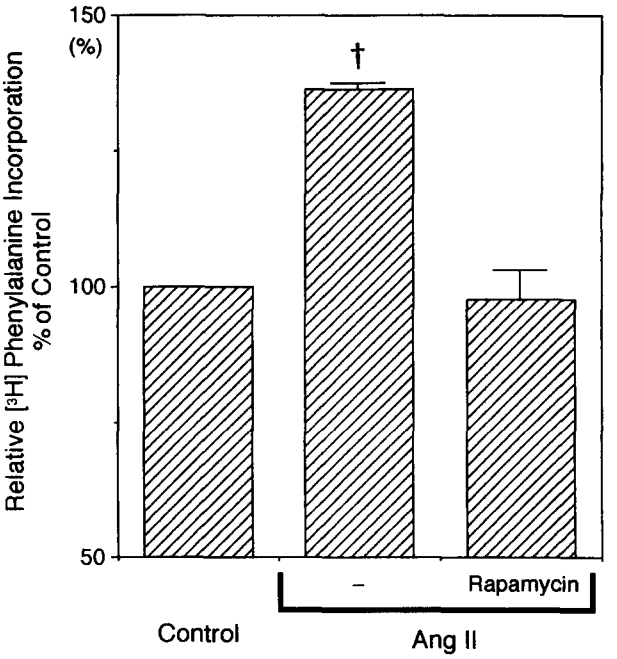

Fig. 4. Rapamycin inhibits Ang II-induced phenylalanine incorporation. After pretreatment with rapamycin (0.5 ng/ml), cardiac myocytes were stimulated by 10^{-6} M Ang II for 24 h and [^{3}H]phenylalanine (1 μCi/ml) was added 2 h before harvest. The total radioactivity of incorporated [^{3}H]phenylalanine was determined by liquid scintillation counting. Data represent the average percentage of control (= 100%, vehicle) from three independent experiments (mean ± S.E.M.) ($^{\dagger}P < 0.01$ vs. control).

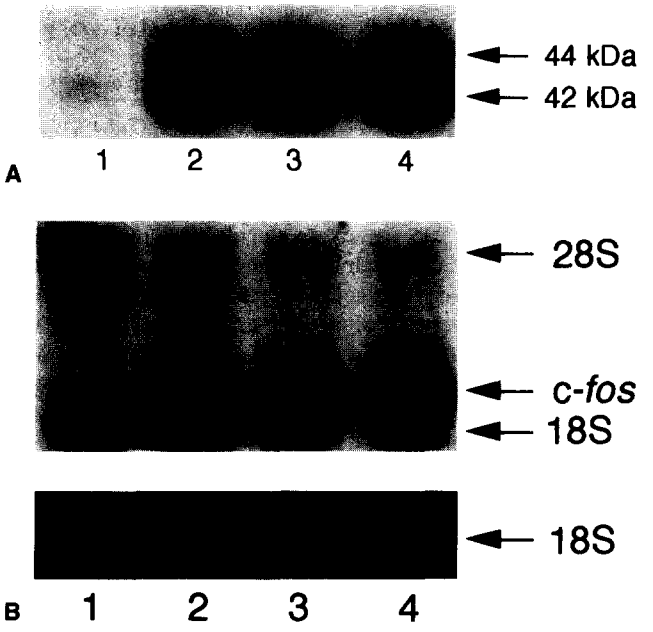

Fig. 5. Rapamycin does not inhibit Ang II-induced MAP kinase activation and *c-fos* gene expression. (A, above) MAP kinase assays using MBP-containing gels. Cardiac myocytes were pretreated for 30 minutes with rapamycin (0.5 ng/ml) or FK506 (10 ng/ml) and stimulated with 10^{-6} M Ang II for 8 min. lane 1, control; lane 2, Ang II; lane 3, Ang II+rapamycin; lane 4, Ang II + FK506. MAP kinase activity was measured using MBP-containing gels as described. (B, below) *c-fos* gene expression. Cardiac myocytes were pretreated for 30 min with rapamycin (0.5 ng/ml) or FK506 (10 ng/ml) and stimulated with 10^{-6} M Ang II for 30 min. lane 1, control; lane 2, Ang II; lane 3, Ang II + rapamycin; lane 4, Ang II + FK506. Northern blot analysis was performed as described. Ethidium bromide staining of 18 S rRNA is presented to show that an equal amount of RNA (8 μg) was loaded in each lane. Similar results were obtained from two independent experiments.

are necessary for $p70^{S6K}$ activation are same as those for the activation of MAP kinases and $p90^{rsk}$ [7–9].

The immunosuppressant agents rapamycin and FK506 are structurally related macrolides that have been reported to inhibit proliferation and activation of T cells by binding the same cellular receptor, FK506-binding protein (FKBP) [20,25–27]. It has been demonstrated that these two agents interfere with distinct signaling pathways after binding FKBP [21–23]. The FK506-FKBP complex binds Ca^{2+}/calmodulin-dependent phosphatase, calcineurin, and inhibits its phosphatase activity [21]. In contrast, the rapamycin-FKBP complex inhibits the IL-2 signaling pathway which leads to activation of $p70^{S6K}$ and cyclin-dependent kinases (CDKs), by binding to FKBP-rapamycin-associated protein (FRAP)/rapamycin and FKBP12 targets (RAFT) [22,23]. A recent report has demonstrated that FRAP is a rapamycin-sensitive regulator of $p70^{S6K}$ in vivo and that both the kinase activity and the amino-terminal domain of FRAP are required for control of $p70^{S6K}$ [28]. Rapamycin blocked the Ang II-induced $p70^{S6K}$ activation and also inhibited the increase in protein synthesis induced by Ang II at the same range of concentration in cardiac myocytes. These results suggest that activation of $p70^{S6K}$ is necessary for the increase in protein synthesis induced by Ang II.

Many reports have suggested that MAP kinases are activated by many growth factors and cytokines and play important roles in cell proliferation and differentiation [29]. Cowley et al. have reported that MAP kinase pathway mediates both differentiation of PC12 pheochromocytoma cells and transformation of NIH 3T3 cells [30]. It has been reported that activated MAP kinases translocate into the nucleus and activate nuclear transcription factor Elk-1 resulting in the induction of *c-fos* gene expression [31]. $p90^{rsk}$ has been reported to exist in downstream of MAP kinases and to phosphorylate nuclear lamins [32]. Rapamycin blocked the Ang II-induced $p70^{S6K}$ activation but did not inhibit the activation of MAP kinases or the expression of *c-fos* gene induced by Ang II in cardiac myocytes. These results suggest that Ang II activates $p70^{S6K}$ through the pathway different from that of MAP kinases/$p90^{rsk}$. Recently it has been reported that MAP kinases phosphorylate PHAS-I, which is a heat-stable protein found in many tissues including the heart and the phosphorylated PHAS-I dissociates from translation initiation factor 4E, resulting in the initiation of translation [33]. Therefore, it is possible that the activations of both $p70^{S6K}$ and MAP kinases are necessary for Ang II-induced cardiac hypertrophy. AT1 receptor is associated with the tetrameric GTP-binding protein, G_q. Further studies will be required to determine how G_q coupled Ang II receptor activates $p70^{S6K}$ in cardiac myocytes.

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References

[1] Linz, W., Schoelkens, B.A. and Ganten, D. (1989) Clin. Exp. Hypertens. 11, 1325–1350.
[2] Baker, K.M., Cherin, M.I., Wixon, S.K. and Aceto, J.F. (1990) Am. J. Physiol. 259, H324–332.
[3] Baker, K.M. and Aceto, J.F. (1990) Am. J. Physiol. 259, H610–618.
[4] Schunkert, H., Dzau, V.J., Tang, S.S., Hirsch, A.T., Apstein, C.S. and Lorell, B.H. (1990) J. Clin. Invest. 86, 1913–1920.
[5] Lindpaintner, K. and Ganten, D. (1991) Circ. Res. 68, 905–921.
[6] Baker, K.M., Booz, G.W. and Dostal, D.E. (1992) Annu. Rev. Physiol. 54, 227–241.
[7] Sadoshima, J. and Izumo, S. (1993) Circ. Res. 73, 413–423.
[8] Yamazaki, T., Komuro, I., Kudoh, S., Zou, Y., Shiojima, I., Mizuno, T., Takano, H., Hiroi, Y., Ueki, K., Tobe, K., Kadowaki, T., Nagai, R. and Yazaki, Y. (1995) J. Clin. Invest. 96, 438–446.
[9] Yamazaki, T., Komuro, I., Kudoh, S., Zou, Y., Shiojima, I., Mizuno, T., Takano, H., Hiroi, Y., Ueki, K., Tobe, K., Kadowaki, T., Nagai, R. and Yazaki, Y. (1995) Circ. Res. 77, 258–265.
[10] Erikson, R.L. (1991) J. Biol. Chem. 266, 6007–6010.
[11] Flotow, H. and Thomas, G. (1992) J. Biol. Chem. 267, 3074–3078.
[12] Jefferies, H.B.J., Reinhard, C., Kozma, S.C. and Thomas, G. (1994) Proc. Natl. Acad. Sci. USA 91, 4441–4445.
[13] Simpson, P. and Savion, S. (1982) Circ. Res. 50, 101–116.
[14] Komuro, I., Kaida, T., Shibazaki, Y., Kyrabayashi, M., Takaku, F. and Yazaki, Y. (1990) J. Biol. Chem. 265, 3595–3598.
[15] Murphy, T.J., Alexander, R.W., Griendling, K.K., Runge, M.S. and Bernstein, K.E. (1991) Nature (London) 351, 233–236.
[16] Sasaki, K., Yamano, Y., Bardhan, S., Iwai, N., Murray, J.J., Hasegawa, M., Matsuda,Y. and Inagami, T. (1991) Nature (London) 351, 230–233.
[17] Mukoyama, M., Nakajima, M., Horiuchi, M., Sasamura, H., Pratt, R.E. and Dzau, V.J. (1993) J. Biol. Chem. 268, 24539–24542.

[18] Kambayashi, Y., Bardhan, S., Takahashi, K., Tsuzuki, S., Inui, H., Hamakubo, T. and Inagami, T. (1993) J. Biol. Chem. 268, 24543–24546.
[19] Matsubara, H., Kanasaki, M., Murasawa, S., Tsukaguchi, Y., Nio, Y. and Inada, M. (1994) J. Clin. Invest. 93, 1592–1601.
[20] Kuo, C.J., Chung, J., Fiorentino, D.F., Flanagan, W.M., Blenis, J. and Crabtree, G.R. (1992) Nature (London) 358, 70–73.
[21] Liu, J., Farmer Jr., J.D., Lane, W.S., Friedman, J., Weissman, I. and Schreiber, S.L. (1991) Cell 66, 807–815.
[22] Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P. and Snyder, S.H. (1994) Cell 78, 35–43.
[23] Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keit, C.T., Lane, W.S. and Schreiber, S.L. (1994) Nature (London) 369, 756–758.
[24] Chung, J., Kuo, C.J., Crabtree, G.R. and Blenis, J. (1992) Cell 69, 1227–1236.
[25] Calvo, V., Crews, C.M., Vik, T.A. and Bierer, B.E. (1992) Proc. Natl. Acad. Sci. USA 89, 7571–7575.
[26] Price, D.J., Grove, J.R., Calvo, V., Avruch, J. and Bierer, B.E. (1992) Science 257, 973–977.
[27] Schreiber, S.L. (1991) Science 251, 283–287.
[28] Brown, E.J., Beal, P.A., Keith, C.T., Chen, J., Shin, T.B. and Schreiber, S.L. (1995) Nature (London) 377, 441–446.
[29] Nishida, E. and Gotoh, Y. (1993) Trends. Biochem. Sci. 18, 128–131.
[30] Cowley, S., Paterson, H., Kemp, P. and Marshall, C.J. (1994) Cell 77, 841–852.
[31] Davis, R.J. (1993) J. Biol. Chem. 268, 14553–14556.
[32] Sturgill, T.W., Ray, L.B., Erikson, E. and Maller, J.L. (1988) Nature (London) 334, 715718.
[33] Lin, T.A., Kong, X., Haystead, T.A.J., Pause, A., Belsham, G., Sonenberg, N. and Lawrence Jr., J.C. (1994) Science 266, 653–656.