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Transactivation of the Insulin-Like Growth Factor-I Receptor by Angiotensin II Mediates Downstream Signaling from the Angiotensin II Type 1 Receptor to Phosphatidylinositol 3-Kinase

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Angiotensin II (AngII) activates phosphatidylinositol 3-kinase (PI3-kinase), a known effector of receptor tyrosine kinases. Treatment of smooth muscle cells with AngII has also been shown to promote phosphorylation of various tyrosine kinase receptors. We therefore investigated the relationship between AngII and IGF-I receptor activation in smooth muscle cells with a phosphorylation-specific antibody. Our experiments showed that IGF-I receptor phosphorylation was maximally stimulated within 10 min by AngII. Inclusion of an IGF-I-neutralizing antibody in the culture media did not prevent IGF-I receptor phosphorylation after AngII treatment, which argues that a paracrine/autocrine loop is not required. Furthermore, this process was blocked by losartan and 1-(1,1-dimethylethyl)-1-(4-methylphenyl)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine (PP-1), indicating stimulation of IGF-I receptor phosphorylation occurs via AngII type 1 receptor-dependent activation of Src kinase. The functional significance of IGF-I receptor transactivation was examined with selective inhibitors of the IGF-I receptor kinase (AG1024, AG538). When AngII-treated cells were incubated with AG1024 or AG538, phosphorylation of the regulatory p85 subunit of PI3-kinase was blocked. Furthermore, phosphorylation of the downstream factor p70^{S6K} did not occur. In contrast, AG1024 did not prevent MAPK or Src kinase activation by AngII. AG1024 also did not inhibit AngII-dependent cell migration, although this process was blocked by inhibitors of the epidermal growth factor and platelet-derived growth factor receptors. Transactivation of the IGF-I receptor is therefore a critical mediator of PI3-kinase activation by AngII but is not required for stimulation of the MAPK cascade. (*Endocrinology* 145: 2978–2987, 2004)

ANGIOTENSIN II (ANGII) is a peptide hormone that promotes the growth and proliferation of vascular smooth muscle cells (SMCs). Although AngII associates with high affinity to two distinct G protein-coupled receptors, angiotensin II types 1 and 2 (AT_1 and AT_2), the mitogenic actions of AngII are primarily exerted through the AT_1 receptor (1). Coupling of the AT_1 receptor to both Gi and Gq indicates why decreased cAMP levels and hydrolysis of phosphoinositides by phospholipase C are important routes by which stimulation of cell proliferation is accomplished, as indicated by the need for Ca^{2+} mobilization and protein kinase C activation to induce c-*fos* gene expression (2) Nevertheless, evidence of increased protein tyrosine phosphorylation after AngII stimulation (3, 4) suggests tyrosine kinase activation is also essential for SMC proliferation (5).

Activation of Src kinase by AngII (6, 7) has been implicated in the tyrosine phosphorylation of FAK and Pyk2, key players in the assembly of focal adhesions. Whereas concurrent modification of paxillin, tensin, and p130^{Cas} (8–10) produces changes in cytoskeletal organization typically associated with cell shape, modification of Pyk2 has also been reported to activate ERK1/2, c-Jun N-terminal kinase, and p70^{S6K} (9, 11, 12), critical mediators of cell proliferation. Likewise, activation of the Janus kinase family of tyrosine kinases in response to AngII treatment, and subsequent phosphorylation of signal transducer and activator of transcription factors, is required for induction of c-*fos* and c-*jun* gene expression. More recently several studies have suggested that tyrosine kinase receptors function as intermediates for G protein-coupled receptor signaling. For example, Voisin *et al.* (13) have reported activation of the epidermal growth factor (EGF) receptor by AngII is necessary for increasing the rate of protein synthesis. Whereas similar roles have also been suggested for both platelet-derived growth factor (PDGF) and IGF-I receptors (14–17), detailed functional and mechanistic studies examining the role of these receptors in AngII-dependent processes have yet to be reported.

Our laboratory was the first to demonstrate that activation of phosphatidylinositol 3-kinase (PI3-kinase) occurred in SMCs treated with AngII and that PI3-kinase was required for cell proliferation (18). In that study, we demonstrated that formation of phosphatidylinositol-3,4,5-P_3 was correlated with tyrosine phosphorylation of the p85 subunit and its translocation to the nuclear periphery. Furthermore, immu-

Abbreviations: AngII, Angiotensin II; AT_1 and AT_2, angiotensin II types 1 and 2 receptors; EGF, epidermal growth factor; IRS, insulin receptor substrate; PDGF, platelet-derived growth factor; PDK1, phosphatidylinositide-dependent protein kinase; PI3-kinase, phosphatidylinositol 3-kinase; PP-1, 1-(1,1-dimethylethyl)-1-(4-methylphenyl)-1*H*-pyrazolo[3,4-d]pyrimidin-4-amine; RIPA, radioimmunoprecipitation assay; SMC, smooth muscle cell.

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noprecipitation conclusively showed that the p110α isoform typically associated with tyrosine kinase receptors was responsible for catalyzing formation of phosphatidylinositol-3,4,5-P_3. Although these observations implicated tyrosine kinase receptors in the activation of PI3-kinase by AngII, a mechanism for linking these distinct events was not apparent.

PI3-kinase has been shown to interact with phosphorylated Src homology 2-domain proteins such as Grb2 and insulin receptor substrate (IRS-1) (19). Because treatment with AngII results in tyrosine phosphorylation of both IRS-1 and Shc (17, 20, 21), the insulin or IGF-I receptor may be implicated in downstream signaling from the AT_1 receptor. Furthermore, these observations suggest a mechanism by which PI3-kinase activation can be achieved by G protein-coupled receptors. We therefore examined the possibility that the IGF-I receptor has a role in AngII-dependent signal transduction. A direct link between the IGF-I receptor and AngII-dependent PI3-kinase activation was identified. Furthermore, our data show that IGF-I receptor transactivation is essential for the activation of specific signal transduction pathways by AngII.

Materials and Methods

Cell culture

Primary cultures of SMCs were generated by an explant organ culture method from the left anterior descending coronary artery of porcine hearts obtained from Maple Leaf Meats, Inc. (Winnipeg, Canada) as previously described (22). To maintain consistency between cultures, only second-passage cells with confirmed expression of smooth muscle markers were used for all experiments. Quiescent SMC populations were prepared by placing the cells in supplemented serum-free DMEM for 5 d (22). Cells were pretreated for 10 min with inhibitors before stimulation with AngII, IGF-I, or EGF (all prepared in water). Appropriate vehicle controls were included when materials added to the cultures were dissolved in dimethylsulfoxide (maximum 0.1% vol/vol), including AG1024 (Calbiochem, La Jolla, CA), AG538 (Calbiochem), AG1295 (BioMol, Plymouth Meeting, PA), and AG1478 (BioMol).

Western blot analysis

Western blotting of cellular proteins (10 μg) separated by SDS-PAGE in a 7.5% gel and transferred to polyvinyl difluoride membrane (Roche, Laval, Quebec, Canada) was conducted as previously described (23). Antibodies employed during the course of this investigation were obtained from Cell Signaling [Beverly, MA; p44/p42 MAPK, phospho-p44/42 MAPK (thr202/tyr204), $p70^{S6K}$, phospho-$p70^{S6K}$ (thr389), phospho-EGF receptor (tyr1068), phospho-insulin (tyr1146)/IGF-I (tyr1131) receptor, PY100], Upstate Biotechnology (Lake Placid, NY; PI3-kinase p85 subunit), Biosource (Camarillo, CA; phosphor-Src, tyr215), or Santa Cruz Biotechnology (Santa Cruz, CA; IGF-I receptor β-subunit, insulin receptor β-subunit).

Immunoprecipitation

Cell lysates prepared from 100-mm culture dishes by addition of either 1.0 ml lysis buffer [1% Nonidet P-40, 20 mM Tris-HCl (pH 7.5), 10% glycerol, 137 mM NaCl, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.4 mM orthovanadate, 1 mM NaF] or radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl (pH 8.0), 1 mM EGTA, and 1 mM EDTA] were cleared by centrifugation (10 min, 12,000 × *g* at 4 C). Aliquots of 100 μg protein were then immunoprecipitated with 4 μg antibody as previously described (18) and analyzed by Western blotting.

Cell migration

Proliferating SMCs (10^4 cells/well) were incubated for 48 h in a Boyden chamber, with chemoattractant (1 μM AngII) added to the lower compartment and inhibitors added to the upper compartment. Cells migrating to the underside of the membrane separating these compartments were stained with Giemsa and counted as described previously (24).

Data analysis

Quantification of data obtained on film or autoradiographs was accomplished with a model GS800 imaging densitometer (Bio-Rad Laboratories, Hercules, CA) under nonsaturating conditions. Background subtraction was achieved by reading the absorbance of an equal-sized region directly adjacent (above, below, or beside) to the band. Although multiple exposures were acquired to ensure the absence of film saturation, the experimental figures typically show longer exposures selected specifically for visual presentation and not used for data analysis. Data were quantified and graphically represented as means ± SEM. Most experiments employed a sample size of at least three, with n = 6/treatment group for migration experiments. All experiments were replicated at least three times, with each replicate employing independent cell or vessel isolations. Treatment means were compared using ANOVA, whereas all other data were analyzed using the unpaired Student's *t* test. Significance was set to $P < 0.05$ in all cases.

Results

AngII stimulates IGF-I receptor phosphorylation

Quiescent SMCs were treated with 1 μM AngII and harvested at various time points over 60 min. Cell extracts were subsequently analyzed by Western blotting for IGF-I receptor modification. The phospho-specific antibody employed recognizes phosphorylation of the β-subunit at Tyr1131. Although a band at 95 kDa was detected in unstimulated cells, presumably due to the presence of insulin in the serum-free culture medium, the intensity of this band increased considerably after stimulation (Fig. 1A). Maximum band intensity (2.2-fold above control) was reached within 10 min and was sustained over a period of 120 min (Fig. 1B). Phosphorylation of this 95-kDa band was also observed after treatment with 0.1 μM IGF-I (data not shown), indicating that this band likely represents the IGF-I receptor.

Because the antibody used to monitor IGF-I receptor phosphorylation cross-reacts with the insulin receptor, an immunoprecipitation approach was employed to verify the identity of the 95-kDa phosphorylated band. Quiescent cells were treated with 1 μM AngII for 10 min, lysed in RIPA buffer, and the samples were subsequently immunoprecipitated with antibody recognizing the phosphorylated IGF-I receptor. The protein present in the immunoprecipitate was visualized by Western blotting with antibody to the IGF-I receptor β-subunit (Fig. 1C). An increase in immunoprecipitated receptor protein from the AngII sample relative to untreated control was observed. In contrast, a much less intense band was detected with antibody specific for the insulin receptor β-subunit (Fig. 1C), which may be attributable to a low level of expression in SMCs (25, 26). As well, there was no obvious difference in band intensity between the treated and untreated samples. These data indicate that AngII activates the IGF-I receptor rather than the insulin receptor.

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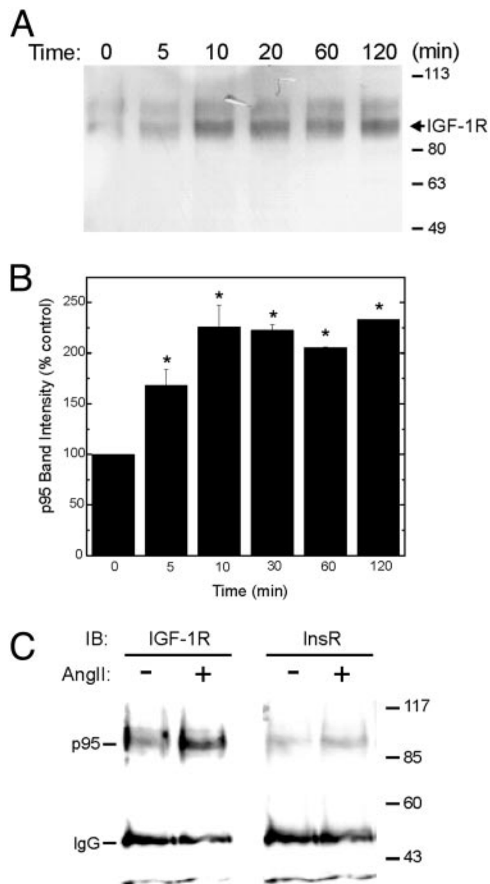

FIG. 1. AngII stimulates tyrosine phosphorylation of the IGF-I receptor. A, Quiescent SMCs were stimulated with AngII (1 μM) and the cells lysed at the indicated time points. IGF-I receptor phosphorylation was monitored by Western blotting with phosphorylation-specific antibody (dilution 1:1000). B, Intensities of phosphorylated IGF-I receptor bands from three independent experiments were quantified by scanning densitometry and normalized to the intensity of the band in the sample at 0 min for each experiment. Values were subsequently averaged and plotted as means ± SEM. Statistically significant ($P < 0.05$) differences from the band intensity at 0 min are shown (*). C, SMCs treated with and without 1 μM AngII for 10 min were lysed with RIPA buffer and phosphorylated IGF-I and insulin receptors precipitated with phospho-specific antibody. Two immunoprecipitations were performed for each sample, and the recovered protein was blotted onto the same membrane. The membrane was subsequently divided, with one portion probed with antibody to the unphosphorylated IGF-I receptor (1:1000 dilution) and the other section probed with antibody to the unphosphorylated insulin receptor (1:1000 dilution). The blots were subsequently stripped and probed with the other antibody. The results from one experiment are shown. Two independent experiments were conducted to verify the results.

IGF-I receptor phosphorylation is AT_1 receptor and Src kinase dependent

Receptor-driven processes demonstrate both concentration dependence and saturation kinetics. To establish that AngII-dependent phosphorylation of the IGF-I receptor is receptor mediated, the effect of AngII concentration was examined. Quiescent SMCs were treated with varying amounts of AngII (10^{-9} to 10^{-5} M) for 10 min, and cell extracts were subsequently examined by Western blotting. Increased phosphorylation of the 95-kDa β-subunit was observed with increasing AngII (Fig. 2A, *inset*). Quantitative analysis of the data by scanning densitometry indicated maximal phosphorylation was reached with 1 μM AngII (Fig. 2A). These results support a role for the AngII receptor in IGF-I receptor transactivation.

AngII operates via two distinct receptors that can be distinguished by selective receptor antagonists. Quiescent SMCs were pretreated with either losartan (AT_1 receptor antagonist) or PD123319 (AT_2 receptor antagonist) for 15 min before addition of AngII (1 μM). Stimulation with AngII increased IGF-I receptor phosphorylation after 10 min relative to untreated control (Fig. 2B). The intensity of this band was reduced in the presence of 10 μM losartan but not 10 μM PD123319 (Fig. 2B). Although losartan at this concentration did not reduce band intensity to basal levels, these results implicate the AT_1 receptor rather than the AT_2 receptor as the route by which transactivation of the IGF-I receptor occurs. Additionally, the signaling intermediates coupling these receptors were examined with inhibitors selective for PI3-kinase, protein kinase C, and Src kinase (27–29). It was observed that only 1-(1,1-dimethylethyl)-1-(4-methylphenyl)-*1H*-pyrazolo[3,4-d]pyrimidin-4-amine (PP-1), a Src kinase inhibitor, prevented IGF-I receptor phosphorylation in the presence of AngII (Fig. 2B). These data agree with other studies that have indicated Src kinase has a pivotal role in the transactivation of receptor tyrosine kinases (*e.g.* EGF receptor) by G protein-coupled receptor (*e.g.* AngII receptor) (30, 31).

Neutralizing antibody treatment does not prevent IGF-I receptor phosphorylation

Paracrine/autocrine stimulation by IGF-I and IGF-I-independent transactivation represent the two mechanisms by which AngII treatment could induce phosphorylation of the IGF-I receptor (32). To distinguish between these possibilities, we employed a neutralizing antibody to IGF-I derived from clone Sm1.2 (Upstate Biotechnology) that has previously been shown to inhibit IGF-I receptor activation (33). It was assumed that the antibody would interfere with the actions of IGF-I if it is secreted in response to AngII treatment. As seen previously, AngII stimulated phosphorylation of a 95-kDa band (Fig. 2C). Pretreatment of the cells with Sm1.2 antibody, however, did not reduce the intensity of this band after AngII treatment (Fig. 2C). The effectiveness of the antibody was confirmed by adding it to cells before treatment with IGF-I, and under these conditions phosphorylation of the 95-kDa band was reduced to near basal levels (Fig. 2C). This visual evidence was confirmed by plotting the aggregate data obtained from three independent experiments (Fig. 2D). Similar results (Fig. 2E) were obtained with a second neutralizing antibody (Ab-1, clone αIR-3, Oncogene Research Products, San Diego, CA), which blocks binding of IGF-I to the receptor (34).

The IGF-I receptor tyrosine kinase can be selectively inhibited

Although inhibitors of tyrosine kinases are commercially available, few of these compounds have shown selectivity for either the insulin or IGF-I receptors. We therefore tested several compounds that have been reported to inhibit the

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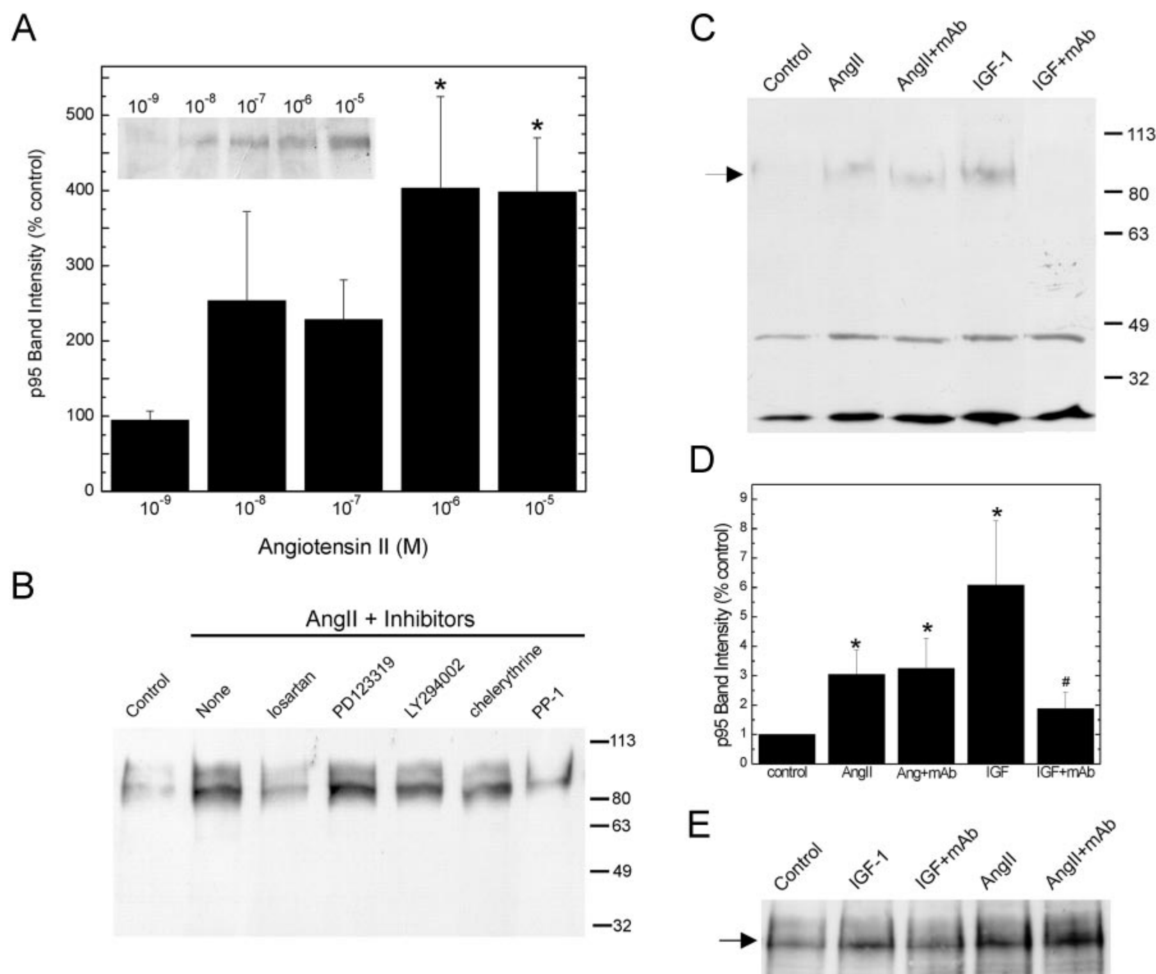

FIG. 2. Characteristics of IGF-I receptor phosphorylation after AngII stimulation of quiescent SMCs. A, SMCs were stimulated for 10 min with varying concentrations of AngII. IGF-I receptor phosphorylation in the lysates was assessed by Western blot analysis with both PY100 and phospho-specific antibody. The data for PY100 are shown in the *inset*. The band intensities were quantified by scanning densitometry and means ± SEM (derived from three independent experiments as described for Fig. 1) were plotted. Statistically significant differences from unstimulated control ($P < 0.05$) are shown (*). B, Quiescent SMCs were pretreated for 15 min with inhibitors before stimulation with 1 μM AngII for 10 min. IGF-I receptor phosphorylation was monitored by Western blotting as previously described for Fig. 1. All inhibitors were used at a concentration of 10 μM. C–E, Neutralizing antibody (30 μg/ml Sm1.2 or 1 μg/ml Ab-1) was added to the culture media 15 min before stimulants (1 μM AngII, 0.1 μM IGF-I). Cells were lysed 10 min after addition of stimulants and IGF-I receptor phosphorylation was monitored by Western blotting. The intensities of the phosphorylated IGF-I receptor bands obtained with Sm1.2 (C) were quantified by scanning densitometry and normalized to the intensity of the band in the unstimulated control sample. Data from three independent experiments were used and are presented as means ± SEM (D). Statistically significant ($P < 0.05$) intensity differences relative to unstimulated control sample are shown (*). Significant decreases resulting from antibody treatment relative to the respective stimulus are also indicated (#). E, Results obtained with antibody Ab-1 in one of two replicate experiments are illustrated.

IGF-I receptor kinase and compared their actions with those of other receptor tyrosine kinase inhibitors. The panel of compounds included a PDGF receptor inhibitor (AG1295), an EGF receptor inhibitor (AG1478), and two putative IGF-I receptor inhibitors (AG538, AG1024) (35–38). Quiescent SMCs were stimulated with IGF-I (0.1 μM), which resulted in an increase in IGF-I receptor phosphorylation relative to control (Fig. 3A). Addition of EGF and PDGF receptor kinase inhibitors before IGF-I receptor treatment had no effect on IGF-I receptor phosphorylation. In contrast, both IGF-I receptor kinase inhibitors decreased phosphorylation to near control levels. Because AG1024 and AG538 effectively inhibited IGF-I receptor activation, we tested their ability to block receptor phosphorylation after stimulation with AngII. Quiescent cells were pretreated for 15 min with varying concentrations of AG1024 or AG538 (1 nM to 10 μM) and then exposed to 1 μM AngII. Western blot analysis of samples prepared 10 min after AngII addition confirmed that the IGF-I receptor was phosphorylated (Fig. 3B). AG1024 inhibited IGF-I receptor transactivation with an EC_{50} of 0.23 ± 0.07 μM, exhibiting the greatest potency at 1–10 μM. Similar results were obtained with AG538 (data not shown). Because 10 μM was slightly toxic to the cells over the maximum 30-min exposure period, a concentration of 5 μM was used for all subsequent experiments. The putative insulin/IGF-I receptor inhibitor hydroxy-2-naphthalenylmethylphosphonic acid Tris-acetoxymethyl ester (39) was also tested in these experiments. This compound consistently killed the cells when concentrations capable of preventing receptor phosphorylation (>100 μM) were employed, even with only a 15-min preincu-

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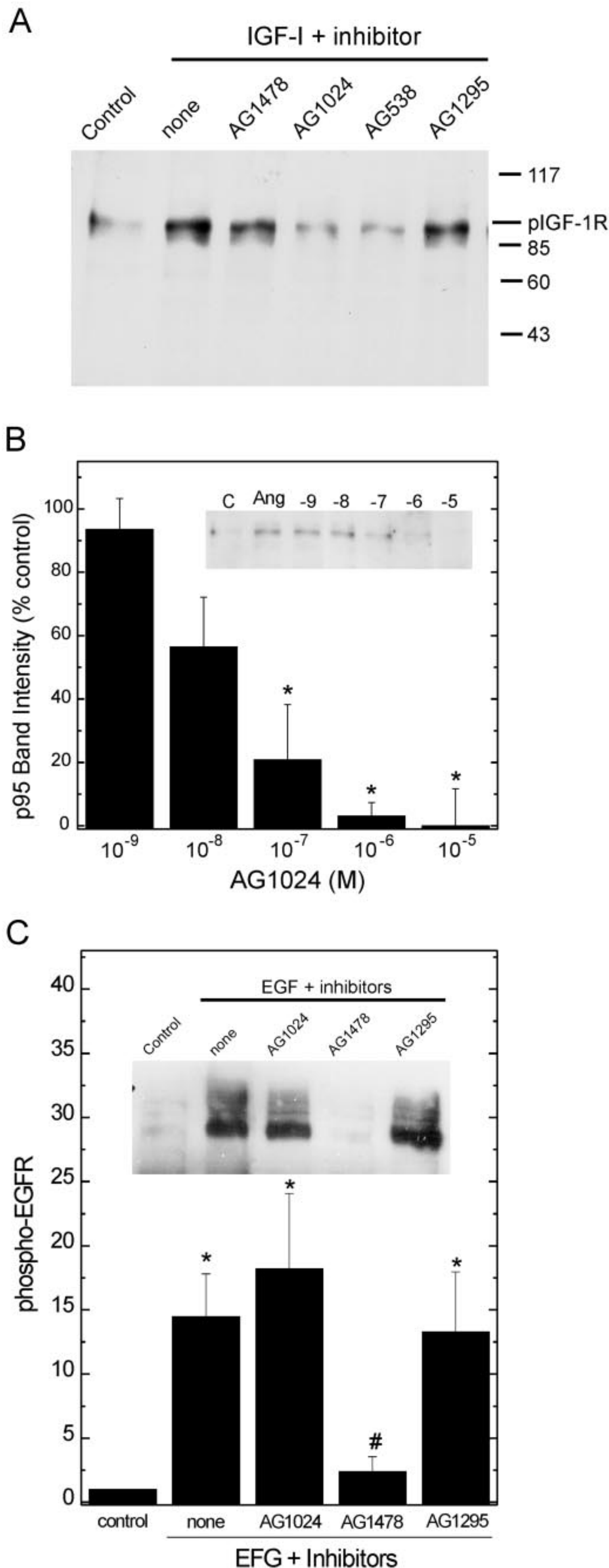

Fig. 3. AG1024 and AG538 are specific inhibitors of the IGF-I receptor kinase and block activation by AngII. Quiescent SMCs were pretreated for 15 min with various receptor tyrosine kinase inhibitors as indicated, and each inhibitor was used at a concentration of 5 μM. A, Phosphorylation of the IGF-I receptor was monitored in samples stimulated with 0.1 μM IGF-I Cells were lysed for Western blot analysis at 10 min post treatment. Phosphorylation-specific antibodies were diluted 1:1000 before use. B, Quiescent SMCs were stimulated with 1 μM AngII for 10 min after 15 min pretreatment with varying concentrations of AG1024. IGF-I receptor phosphorylation was monitored by Western blot analysis. A representative blot is shown in the *inset*. Band intensity was quantified by scanning densitometry and means ± SEM were plotted. Statistically significant differences ($P < 0.05$) from AngII-stimulated control in the absence of inhibitor (*) are indicated. C, Quiescent SMCs were stimulated with 1 μg/ml EGF for 10 min after 15 min pretreatment with 5 μM tyrosine kinase inhibitor. EGF receptor phosphorylation was monitored by Western blot analysis. A representative blot is shown in the *inset*. Band intensity was quantified by scanning densitometry, and means ± SEM were plotted. Statistically significant differences ($P < 0.05$) from unstimulated control (*) and EGF-stimulated (#) samples are shown.

bation (data not shown). Hydroxy-2-naphthalenylmethylphosphonic acid Tris-acetoxymethyl ester was therefore not used.

To verify that the IGF-I receptor kinase inhibitors did not affect other receptor tyrosine kinases, SMCs were treated with EGF (1 μg/ml), and EGF receptor phosphorylation was monitored in the presence of these inhibitors. Under these conditions, only the selective EGF receptor kinase inhibitor, AG1478, blocked phosphorylation of the EGF receptor (Fig. 3C). Neither AG1024 nor AG538 influenced this process, which suggests these compounds are specific for the IGF-I receptor kinase.

IGF-I receptor kinase inhibitors prevent PI3-kinase activation

AT_1 receptor stimulation by AngII increases PI3-kinase activity in SMCs (18). Concurrent with this change in activity, the p85 regulatory subunit undergoes tyrosine phosphorylation (18). To determine whether p85 phosphorylation was IGF-I receptor dependent, quiescent SMCs were stimulated with AngII in the presence and absence of AG1024. Cells were lysed after 10 min, and p85 was immunoprecipitated. Western blotting with PY100, an antibody selective for phosphorylated tyrosine residues, was then used to compare levels of p85 modification. This experiment revealed that p85 was phosphorylated in response to 1 μM AngII treatment (Fig. 4A). In the presence of either AG1024 or AG538, however, p85 phosphorylation was reduced to basal levels (Fig. 4A). These data suggest that activation of PI3-kinase requires transactivation of the IGF-I receptor.

PI3-kinase mediates a variety of distinct intracellular signaling pathways, including those leading to protein synthesis. PI3-kinase controls this process through the sequential activation of PDK-1 and $p70^{S6K}$ (40). We therefore monitored $p70^{S6K}$ to determine whether its phosphorylation was inhibited with AG1024. As expected, the phosphorylation of $p70^{S6K}$ was increased in response to AngII and inhibited by LY294002, a selective inhibitor of PI3-kinase (Fig. 4B). Like LY294002, AG1024 also prevented $p70^{S6K}$ phosphorylation (Fig. 4B). Quantification of these data confirmed $p70^{S6K}$ is downstream of both Src kinase and PI3-kinase (Fig. 4C). As well, neither AG1478 nor AG1295 had the same inhibitory

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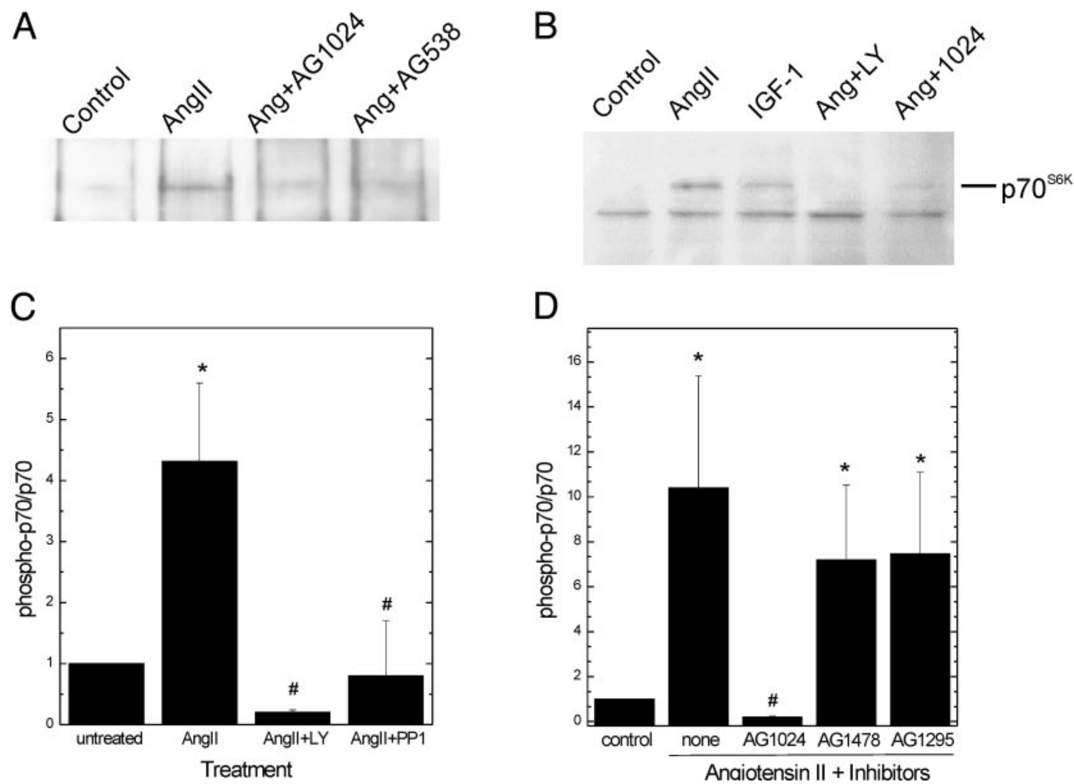

FIG. 4. AngII-dependent activation of PI3-kinase and its downstream effectors is blocked by inhibitors of the IGF-I receptor kinase. A, Quiescent SMCs were stimulated with 1 μM AngII for 10 min and then lysed. Inhibitors (5 μM) were added 15 min before AngII. Tyrosine phosphorylation of the PI3-kinase p85 subunit was determined by Western blot analysis after immunoprecipitation. B, Activation of $p70^{S6K}$ was measured by Western blot analysis with phosphorylation-specific antibodies (diluted 1:1000) after treatment with 1 μM AngII or 0.1 μM IGF-I in the presence of 5 μM inhibitors. C, The effect of PI3-kinase (10 μM LY294002) and Src kinase (10 μM PP-1) inhibitors on AngII-stimulated $p70^{S6K}$ phosphorylation was monitored by Western blot analysis. The same blot was subsequently probed with antibody to total (unphosphorylated + phosphorylated) $p70^{S6K}$. The intensity of the phospho-$p70^{S6K}$ and total $p70^{S6K}$ bands was quantified by scanning densitometry and means ± SEM of the phospho-$p70^{S6K}$/total $p70^{S6K}$ ratio from three independent experiments were plotted. Statistically significant differences ($P < 0.05$) from untreated control (*) and AngII-stimulated sample in the absence of inhibitor (#) are indicated. D, The effect of tyrosine kinase inhibitors (5 μM) on AngII-stimulated $p70^{S6K}$ phosphorylation were measured as described for C.

effect as AG1024 (Fig. 4D). These results indicate that IGF-I receptor transactivation is required for activation of PI3-kinase-dependent signaling pathways and that these pathways are not coupled to either EGF or PDGF receptors.

AT_1 receptor-dependent stimulation of MAPK is IGF-I receptor independent

Our studies indicated that PI3-kinase activation mediates stimulation of the MAPK cascade in response to AngII (24). Activation of ERK1/2 MAPK by PI3-kinase, however, apparently involves a cascade that is distinct from the PDK-1- and $p70^{S6K}$-dependent pathway that leads to protein synthesis (40). Therefore, to determine whether IGF-I receptor transactivation participates in all PI3-kinase-mediated processes, we examined the effect of IGF-I receptor inhibition with AG1024 on MAPK activation by AngII. Quiescent SMCs were treated with 1 μM AngII, and harvested after 5 min for assessment of MAPK phosphorylation by Western blotting. The degree of phosphorylation is directly linked to activity (41), with 5 min being close to the peak of activation. As we have seen previously (24), AngII stimulated a rapid increase in MAPK phosphorylation, and this response was also obtained with IGF-I (Fig. 5A). However, the degree of MAPK phosphorylation was not reduced in the presence of AG1024. Interestingly, inhibition of Src kinase with PP-1 also prevented MAPK phosphorylation (Fig. 5A), suggesting the existence of a Src kinase-dependent pathway that branches before reaching the IGF-I receptor. The existence of divergent pathways is supported by evidence showing phosphorylation of Src kinase after AngII treatment was unaffected by AG1024 (Fig. 5B). This figure also demonstrates that activation of Src kinase by AngII occurs independently of other receptor tyrosine kinases as well.

IGF-I receptor transactivation is not coupled to AngII-dependent cell migration

AngII functions as a chemoattractant for SMCs (40). To assess the contribution of IGF-I receptor transactivation to SMC migration in response to AngII (1 μM), the movement of cells through a membrane with 5-μm pores was quantified with a Boyden chamber in the presence of selective receptor tyrosine kinase inhibitors. Basal migration was measured in the absence of AngII and set to unity. AngII increased cell migration by a statistically significantly 4.6-fold (Fig. 6). Addition of AG1024 did not result in a significant decrease in migration. In contrast, both AG1478 and AG1295 reduced

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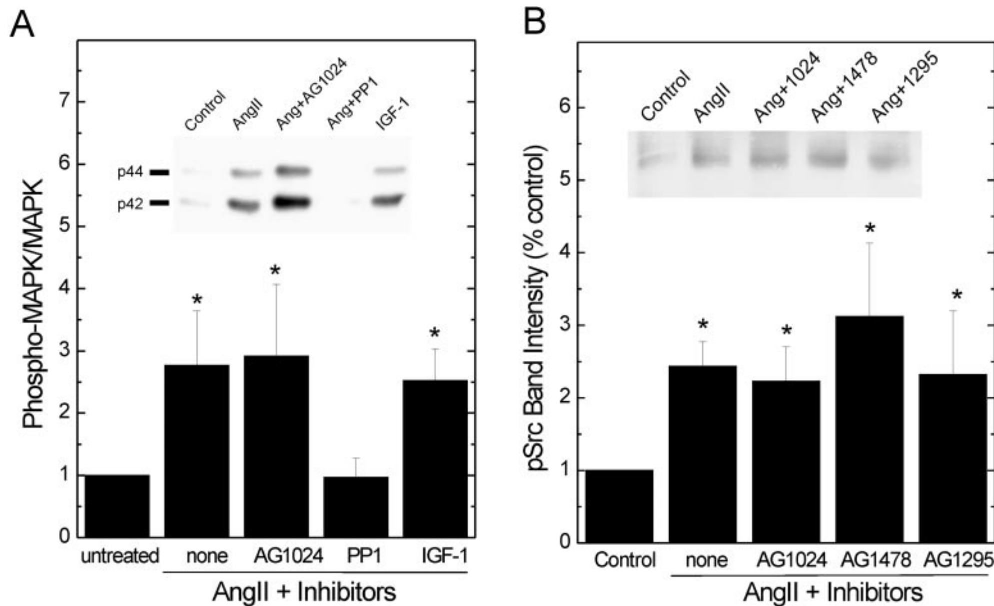

FIG. 5. AG1024 does not inhibit MAPK or Src kinase activation by AngII. A, Phospho-specific antibodies were employed to monitored MAPK activation in response to 10 min incubation with 1 μM AngII or 0.1 μM IGF-I. Inhibitors (5 μM AG1024, 10 μM PP-1) were added 15 min before stimulation. Samples were analyzed by Western blotting, with antibodies diluted 1:1000. A representative blot is shown in the *inset*. The same blot was probed with antibody to total (phosphorylated + unphosphorylated) MAPK. Band intensity (p42 + p44) was quantified by scanning densitometry and means ± SEM of the phospho-MAPK/total MAPK ratio were plotted. Statistically significant differences ($P < 0.05$) from unstimulated control (*) are indicated. B, Phospho-specific antibodies (diluted 1:1000) were employed to monitored Src kinase activation in response to 10 min incubation with 1 μM AngII ± 5 μM inhibitors (added 15 min before stimulation) by Western blotting. A representative blot is shown in the *inset*. Band intensity was quantified by scanning densitometry and means ± SEM were plotted. Statistically significant differences ($P < 0.05$) from unstimulated control (*) are indicated.

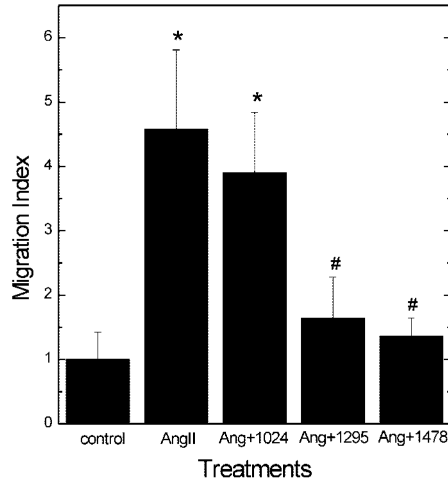

FIG. 6. SMC migration is reduced by inhibitors of receptor tyrosine kinases. Directional movement of growing SMCs was measured with a Boyden chamber as described in *Materials and Methods*. SMCs on the underside of the membrane were fixed, stained, and counted. Chemoattractant (1 μM AngII) was placed into the lower reservoir of the chamber, whereas inhibitors (5 μM) were added to the upper reservoir of the chamber. *Bars* show means ± SEM (n = 6). Values represent the ratio of cells migrated for each treatment relative to untreated control (migration index). Statistically significant differences ($P < 0.05$) from control (*) and AngII-treated cells (#) are indicated.

cell migration to near basal levels. These data suggest that IGF-I receptor transactivation does not functionally contribute to AngII-mediated migration. Rather, activation of the EGF and PDGF receptors is required.

Discussion

AngII is a critical element in the vascular response to injury, operating primarily to promote conversion of SMCs to a phenotypic state, which permits migration and proliferation (42). As part of this process, AngII stimulates tyrosine phosphorylation of key protein mediators of intracellular signaling pathways associated with migration and proliferation (43). However, mechanistic information detailing how G protein-coupled AngII receptors stimulate tyrosine phosphorylation is limited. In this investigation, we examined the role of the IGF-I receptor kinase as an intermediate for the transduction of signals originating from AngII receptors. Our rationale for examining the IGF-I receptor was based on published evidence that showed both the IGF-I receptor and IRS-1 are tyrosine phosphorylated in response to AngII (16, 17). In this study, we employed a phosphorylation-specific antibody to establish that IGF-I receptor activation was time- and AngII concentration-dependent. Furthermore, phosphorylation of the IGF-I receptor was shown to be AT_1 receptor dependent and mediated by Src kinase. However, binding of IGF-I to the receptor was not required for transactivation because neutralizing antibodies did not prevent IGF-I receptor phosphorylation by AngII. The functional significance of IGF-I receptor participation was demonstrated with selective inhibitors of the IGF-I receptor tyrosine kinase, which revealed transactivation was required for the stimulation of PI3-kinase and its downstream effector $p70^{S6K}$, but not MAPK. These results indicate IGF-I receptor transactivation has a critical role in the cellular actions of AngII, although it is apparently not required for cell migration.

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Receptor transactivation is a relatively recent concept, originating from evidence that phosphorylation of receptor tyrosine kinases occurs in response to agonists of G protein-coupled receptors (32, 44, 45). In SMCs treated with AngII, tyrosine phosphorylation of the EGF, PDGF, and IGF-I receptors has been observed (reviewed in Ref. 46, 47), and it is evident from our investigation that these events are critical for cellular functions such as migration. However, the initial reports examining this phenomenon suggested that receptor tyrosine kinase activation was triggered by the release of growth factors in response to G protein-coupled receptor stimulation (48). This mechanism does not appear to operate for AngII-dependent activation of the PDGFβ receptor because neither conditioned media nor neutralizing antibody blocked PDGF receptor phosphorylation in SMCs (49). Even so, there are indications that the PDGF receptor is not activated in response to AngII treatment of SMCs (30), and this disparity remains unresolved. In contrast, EGF receptor phosphorylation has been independently confirmed and shown to mediate, ERK1/2 phosphorylation, c-*fos* expression, and protein synthesis (13, 30, 50) as well as migration (Fig. 6). The mechanism that mediates EGF receptor activation, however, has yet to be clarified. The failure of neutralizing antibodies to prevent EGF receptor phosphorylation (46) suggests that release of EGF is not a prerequisite for this process. On the other hand, metalloproteinase cleavage of proheparin-binding EGF has been reported to precede receptor dimerization and autophosphorylation (51).

Delafontaine and colleagues (16) were the first to identify IGF-I receptor activation in response to stimulation with AngII. Because neutralizing IGF-I antibody was found to prevent the mitogenic effects of AngII, it was concluded that secretion of IGF-I was essential for receptor activation (52). Although this observation does not agree with our data, it may be argued that two distinct events were being compared. It has been previously established that the IGF-I receptor has a role in mitogenesis because IGF-I receptor activation is necessary for advancing past the G_1 restriction point (53). Furthermore, the temporal separation between transactivation (minutes post stimulus) and cell cycle progression (hours post stimulus) unequivocally demonstrates that these are independent processes. Thus, the ability to inhibit cell proliferation with a neutralizing antibody cannot be equated with the lack of an effect on receptor transactivation, which we assayed directly in this study. Rather, transactivation of the IGF-I receptor by the AT_1 receptor occurs in the absence of IGF-I secretion, whereas progression through G_1 phase may require IGF-I synthesis and secretion before IGF-I receptor activation. The latter view is supported by evidence showing AngII stimulates both IGF-I and IGF-I receptor gene expression (54).

The AT_1 receptor is a member of the G protein-coupled receptor family that operates through specific heterotrimeric G proteins. Although the AT_1 receptor involves Gq-mediated activation of various phospholipases (reviewed in Ref. 1), participation of the nonreceptor tyrosine kinases Src and Janus kinase has also been reported (43). Src kinase has been identified as the leading candidate for mediating receptor tyrosine kinase transactivation in response to AngII (30, 31, 55), and we have similarly shown Src kinase is required for

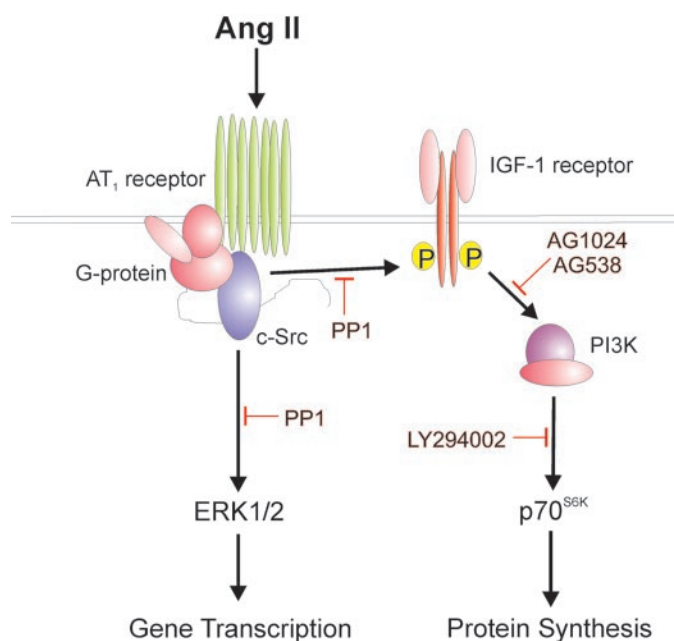

FIG. 7. Signaling pathways activated by AngII and mediated by IGF-I receptor transactivation.

IGF-I receptor transactivation. But how does Src kinase operate? It has been speculated that Src kinase may phosphorylate these receptors directly (56, 57). Recently, however, Seta and Sadoshima (58) reported that tyrosine phosphorylation of the AT_1 receptor (Y-319) is required for EGF receptor transactivation. Mutation of this amino acid, which prevented EGF receptor phosphorylation, did not decrease Src kinase activation in response to AngII. These data therefore imply that Src kinase functions indirectly, whereas also confirming that activation of Src kinase must precede transactivation of receptor tyrosine kinases. However, the link between Src kinase and G proteins (either G_α- or $G_\beta\gamma$-subunits) still remains to be resolved, and there is speculation that this step may involve a G protein-independent process (59). Our data also implicate Src kinase in the activation of MAPK, and similar conclusions have been reached by other investigators studying both SMCs and other cell types (60–62). Interestingly, it has been recently shown that MAPK activation in hepatocytes requires transactivation of the PDGF receptor (60).

There has been limited study of IGF-I receptor function in relation to AngII. Our data indicate IGF-I receptor phosphorylation is required for the activation of PI3-kinase by AngII. Velloso *et al.* (21) previously showed that PI3-kinase associates with phosphorylated IRS-1 and IRS-2 after AngII stimulation of heart tissue, and AngII also triggers phosphorylation of IRS-1 in SMCs (17). Although we did not examine IRS-1, inhibition with AG1024 established that PI3-kinase was downstream of the IGF-I receptor. Furthermore, experiments with AG1024 and LY294002 indicated that $p70^{S6K}$ also follows PI3-kinase. Based on these results, Src kinase can be viewed as controlling two distinct pathways leading from the AT_1 receptor (Fig. 7). First, Src kinase controls MAPK activity and nuclear translocation. This process is necessary for immediate early gene expression. However, tyrosine kinase-independent activation of MAPK by the

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IGF-I receptor could also explain why this event is both Src kinase dependent and AG1024 insensitive (23). Second, Src kinase mediates IGF-I receptor transactivation, which is required for PI3-kinase activation. In this pathway, the IGF-I receptor likely functions as a scaffold for p85 binding, and synthesis of PI3-P results in PDK-1 activation, $p70^{S6K}$ phosphorylation, and increased protein synthesis. How does this scheme reconcile our evidence that MAPK phosphorylation is blocked by inhibition of PI3-kinase (24) and that PI3-kinase is essential for cell migration (Zahradka P., G. E. J. Harding, S. Thomas, J. P. Werner, D. P. Wilson, and N. Yurkova, submitted for publication)? It is becoming obvious that each PI3-kinase isoform has a distinct function in the transduction of signals within cells. Whereas we specifically examined the classical tyrosine kinase receptor-activated p85/p110 PI3-kinase (class I_A) during the course of this study, it is recognized that other PI3-kinase isoforms are equally sensitive to LY294002 (19). Consequently, inhibition of MAPK by LY294002 could implicate another class of PI3-kinases in the modulation of MAPK cascades. The p110γ isoform of PI3-kinase (class I_B) may be particularly relevant to AngII stimulation because its activation is mediated by $G_{\beta\gamma}$-subunits (19). A role for both PI3-kinase isoforms in the transduction of signals from the AT_1 receptor may therefore be projected. Appreciating these distinctions may also help alleviate the confusion surrounding the roles of individual PI3-kinase isoforms in signaling by G protein-coupled receptors.

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