ANG II activates effectors of mTOR via PI3-K signaling in human coronary smooth muscle cells

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1Department of Cardiothoracic Surgery, National Heart and Lung Institute, Imperial College of Science, Technology, and Medicine, Heart Science Centre, Harefield Hospital, Middlesex, UB9 6JH; and 2Division of Molecular Physiology, Faculty of Life Sciences, University of Dundee, Dundee, DD1 5EH, United Kingdom

Hafizi, Sassan, Xuenin Wang, Adrian H. Chester, Magdi H. Yacoub, and Christopher G. Proud. ANG II activates effectors of mTOR via PI3-K signaling in human coronary smooth muscle cells. Am J Physiol Heart Circ Physiol 287: H1232–H1238, 2004; 10.1152/ajpheart.00040.2004.—We have previously shown that the vasoconstrictive peptide angiotensin II (ANG II) is a hypertrophic agent for human coronary artery smooth muscle cells (cSMCs), which suggests that it plays a role in vascular wall thickening. The present study investigated the intracellular signal transduction pathways involved in the growth response of cSMCs to ANG II. The stimulation of protein synthesis by ANG II in cSMCs was blocked by the immunosuppressive rapamycin, which is an inhibitor of the mammalian target of rapamycin (mTOR) signaling pathway that includes the 70-kDa S6 kinase (p70^S6K) and plays a key role in cell growth. The inhibitory effect of rapamycin was reversed by a molar excess of FK506; this indicates that both agents act through the common 12-kDa immunophilin FK506-binding protein. ANG II caused a rapid and sustained activation of p70^S6K activity that paralleled its phosphorylation, and both processes were blocked by rapamycin. In addition, both of the phosphatidylinositol-3-kinase inhibitors wortmannin and LY-294002 abolished the ANG II-induced increase in protein synthesis, and wortmannin also blocked p70^S6K phosphorylation. Furthermore, ANG II triggered dissociation of the translation initiation factor, eukaryotic initiation factor 4E, from its regulatory binding protein 4E-BP1, which was also inhibited by rapamycin and wortmannin. In conclusion, we have shown that ANG II activates components of the rapamycin-sensitive mTOR signaling pathway in human cSMCs and involves activation of phosphatidylinositol 3-kinase, p70^S6K, and eukaryotic initiation factor-4E, which leads to activation of protein synthesis. These signaling mechanisms may mediate the growth-promoting effect of ANG II in human cSMCs.

mammalian target of rapamycin; S6 kinase; phosphatidylinositol 3-kinase; angiotensin II

ABNORMAL AND EXCESSIVE GROWTH of vascular smooth muscle cells (VSMCs) such as those in the coronary arteries of the human heart is a hallmark of vascular occlusive disorders such as atherosclerosis and restenosis after balloon angioplasty. In these disorders, VSMCs can undergo hyperplasia or hypertrophy (increases in cell number and size, respectively) in response to a variety of humoral and mechanical stimuli. The vasoconstrictive octapeptide angiotensin II (ANG II) has been implicated as a potential VSMC growth-inducing agent and acts via at least two G protein-coupled receptor subtypes (46), AT1 and AT2. Experimentally, ANG II has been demonstrated to stimulate growth of cultured VSMCs independent of its hemodynamic properties, although the type of growth response varies depending on the species and vessel of origin. For example, ANG II was shown to be mitogenic for rat renal arteriolar VSMCs (10), whereas its growth effect was limited to hypertrophy in rat aortic VSMCs (2, 13). We have previously shown that in VSMCs isolated from human coronary artery smooth muscle cells (cSMCs), ANG II does not stimulate proliferation but instead induces protein synthesis that is indicative of hypertrophy via the AT1 receptor (15). A lack of proliferative response to ANG II has also been observed in human saphenous vein VSMCs (28). Therefore, the ineffectiveness of ANG II as a mitogen appears to be a universal finding at least in human VSMC cultures.

The intracellular mechanisms that mediate the potential growth response of human VSMCs to ANG II are unclear. In cultured human saphenous vein VSMCs, ANG II was shown to only weakly and transiently activate the 42- and 44-kDa extracellular signal-regulated kinases (ERKs; Ref. 27). In contrast, growth factors such as platelet-derived growth factor (PDGF) induced prolonged activation of ERK as well as its translocation to the nucleus. This difference in ERK activation was associated with the strong mitogenic properties of growth factors and the relatively weak growth-promoting effects of ANG II on these cells. Furthermore, ANG II was also shown to induce expression of the immediate early protooncogene c-fos in human saphenous vein VSMCs (33). Therefore, these studies suggest that alternative intracellular events that involve signaling cascade(s) other than ERK may mediate a selective hypertrophic response in human VSMCs as opposed to hyperplasia.

In rat aortic VSMCs (in which ANG II induces hypertrophy), a signaling cascade that involves the 70-kDa ribosomal protein S6 kinase (p70^S6K) was implicated in mediating the activation of protein synthesis elicited by ANG II (14). This signaling pathway is involved in a number of aspects of the regulation of mRNA translation (36), one of these being the phosphorylation of the translational regulatory protein 4E-binding protein-1 (4E-BP1; Ref. 48). Nonphosphorylated 4E-BP1 binds tightly to and inhibits eukaryotic initiation factor-4E (eIF4E), which is the mRNA cap-binding protein involved in translation initiation. Phosphorylation of 4E-BP1 results in its dissociation from eIF4E and thus facilitates mRNA translation. Additional evidence for this was provided by inhibition of ANG II-induced protein synthesis by the immunosuppressive macrolide rapamycin that specifically inhibits the 289-kDa
protein kinase mammalian target of rapamycin (mTOR), which is an activator of p70^{S6k} (5). In addition, phosphatidylinositol 3-kinase (PI3-K) was implicated in the activation of p70^{S6k} (7, 54) and protein synthesis (26) and was also shown to be activated by ANG II in VSMCs (39). Therefore, it is of interest to investigate the role of the mTOR signaling pathway and its putative up- and downstream components, PI3-K and 4E-BP1, respectively, in the specific hypertrophic growth response that ANG II elicits in human coronary artery VSMCs.

In the present study, we demonstrate that in human cSMCs, ANG II regulates different targets of the mTOR signaling complex, including p70^{S6k} 4E-BP1, and eIF4E, and that it does so via PI3-K. These results provide insight into the intracellular mechanisms that underlie ANG II-induced growth of arterial cSMCs and hence its potential role in coronary artery disease.

MATERIALS AND METHODS

Materials. Human ANG II acetate, recombinant human platelet-derived growth factor-AB (PDGF-AB), DMEM, FCS, PBS, l-glutamine, penicillin-streptomycin, and TCA were purchased from Sigma Chemical (Poole, UK) and reconstituted in DMEM. Rapamycin and FK506 (gifts from Wyeth-Ayerst Research; Princeton, NJ and Fujisawa; Munich, Germany, respectively) were dissolved in ethanol and FK506 (gifts from Wyeth-Ayerst Research; Princeton, NJ and Fujisawa; Munich, Germany, respectively) were dissolved in ethanol to form stock 2 mg/ml concentrations. When used, rapamycin and FK506 were diluted 1:1,000 to achieve final concentrations, and ethanol (as vehicle) was added to controls at the same dilution. Wortmannin and LY-294002 were purchased from Sigma and Alexis Biochemicals (Nottingham, UK), respectively, and were dissolved in DMSO and diluted 1:1,000 in DMEM for experiments; DMSO was used as vehicle and was diluted to the same extent in controls. All stock solutions were stored at −20°C. Tissue culture plastics (Falcon) were from Marathon Laboratory Supplies (London, UK). The [2,3,4,5-3H]-leucine (160 Ci/mmol) was from ICN Biomedicals (Thame, UK). Liquid scintillation cocktail was from Canberra Packard (Groningen, The Netherlands). Rabbit polyclonal antibodies against 4E-BP1 (51), eIF4E (17), and p70S6k (32) were generated in house and were used previously in the studies referenced.

Isolation and culture of human cSMCs. In accordance with institutional guidelines, nonatherosclerotic epicardial coronary arteries were isolated from cardiac transplant-recipient hearts after informed consent was obtained. The cSMCs were isolated from the vessel media as previously described (15). Cells were maintained in DMEM supplemented with l-glutamine (20 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and 20% (vol/vol) heat-inactivated FCS (Sigma). Cultured human cSMCs (passages 3–9) exhibited the characteristic “hill and valley” morphology when viewed under light microscopy. Cells were also characterized by immunocytochemical staining for α-smooth muscle actin and smooth muscle myosin and were negative for platelet endothelial cell adhesion molecules as expected. A total of 16 separate human cSMC preparations were used in the experiments.

Protein synthesis measurement. Cells were seeded into 24-well plates at 4 × 10^4 cells/well and incubated overnight after which they were made quiescent by incubation for 48 h in DMEM that contained only 0.4% FCS. After this period, test agents were added in fresh DMEM with 0.4% FCS (1 ml/well) and plates were incubated for 48 h. In experiments using inhibitors, cells were preincubated with rapamycin, wortmannin, or LY-294002 for 30 min before the start of the experiment. At time 0, [3H]leucine was added to each well (final concentration, 0.5 µCi/ml), and it remained in the medium for the rest of the 2-h incubation period. At the end of the incubation period, the supernatant was removed, and ice-cold 10% TCA was added for 20 min at 4°C. The acid-insoluble material was solubilized by incubation in 0.3 mol/l NaOH with 0.1% SDS at 37°C for 2 h and was then added to 3 ml of liquid scintillant. The incorporated radioactivity (in cpm) was measured in a beta counter.

Immunoblot analysis. Human cSMCs were grown to near confluence in six-well plates and were made quiescent by incubation for 72 h in DMEM that contained 0.4% FCS. Afterward, fresh medium that contained ANG II (10^{-7} M) in the presence and absence of the inhibitors rapamycin (20 ng/ml) and wortmannin (100 nM) was incubated with the cells for ±2 h. At individual time points, cells were rinsed twice with ice-cold PBS and lysed in lysis buffer (150 µl/well) containing 0.5% Triton X-100, 500 µM PMSF, 2.5 µg/ml aprotinin, 2.5 µg/ml leupeptin, 1 mM β-glycerophosphate, and 1 mM sodium orthovanadate (Na$_{3}$VO$_{4}$; Sigma). Lysates were passed though a syringe needle 12 times before being centrifuged for 5 min at 14,000 rpm. Equal amounts of cell extracts (20 µg) were resolved by 10% SDS-PAGE and transferred onto a Hybond-C nitrocellulose membrane (Amersham; Little Chalfont, UK). The membrane was blocked for 1 h at room temperature in PBS that contained 0.05% Tween 20 and 3% milk powder and then for 2 h with rabbit polyclonal anti-p70^{S6k} at a 1:800 dilution. The membrane was washed several times with blocking solution and was incubated for an additional 1 h with a horseradish peroxidase-conjugated recombinant anti-rabbit secondary antibody (Dako; High Wycombe, UK). After the membrane was washed, the immunoreactive protein bands were detected using enhanced chemiluminescence (ECL) (Amersham; Amersham). Immunoprecipitation and immune complex p70^{S6k} activity assay. Human cSMCs were grown to near confluence in 10-cm dishes, and quiescence was achieved by 72 h of incubation in DMEM that contained 0.4% FCS. Afterward, fresh medium that contained ANG II (10^{-7} M) was added at 37°C for ±2 h. At individual time points during this period, cells were rinsed twice with ice-cold PBS and scraped into 150 µl of ice-cold kinase buffer (pH 7.4) that contained (in mM) 70 β-glycerophosphate, 1 benzamidine, 0.5 Na$_{3}$VO$_{4}$, 10 MgCl$_{2}$, 1.5 EGTA, and 20 HEPES (Sigma). Samples were passed several times though a syringe needle to aid disruption and were then centrifuged at 14,000 rpm for 5 min at 4°C. For immunoprecipitation, supernatants that contained equal amounts of protein (500 µg) were incubated for 2 h at 4°C with a rabbit polyclonal anti-p70^{S6k} antibody (1:100 dilution) that was preadsorbed to protein A-Sepharose beads. The immune complexes were washed three times with and then resuspended in 25 µl of kinase buffer to which was added 10 µl of S6 substrate peptide to yield a final concentration of 0.2 mM. To start the reaction, 5 µl of ATP mixture, which contained 20 µM cold ATP and 5 µCi [γ-32P]ATP (4,500 Ci/mmol; ICN) was added to the substrate and incubated at 30°C for 20 min. The reaction was terminated by spotting the mixtures onto phosphocellulose P-81 paper (Whatman; Maidstone, UK). The filters were washed three times with 1% phosphoric acid and were placed into separate scintillation vials that contained deionized water. The radioactivity (in cpm) was measured using a beta counter. Each sample was processed in duplicate with a third sample (in which the S6 peptide was absent) that served as a blank reaction.

Determination of association between 4E-BP1 and eIF4E. Quiescent human cSMCs were incubated with ANG II (10^{-7} M) alone or together with either rapamycin (20 ng/ml) or wortmannin (100 nM) and were preincubated for 30 min. Cells were incubated for 30 min, which is a time point when p70^{S6k} is maximally activated. Cells were then rinsed in ice-cold PBS and lysed in 1% Triton X-100 that contained 500 µM PMSF, 2.5 µg/ml aprotinin, 2.5 µg/ml leupeptin, 1 mM β-glycerophosphate, and 1 mM Na$_{3}$VO$_{4}$. Lysis was aided by rapidly freeze-thawing the cells using liquid nitrogen before passing them though a syringe needle several times; after this, the lysates were centrifuged for 5 min at 14,000 rpm. The cell extracts were incubated with m’GTP-Sepharose (Pharmacia Biotech; St. Albans, UK) for affinity purification of eIF4E as previously described (2). The purified extracts were resolved by 15% SDS-PAGE and then transferred onto membranes as before and probed simultaneously with anti-4E-BP1 and anti-eIF4E rabbit polyclonal antibodies. The ratio of 4E-BP1 to 4E-BP1.
cIif-4E was determined from the relative densities of the bands detected by ECL reaction.

Statistical analysis. Unless otherwise stated, experiments were carried out on multiple human cSMC cultures derived from different hearts. Data underwent a one-way ANOVA followed by a Bonferroni t-test for comparison between individual treatments. A P value of <0.05 was considered statistically significant.

RESULTS

Inhibition of ANG II-induced protein synthesis by rapamycin. The immunosuppressant rapamycin, which is an inhibitor of mTOR, completely suppressed the increase in human cSMC protein synthesis induced by ANG II (Fig. 1). Slight inhibition of the ANG II effect was apparent with rapamycin concentrations beginning at 0.001 ng/ml ([3H]leucine incorporation for ANG II with rapamycin, 108.2 ± 2.2%; P < 0.05 vs. ANG II only), whereas 0.01 ng/ml rapamycin caused maximal inhibition with no additional concentration-dependent inhibition occurring at higher concentrations ([3H]leucine incorporation for ANG II with rapamycin, 96.6 ± 3.4%; P < 0.05 vs. ANG II only). Rapamycin also decreased basal levels of protein synthesis over the 48-h incubation period. These results suggest that maintenance of protein synthetic rates in VSMCs requires signaling over the 48-h incubation period. These results suggest that maintenance of protein synthetic rates in VSMCs requires signaling through the rapamycin-sensitive mTOR kinase.

Reversal of rapamycin inhibitory effect by FK506. To investigate the site of action of rapamycin and its specificity, we coincubated the cells with rapamycin and various concentrations of FK506, which is a structurally related immunosuppressant that acts on the same cytosolic receptor as rapamycin, the immunophilin termed FK506-binding protein (FKBP)12. The inhibitory effect of rapamycin on protein synthesis was reversed in a concentration-dependent manner by coincubation with a molar excess of FK506. A 250-fold excess of FK506 was sufficient to achieve complete restoration of protein synthesis to the levels in ANG II-stimulated cells (Fig. 2). This therefore indicates that the inhibitory effect of rapamycin may be mediated through binding to FKBP12.

ANG II stimulates p70^S6 kinase activity and phosphorylation. The phosphotransferase activity of immunoprecipitated p70^S6k was measured by immune complex kinase assay using an S6 peptide sequence as substrate (Fig. 3A). Stimulation of human cSMCs with ANG II (10^-7 M) caused an increase in the enzymatic activity of p70^S6k. This increase was observed as early as 5 min after stimulation and reached a peak at 20 min with a sixfold increase above control, which was sustained for 1–2 h. These peak times are in accordance with the reported time course of p70^S6k activation in rat aortic VSMCs (14). The ANG II-enhanced activity at 20 min was completely suppressed by pretreatment of human cSMCs with rapamycin (20 ng/ml; Fig. 3B). The efficacy of rapamycin was also demonstrated by its ability to block completely the PDGF-induced

Fig. 1. Rapamycin inhibits ANG II-induced protein synthesis. Quiescent human coronary smooth muscle cells (cSMCs) were preincubated with rapamycin (Rapa, 20 ng/ml) or vehicle for 20 min before addition of ANG II (10^-7 M) and were then incubated for 48 h in the continued presence of the drug. Incorporation of [3H]leucine over the whole 48-h incubation period was measured. Bars represent mean ± SE of quadruplicate treatments from three independent experiments on separate human cSMC cultures. *P < 0.05 vs. control.

Fig. 2. Excess FK506 antagonizes the inhibitory capacity of rapamycin. Quiescent human cSMCs were pretreated with rapamycin (2 ng/ml) alone or together with increasing concentrations of FK506 (2–500 ng/ml) for 20 min before addition of ANG II (10^-7 M). Cells were then incubated with these agents for 48 h, after which [3H]leucine incorporation was measured. Bars represent means ± SD of quadruplicate treatments from a representative of three experiments. cpm, Counts/min. *P < 0.05 vs. ANG II alone and vs. ANG II with rapamycin and FK506 (100–500 ng/ml). **P < 0.01 vs. ANG II with rapamycin only. FK506 alone had no effects at these concentrations (data not shown).

Fig. 3. Activation of 70-kDa S6 kinase (p70^S6k) by ANG II in human cSMCs and its inhibition by rapamycin. A: time course of activation of p70^S6k by ANG II. ANG II (10^-7 M) was incubated with quiescent cells for ≤120 min. Lysates were subjected to immunoprecipitation and subsequent immune complex kinase activity assay (see MATERIALS AND METHODS). Points are determined from duplicate samples corrected by subtraction of a third sample that served as control from which the substrate was absent. Results are representative of two experiments from separate cultures. B: complete inhibition of ANG II-stimulated p70^S6k enzyme activity at 20 min by rapamycin (20 ng/ml).
activation of p70S6k [PDGF-treated activity, 6.3, reduced to 0.3 (measured in arbitrary units of activity relative to control) in the presence of rapamycin; data not shown]. As with protein synthesis, rapamycin treatment alone also caused slight inhibition of the basal level of p70S6k activity, which indicates that rapamycin acts by either attenuating basal phosphorylation of the kinase or by bringing about its dephosphorylation.

The activation of p70S6k involves its phosphorylation on multiple serine and threonine residues (1). This multiple phosphorylation results in reduced mobility of the protein on SDS-PAGE. Therefore, we used Western blotting as another method of examining the activation state of p70S6k. As judged in this way, ANG II (10^{-7} M) over a period of 2 h caused an increase in the phosphorylation state of p70S6k by 20 min, the time at which activation was maximal (Fig. 4, cf. Fig. 3). The appearance of the upper hyperphosphorylated band was abolished when cells were pretreated with rapamycin (20 ng/ml) or the PI3-K inhibitor wortmannin (100 nM) for 30 min before addition of ANG II. Furthermore, the strong VSMC growth factor PDGF, which is expected to markedly activate all growth-related signaling pathways, caused clear phosphorylation of p70S6k that coincided with complete disappearance of the lower band. These results therefore identify p70S6k as a target for activation by ANG II and inhibition by rapamycin. Furthermore, they suggest that the activation of p70S6k by ANG II is mediated by PI3-K.

Dissociation of 4E-BP1 from eIF4E in response to ANG II. Stimulation of human cSMCs with ANG II caused dissociation of 4E-BP1 from eIF4E (Fig. 5). ANG II stimulation (10^{-7} M) for 30 min resulted in a decrease in the amount of 4E-BP1 protein bound to affinity-purified eIF4E. Relative amounts of eIF4E and 4E-BP1 were assessed by densitometry of the Western blot (Fig. 5). This effect was completely prevented by either rapamycin or wortmannin, which implicates p70S6k and PI3-K, respectively, in the regulation of eIF4E in response to ANG II. Although wortmannin was previously reported to inhibit the kinase activity of mTOR in vitro (6), our earlier data indicate that it does not do so within cells even at concentrations much higher than those used here (50). Effects of wortmannin thus reflect its ability to inhibit PI3-K rather than mTOR, and the present data thus strongly suggest that PI3-K is required for signaling from the ANG II receptor to 4E-BP1.

Inhibitors of PI3-K block ANG II-induced protein synthesis. Two pharmacological inhibitors of PI3-K, the fungal metabolite wortmannin and LY-294002, were used to assess the role of PI3-K in the hypertrophic response of human cSMCs to ANG II (Fig. 6). Pretreatment of cells with wortmannin (50 nM) or LY-294002 (10 μM) at concentrations reported to be nontoxic to VSMCs over 48 h (39) abolished the ANG II-induced increase in [3H]leucine incorporation, which suggests that the activation of protein synthesis in response to ANG II requires signaling through PI3-K.

DISCUSSION

Although ANG II is well recognized in humans as a potent vasoconstrictor, its role in more prolonged processes such as VSMC growth, including the relevant signaling mechanisms involved, has remained obscure. ANG II has emerged as a prototypical molecule in studies aimed at elucidating signaling mechanisms that act downstream of seven-transmembrane G protein-coupled receptors as opposed to classical growth factors that act via tyrosine kinase receptors (16). Given the variability in the effects of ANG II as a growth factor in different species, cell types, and models, it is of particular interest to investigate the growth effects of ANG II on VSMCs derived from human coronary arteries. We previously reported (15) that ANG II is not a mitogen for human cSMCs but does stimulate protein synthesis indicative of hypertrophy in these cells via the AT1 receptor. In the present study, we have attempted to elucidate the intracellular signaling mechanisms behind the observed activation of protein synthesis. We report here that the mTOR signal transduction pathway, which includes the ribosomal p70S6k, is activated by ANG II and plays a key role in modulating the rate of protein synthesis in...
VSMCs in response to ANG II. This cascade begins with ANG II activation of the AT₁ receptor, which is a pertussis toxin-insensitive G protein-coupled receptor (15). Receptor activation is coupled to activation of PI3-K, and the present data show this subsequently leads to activation of mTOR signaling and, further downstream, of eIF4E and p70S6k.

MAPK or ERK, which has also been detected in the nuclear compartment (8, 38), is believed to figure highly as a component in mitogenic signal transduction cascades. In particular, it is sustained ERK activation over a prolonged period that has been shown as essential for cell cycle progression and mitogenicity induced by proliferative agents (25). ANG II, in contrast, induces only transient ERK activation in human VSMCs (27) and thereby highlights a distinction between a G protein-coupled receptor agonist and growth factors that act at tyrosine kinase receptors. It is interesting to note that two other hypertrophic agents that act through G protein-coupled receptors, phenylephrine and endothelin-1, also elicit only transient ERK activation (49).

In the present study, we have demonstrated activation by ANG II of an additional signal transduction pathway in human cSMCs that involves phosphorylation and concomitant activation of p70S6k. The immunosuppressive macrolide rapamycin completely blocked both ANG II-induced activation and phosphorylation of p70S6k. It reduced both basal and ANG II-stimulated rates of protein synthesis in VSMCs. ANG II also promoted dissociation of the regulatory protein 4E-BP1 from eIF4E, a component of the translation initiation complex. In unstimulated conditions, 4E-BP1 binds tightly to eIF4E and thus renders it inactive. Dissociation of 4E-BP1 by ANG II through phosphorylation liberates eIF4E and thereby allows it to bind eIF4G and form functional initiation complexes (21). Furthermore, both rapamycin and wortmannin completely inhibited dissociation of the complex in response to ANG II, additionally implicating mTOR and PI3-K in this signaling process. The time courses for p70S6k phosphorylation and 4E-BP1 dissociation match closely those of p70S6k activation, all of which constitute an acute response that subsequently drives the mechanisms for the longer-term cellular response of protein synthesis.

The inhibitory effect of rapamycin on ANG II-induced protein synthesis was completely reversed by a molar excess of FK506. This observation of reciprocal antagonism strengthens the hypothesis that FKBP proteins, which form complexes with FK506, are also the intracellular molecules to which rapamycin binds. The picolic acid portions of the rapamycin and FK506 molecules are structurally identical, and it is through this common structure that both drugs form complexes with FKBP proteins (30). However, the additional ability of rapamycin to interact extensively with hydrophobic pockets in the mTOR structure may explain the clearly divergent signaling effects observed with the two immunosuppressive agents (9). Nevertheless, other possibilities for the restorative effect in this experiment should also be considered such as antagonism at a site other than FKBP12 or hitherto unidentified interference of FK506 with rapamycin signaling.

Activation of PI3-K by ANG II was previously demonstrated in porcine VSMCs (39) with the synthetic compound LY-294002 being shown as effective in inhibiting ANG II-induced VSMC growth. However, ANG II was found to cause proliferation of porcine VSMCs in that study. Therefore, it was not clear whether the PI3-K activation was necessary for the proliferative effect or whether it also served to mediate the hypertrophic response seen in human cSMCs. Moreover, the inhibitory influence of LY-294002 at concentrations previously thought to be specific to PI3-K activity appears also to extend to mTOR activity (6). In the present study, we studied the role of PI3-K in human cSMC protein synthesis by also using the fungal metabolite wortmannin as a pharmacological inhibitor of PI3-K in addition to LY-294002. We observed in cultured adult rat cardiac myocytes that LY-294002 blocks p70S6k activation, whereas wortmannin fails to do so even at concentrations higher than those required for PI3-K inhibition (50), which indicates that wortmannin is a more useful reagent than LY-294002 for studying the role of PI3-K-signaling in the control of targets of the mTOR pathway. In human cSMCs, ANG II-induced protein synthesis, p70S6k phosphorylation, and 4E-BP1-eIF4E dissociation were completely inhibited by both agents, which suggests that PI3-K activation is indeed involved in the ANG II-induced hypertrophic signal transduction pathway and strengthens the hypothesis that PI3-K lies upstream of p70S6k and 4E-BP1 in signaling from the ANG II receptor (54).

Further downstream, the serine-threonine kinase Akt, which is also known as protein kinase B (PKB), has been identified as a target of PI3-K (4). Recent studies have also provided a mechanism linking PKB/Akt to mTOR activation through the phosphorylation of tuberous sclerosis complex-2 (TSC2, also known as tuberin), which is a member of the tuberous sclerosis complex that also comprises TSC1 (also known as hamartin; Ref. 24). Together with TSC1, TSC2 acts to repress mTOR function by acting at the GTPase-activator protein for the small G protein Rheb (22). Phosphorylation of TSC2 by PKB/Akt alleviates its inhibitory action on Rheb. Overexpression studies in Drosophila melanogaster and in cells have shown that active Rheb controls mTOR and positively regulates cell size (34, 43). Thus in VSMCs, TSC1, TSC2, and Rheb could be the links between ANG II-induced PI3-K activation (causing Akt activation) and downstream p70S6k activation through mTOR.

More significantly, in the context of hypertrophy, in vivo studies have strengthened the placing of mTOR as downstream of PI3-K and Akt and have implicated them in control of cell size (35, 43). The importance of p70S6k function in cell growth in mice was demonstrated by disruption of the p70S6k gene, which resulted in smaller-sized animals (42). Furthermore, disruption of the S6 kinase gene in D. melanogaster was observed to cause severe delay in development and reduction in body size (29). Moreover, these flies had smaller rather than fewer cells. Therefore, mTOR signaling appears to play a key role in cell growth control with particular bearing on cell size rather than cell number. Thus we envisage activation of this pathway in ANG II-exposed VSMCs to lead to increased cell size but not subsequent cell division as would occur with mitogenic stimulation. In accordance with this hypothesis, mTOR-dependent mechanisms appear to account for ANG II-induced cardiac myocyte hypertrophy via the AT₁ receptor in transgenic mice (55). Furthermore, involvement of the mTOR pathway in pathological states was demonstrated in vivo models of pressure overload-induced cardiac hypertrophy (44) and skeletal muscle hypertrophy (3).

Interestingly, although rapamycin decreased rates of protein synthesis in ANG II-treated cells, it also reduced these rates in
unstimulated cells, and ANG II was still able to activate protein synthesis in the presence of rapamycin. This inhibition of basal protein synthesis likely reflects the importance of p70S6K in promoting ribosome biogenesis (18) and thus maintaining or upregulating cellular capacity for protein synthesis. The ability of ANG II to activate protein synthesis even in the presence of rapamycin indicates that other pathways or rapamycin-insensitive outputs from mTOR are also involved in the stimulation of protein synthesis by ANG II. Such rapamycin-insensitive outputs have been hinted at before. For example, (1) the phosphorylation of certain sites in 4E-BP1 by mTOR in vitro is rapamycin insensitive (23), and (2) the integrity of the yeast TORC2 complex is also insensitive to this drug (20). In contrast, in the present study, both LY-294002 and wortmannin clearly block the ability of ANG II to activate protein synthesis. Because wortmannin (at the concentration used) does not appear to interfere with mTOR signaling per se (50), these effects are likely to reflect a role for PI3-K in the activation of protein synthesis through a mechanism that is insensitive to rapamycin. One example of such a mechanism is the activation of the guanine nucleotide-exchange factor, eIF2B. This is required for the recycling of eIF2, which is needed for all translation-initiation events and controls the overall translation rate. The activity of eIF2B is regulated in a PI3-K-dependent but rapamycin-insensitive manner through its phosphorylation by glycogen synthase kinase-3, an effector for PKB/Akt (37, 52, 53). Activation of p70S6K may primarily be important in regulating the translational capacity of the cell so that long-term treatment with rapamycin decreases the overall rate of protein synthesis.

The present findings in human cSMCs are of special significance given the variability in growth response to ANG II observed in studies utilizing cells from different species and vascular beds. Our results may help to explain the apparent ineffectiveness of angiotensin inhibition at reducing coronary restenosis rates in humans (11) and in other higher animals such as pigs (19), which is in contrast to findings in rodents (47). However, our observation of activation of the mTOR pathway in human cSMCs sheds new light on the intracellular mechanisms that underlie ANG II-induced hypertrophy in these cells. Recent work has also brought to light other novel intracellular effects of ANG II in VSMCs that may also apply to the mechanisms involved in the hypertrophic effect in human cSMCs. These include ANG II-induced activation of the nonreceptor tyrosine kinase c-Src, generation of reactive oxygen species, and ensuing transactivation of the receptors for the growth factors EGF (41) and IGF-I (56), which were linked to PI3-K and mTOR activation.

In conclusion, we have shown that ANG II activates components of the rapamycin-sensitive mTOR signal transduction pathway in cultured human cSMCs. Additional elucidation of the molecular mechanisms responsible for the growth-promoting effects of ANG II on human VSMCs may help to identify new targets for therapy in the future. Indeed, rapamycin (sirolimus) has shown promise in clinical trials as a therapeutic tool to inhibit vascular occlusion. For example, implantation of sirolimus-eluting stents as part of coronary revascularization procedures were observed to significantly reduce the risk of vessel restenosis (31, 40). It is therefore indicated that intervention in the mTOR signaling pathway may effectively control ANG II-stimulated VSMC-growth processes that occur in diseases such as hypertension and atherosclerosis.

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REFERENCES


