Angiotensin II-induced activation of p21-activated kinase 1 requires Ca\(^{2+}\) and protein kinase C\(\delta\) in vascular smooth muscle cells


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ANG II, an octapeptide hormone involved in both cardiovascular and renal homeostasis, has been implicated in cardiovascular remodeling associated with the development and progression of hypertension, atherosclerosis, and restenosis after angioplasty (17, 31). A number of in vitro experiments confirmed that ANG II augments hypertrophy and hyperplasia in cultured vascular smooth muscle cells (VSMCs), cardiac myocytes and fibroblasts, and renal mesangial cells (21, 38). However, the detailed signaling pathway(s) by which ANG II may contribute to vascular remodeling is still not fully understood.

Angiotensin II (ANG II) promotes remodeling of vascular smooth muscle cells (VSMCs) in cardiovascular diseases. It has been shown to activate p21-activated kinase (PAK1), a critical component of signaling pathways implicated in growth and migration. However, the detailed signaling mechanism by which ANG II induces PAK1 activation in VSMCs remains unclear. Therefore, we have examined the mechanisms required for activation of PAK1 by ANG II in VSMCs. ANG II, through activation of the ANG II type 1 receptor, rapidly promotes phosphorylation of PAK1 in VSMCs via a pathway independent of transactivation of the epidermal growth factor receptor. Using selective agonists and inhibitors, we demonstrated that mobilization of intracellular Ca\(^{2+}\) and PKC\(\delta\) activation are required for ANG II-induced PAK1 phosphorylation. Rottlerin, a PKC\(\delta\) inhibitor, significantly blocked ANG II-induced PAK1 phosphorylation. Further support for this notion was provided through infection of VSMCs with adenovirus encoding a dominant-negative (dn)PKC\(\delta\), which also markedly reduced phosphorylation of PAK1 by ANG II. In this pathway, Ca\(^{2+}\) acts upstream of PKC\(\delta\) because a Ca\(^{2+}\)-ionophore rapidly induced PKC\(\delta\) phosphorylation at Tyr311 and Ca\(^{2+}\)-dependent PAK1 phosphorylation was blocked by rottlerin. In addition, dnPYK-2, dnRac, and antioxidants inhibited ANG II-induced PAK1 phosphorylation, suggesting that PYK-2, Rac, and reactive oxygen species are involved in the upstream signaling. Finally, dnPAK1 markedly inhibited ANG II-induced protein synthesis in VSMCs. These data provide a novel signaling pathway by which ANG II may contribute to vascular remodeling by triggering the onset of growth and migration of VSMCs remains unclear.

ANG II produces its action by binding to high-affinity G protein-coupled receptors, of which there are two subtypes, ANG II type 1 and type 2 (AT\(_1\) and AT\(_2\)) receptors (3). The AT\(_1\) receptor, the primary ANG II receptor expressed in VSMCs, not only regulates most physiological responses to ANG II but also is the principal ANG II receptor involved in pathophysiological signaling in the cardiovascular system (21, 38). The AT\(_1\) receptor mediates a number of different signaling pathways through activation of the G\(_{q/11}\) family of heterotrimERIC \(G\) proteins (5, 18, 21, 38, 41). Thus activation of the AT\(_1\) receptor results in PLC-catalyzed hydrolysis of phosphatidylinositol 4,5-bisphosphate, generating inositol trisphosphate and diacylglycerol. Formation of inositol trisphosphate and diacylglycerol leads to mobilization of Ca\(^{2+}\) and activation of PKC, respectively (18, 21, 38). The AT\(_1\) receptor is also coupled to the production of reactive oxygen species (ROS) (18, 38), which likely activate both downstream tyrosine and serine/threonine kinases (5, 10, 11, 18, 21, 38, 41).

Several key serine/threonine kinases have been identified in ANG II-induced signaling pathways. These kinases include the MAPK/ERK family (5, 8, 18, 21, 38, 41), Akt/PKB (7, 35), PKC (18, 21, 38), p70S6 and p90S6 kinases (7, 18, 21, 38), and p21-activated kinase (PAK) (32, 33), all of which have been implicated in ANG II-induced cardiovascular remodeling. PAKs are a family of 60- to 65-kDa serine/threonine kinases that have been demonstrated to regulate cellular proliferation, differentiation, transformation, and survival via several downstream signaling pathways (1). They are activated by GTP-bound Rac and Cdc42, protein kinases such as phosphoinositide-dependent kinase (PDK)-1, tyrosine kinase receptors, cytokine receptors, and G protein-coupled receptors (1). Currently, very few studies have reported activation of PAK by ANG II in VSMCs (32, 33); however, a role for PAK in cell migration processes has been established through overexpression of mutant PAK constructs in both endothelial cells (22) and epithelial cancer cells (24). Thus PAK may also have a significant role in ANG II-induced cardiovascular remodeling.

The purpose of the present study was to examine the signaling events in the ANG II-induced regulation of PAK1 activation. We hypothesized that ANG II mediates PAK1 activity through a signaling pathway that involves the elevation...
of intracellular Ca\(^{2+}\) and the activation of PKC\(\theta\). Herein we provide evidence that the G protein-coupled AT\(_1\) receptor, Ca\(^{2+}\), PKC\(\theta\), ROS, PYK-2, and Rac are essential upstream components of the signaling pathway utilized by ANG II to phosphorylate and activate PAK1, leading to protein synthesis in VSMCs.

**MATERIALS AND METHODS**

**Reagents and antibodies.** DMEM, fetal calf serum, penicillin, and streptomycin were purchased from Life Technologies; ANG II and N-acetyl-l-cysteine (NAC) were purchased from Sigma. RNH6270 was a gift from Sankyo. EGF was purchased from Upstate Biotechnology; AG1478, A-23187, PMA, Go6976, rotterlin, 8-(N,N-diethylamino)-octyl-3,4,5-trimethoxybenzoate, HCl (TMB-8), BAPTA-AM, and di(phenyl)hexafluoroacetone (DHE) were purchased from Calbiochem. Anti-PAK1 antibodies, which recognize phosphorylated PAK1 at Ser199/204, phosphorylated PAK1 at Thr423, and total PAK1, were purchased from Cell Signaling Technology. Anti-PKC\(\theta\) antibody, which selectively recognizes PKC\(\theta\) phosphorylated at Tyr311, was purchased from BioSource International. Anti-PYK-2 antibody was obtained from BD Transduction Laboratories. Antibodies directed against total PKC\(\theta\), Rac1, and ERK1/2 phosphorylated at Tyr204 were purchased from Santa Cruz Biotechnology.

**Cell culture.** VSMCs were prepared from the thoracic aortas of 12-wk-old Sprague-Dawley rats (Charles River Breeding Laboratories) by the explant method and cultured in DMEM containing 10% fetal calf serum, penicillin, and streptomycin as previously described (8). Subcultured cells from passages 3–15 were used in the experiments; they showed >99% positive immunostaining of smooth muscle \(\alpha\)-actin antibody and were negative for mycoplasma infection. For each experiment, cells were cultured to 80–90% confluence and then made quiescent by incubation in serum-free medium for 2–3 days. VSMCs were stimulated with ANG II or other agonists at 37°C. The animal protocol (no. M/00/296) was approved by the Vanderbilt University Institutional Animal Care and Use Committee.

**Immunoblotting.** Cell lysates were separated by SDS-PAGE, and proteins were electrophoretically transferred to a nitrocellulose membrane as previously described (9). The membranes were exposed to primary antibodies overnight at 4°C and then incubated with a peroxidase-linked secondary antibody for 1 h at room temperature. An ECL Western blotting detection kit (Amersham Pharmacia Biotech) was used to visualize immunoreactive proteins.

**Protein assay.** Subconfluent VSMCs on 12-well culture plates were incubated with serum-free DMEM for 1 day and were infected with adenovirus encoding empty vector or dnPAK1 at MOI of 100 for 2 days. Adenovirus-infected cells were further incubated with or without 100 nM ANG II for 3 days. After aspiration of the medium, cells were washed twice with ice-cold Hank’s balanced salt solution, and the total amount of cellular protein was measured with a bichinchoninic acid protein assay kit (Pierce) before and after ANG II stimulation.

**Proliferation assay.** Cell proliferation was measured with a Cell Titer 96 AQeueous cell proliferation assay kit (Promega), following the manufacturer’s protocol. In brief, after incubation with serum-free DMEM for 3 days, quiescent VSMCs in 96-well plates were stimulated with ANG II (100 nM) for 72 h. After incubation with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium solution provided with the kit, viable cells were determined at 490-nm absorbance with a 96-well plate reader.

**Statistical analysis.** Unless otherwise stated, the data presented in this study are representative of a minimum of three independent experiments yielding similar results. The data were analyzed using one-way ANOVA with posttest to indicate statistically significant differences.

**RESULTS**

**Phosphorylation of PAK1 by ANG II through AT\(_1\) receptor.** Several PAK autophosphorylation sites have been identified as essential for maintaining PAKs in their active conformational state, thereby augmenting PAK activity (1). These residues include Ser199/204 of PAK1 and Ser192/197 of PAK2, both of which are located in the NH\(_2\)-terminal inhibitory domain (16, 27). Mutation of this domain prevents the kinase from reverting to its inactive conformation (25). To evaluate PAK1 activation in response to ANG II, the phosphorylation of PAK1 was measured by immunoblotting with a phosphospecific antibody that recognizes phosphorylated PAK1 at Ser199/204. ANG II stimulated phosphorylation of PAK1 at Ser199/204 in a time- and concentration-dependent manner. PAK1 was significantly phosphorylated as early as 2 min and maximally at 5 min (Fig. 1A). ANG II enhanced phosphorylation of PAK1 at concentrations as low as 1 nM and maximally at 100 nM (Fig. 1B). In contrast, ANG II (100 nM) minimally phosphorylated PAK1 at Thr423, a PDK-1-dependent phosphorylation site (1) in VSMCs (data not shown). For all subsequent experiments described, unless otherwise stated, we stimulated cells with 100 nM ANG II for 5 min and evaluated the effects on phosphorylation of PAK1 at Ser199/204.

We next determined which receptor(s) is responsible for ANG II-induced phosphorylation of PAK1. The AT\(_1\) receptor is well known to mediate most functions of ANG II in VSMCs. We showed previously (9) that ANG II signaling through its AT\(_1\) receptor leads to rapid transactivation of the EGF receptor in VSMCs in a Ca\(^{2+}\)-dependent manner to activate ERK. In this study, we also observed that activation of the EGF receptor by EGF caused a time-dependent phosphorylation of PAK1 in VSMCs (Fig. 2A). Therefore, to determine the involvement of the AT\(_1\) receptor and the EGF receptor, VSMCs were pretreated with an AT\(_1\) receptor antagonist, RNH6270, or an EGF receptor kinase inhibitor, AG1478, and then stimulated with ANG II (Fig. 2B). Both PAK and ERK phosphorylation by ANG II were significantly reduced when VSMCs were pretreated with an AT\(_1\) receptor antagonist RNH6270 (Fig. 2B). Pretreatment with the EGF receptor kinase inhibitor AG1478 only slightly reduced phosphorylation of PAK1 by ANG II, suggesting that AT\(_1\) receptor-mediated activation of PAK1 does not require transactivation of the EGF receptor. However, as expected, activation of ERK was markedly reduced by AG1478 (Fig. 2B), confirming that AT\(_1\) receptor-mediated ERK activation does require EGF receptor activation.

**Involvement of Ca\(^{2+}\) in ANG II-induced PAK phosphorylation.** It is well known that ANG II signaling through the AT\(_1\) receptor is coupled to the heterotrimeric G protein \(G_i\) (5, 18). Because the signaling of ANG II through \(G_i\) involves mobilization of Ca\(^{2+}\), we determined whether this second messenger-activated signal contributed to ANG II-induced PAK1 phosphorylation. First, we stimulated VSMCs with a Ca\(^{2+}\) ionophore, A-23187. As shown in Fig. 3A, A-23187 induced phosphorylation of PAK1 at 5–10 min, returning to near-basal levels at 20 min.
To further clarify the involvement of intracellular Ca²⁺ elevation, we pretreated VSMCs with TMB-8, an intracellular Ca²⁺ antagonist that blocks the release of Ca²⁺ from its intracellular stores, and BAPTA-AM, an intracellular Ca²⁺ chelator. Although both inhibitors significantly decreased the level of phosphorylated PAK (Fig. 3B), TMB-8 almost completely blocked phosphorylation of PAK1 by ANG II, whereas BAPTA-AM caused partial inhibition of PAK1.

**Involvement of PKCδ in ANG II-induced PAK activation.**

The AT₁ receptor is also coupled to the activation of PKC (18). As shown in Fig. 4A, PMA, a potent PKC activator, can evoke a sustained elevation of PAK phosphorylation from 5 to 20 min. To ascertain whether PKC mediates AT₁ receptor activation of PAK1 and to reveal which isoform(s) of PKC might be involved in ANG II-mediated PAK phosphorylation, we pretreated the cells with rottlerin, a selective PKCδ inhibitor, and Go-6976, a selective PKCα and PKCβ inhibitor (Fig. 4B). Whereas rottlerin significantly reduced levels of phosphorylated PAK1 and only partially inhibited activation of ERK, Go-6976 had little effect on phosphorylated levels of either PAK or ERK.

To further assess the role of PKCδ in ANG II activation of PAK1, we infected VSMCs with an adenovirus construct encoded for dnPKCδ. Figure 4C shows that ANG II-induced PAK1, but not ERK, phosphorylation was significantly re-
inhibited PKC phosphorylation. Moreover, pretreatment with TMB-8 markedly inhibited PKC phosphorylation induced by ANG II in VSMCs (Fig. 5D). Together these data suggest that Ca$^{2+}$ activates PKC\(\delta\) through phosphorylation of Tyr311, leading to PAK1 activation in response to ANG II.

Involvement of PYK-2, ROS, and Rac in ANG II-induced PAK phosphorylation. In VSMCs, PYK-2, a proline-rich tyrosine kinase, can be activated by ANG II through a mechanism involving Ca$^{2+}$, PKC, and ROS (6, 13, 30). We previously showed (12, 14) the essential role of PKC\(\delta\) in ANG II- and ROS-dependent PYK-2 activation in VSMCs. Also, the AT\(_1\) receptor activation leads to a NAD(P)H oxidase-dependent ROS production in VSMCs (18). Interestingly, ANG II has been shown to activate Rac (33, 34), which is an established upstream activator of PAK as well as the NADPH oxidase. To explore the involvement of PYK-2, ROS, and Rac in mediating PAK activation by ANG II in VSMCs, we tested the effect of inhibition of these molecules on PAK1 activation. Inhibition of PYK-2 by overexpression of dnPYK-2 in VSMCs (Fig. 6A) or inhibition of ROS production by an antioxidant, NAC, and a NADPH oxidase inhibitor, DPI (Fig. 6B), significantly reduced phosphorylation of PAK1 by ANG II. Also, dnRac significantly inhibited PKC\(\delta\) phosphorylation by ANG II (Fig. 6C). To explore the relationship between Rac activation and PKC\(\delta\), we examined the effect of dnRac on PKC\(\delta\) phosphorylation. As shown in Fig. 6D, dnRac had no inhibitory effect on ANG II-induced PKC\(\delta\) phosphorylation, suggesting a minimal role of Rac-dependent ROS production in ANG II-induced PKC\(\delta\) activation in VSMCs. These data suggest critical roles for PYK-2, ROS, and Rac in the activation pathway of PAK1 by ANG II in VSMCs.

Overexpression of dnPAK1 inhibits ANG II-induced PAK phosphorylation and protein synthesis. To further verify a functional significance of PAK activation by ANG II in VSMCs, we examined the effect of dnPAK1 on ANG II-induced protein synthesis. We observed that overexpression of dnPAK1 significantly inhibited ANG II-induced PKC\(\delta\) phosphorylation (Fig. 7A) as well as protein synthesis induced by ANG II (Fig. 7B). We did not observe a significant amount of VSMC proliferation by ANG II (data not shown), indicating that ANG II stimulates VSMC hypertrophy but not hyperplasia.

DISCUSSION

The major finding of this study is that ANG II activates PAK1 through the AT\(_1\) receptor and that this signaling pathway is independent of EGF receptor transactivation in VSMCs. Also, the upstream signaling pathway includes mobilization of intracellular Ca$^{2+}$, production of ROS, and activation of PKC\(\delta\), PYK-2, and Rac. These data suggest a novel signaling mechanism by which ANG II and the AT\(_1\) receptor contribute to vascular remodeling (Fig. 8).

Previous studies showed that there is dominant expression of the AT\(_1\) receptor in VSMCs (21, 38) and that PAK is activated by ANG II in Chinese hamster ovary cells expressing the AT\(_1\) receptor (32). Activation of the AT\(_1\) receptor leads to transactivation of the EGF receptor and ERK phosphorylation in VSMCs (4, 5). Therefore, we asked whether transactivation of the EGF receptor is also involved in ANG II-induced phosphorylation of PAK. Although we showed that EGF enhanced PAK phosphorylation, pretreatment with the EGF receptor kinase inhibitor AG1478 did not alter PAK phosphorylation by ANG II. Conversely, AG1478 did reduce ANG II-mediated

![Image](http://ajpcell.physiology.org/ by 10.220.33.6 on November 6, 2017)
phosphorylation of ERK. These observations suggest that ANG II→ERK and ANG II→PAK regulatory signaling pathways are separate.

Both intracellular Ca\textsuperscript{2+} elevation and activation of PKC occur as a result of AT\textsubscript{1} receptor activation through G\textsubscript{q} (18, 21, 38). In our study, phosphorylation of PAK1 by ANG II was markedly reduced by both BAPTA-AM and TMB-8, which suggests that Ca\textsuperscript{2+} is required for ANG II-induced activation of PAK and that release of Ca\textsuperscript{2+} from intracellular storage sites is most critical for phosphorylation of PAK by ANG II. The difference in the degree of inhibition caused by BAPTA and TMB-8 may be due to the fact that these substances used distinct mechanisms to antagonize Ca\textsuperscript{2+}. In support of our findings, Lian et al. (26) implicated a major role for the Ca\textsuperscript{2+}/calmodulin complex in the activation of PAK in neutrophils. Moreover, in a separate study performed in VSMCs by Schmitz et al. (32), Ca\textsuperscript{2+} was again shown to be involved in activation of PAK1 by ANG II.

In the present study, the time course for PAK phosphorylation by A-23187 appears to have been delayed compared with ANG II, perhaps because of the distinct nature of intracellular Ca\textsuperscript{2+} elevation or because ANG II has additional signaling such as PKC activation. The PMA initial time course mimics that of ANG II, but is sustained. This may reflect the stronger and more sustained nature of PKC activation by PMA than ANG II. The involvement of PKC has been shown in the activation of PAK in HepG2 cells (19). Schmitz et al. (32) also previously reported PKC-dependent activation of PAK by ANG II in VSMCs. However, the PKC isoform(s) involved in this PAK activation has not been sufficiently characterized.

PKC\textsubscript{δ}, a member of the novel class of PKC isoforms, is abundantly expressed in VSMCs (15). We found that pretreatment with rottlerin, but not Go-6976, significantly reduced activation of PAK1 by ANG II. Furthermore, overexpression of dnPKC\textsubscript{δ} also diminished the level of phosphorylated PAK1 induced by ANG II. Together, our findings indicate that the PKC\textsubscript{δ} isoform is essential for PAK activation by ANG II.

Although the novel PKCs have no structural site for Ca\textsuperscript{2+}-dependent activation (37), we demonstrate that increasing Ca\textsuperscript{2+} levels in VSMCs by exposing them to A-23187, as well
as to ANG II stimulation, can induce phosphorylation of PKC\(_\delta\) at Tyr311, which has been shown to be an important phosphorylation and activation site for PKC\(_\delta\) in response to other agonists such as H\(_2\)O\(_2\) (23). Our data further support the notion that intracellular Ca\(^{2+}\) is a critical upstream signal for Tyr311 phosphorylation of PKC\(_\delta\) and subsequent activation of PAK1 through phosphorylation of Ser199/204. Thus we provide a novel PAK activation pathway involving Ca\(^{2+}\)-dependent activation of PKC\(_\delta\) through Tyr311 phosphorylation by ANG II in VSMCs.

An earlier study by Schmitz et al. (32) proposed the presence of a Src kinase inhibitor (PP1)-insensitive tyrosine kinase upstream of PAK activation by ANG II in VSMCs. The activation of this kinase may cause an adaptor Nck to activate PAK (33). PYK-2 could be such a kinase because ANG II-induced PYK-2 activation lies downstream of intracellular Ca\(^{2+}\) elevation and PKC activation in VSMCs (6, 30). Together with our previous findings (12, 14) demonstrating the requirement of PKC\(_\delta\) and ROS for ANG II-induced PYK-2 activation in VSMCs, we have investigated the roles of PYK-2 and ROS in ANG II-induced PAK phosphorylation. We found that overexpression of dnPYK-2 or antioxidants inhibits PAK1 phosphorylation by ANG II, thus strongly indicating the involvement of PYK-2 and ROS in ANG II-induced PAK1 activation. Recently, Weber et al. (39) demonstrated that phosphorylation of PAK1 at Thr423 but not Ser199/204 by PDGF is mediated by ROS and PDK1 in cultured VSMCs. We observed that Thr423 was minimally phosphorylated in response to ANG II in VSMCs, whereas ANG II-induced Ser199/204 phosphorylation was markedly inhibited by kinase-inactive dnPAK transfection, supporting our notion that Ser199/204 phosphorylation is a useful marker with which to study the regulation of PAK activation by ANG II in VSMCs. The discrepancy between ANG II and PDGF in regard to Thr423 phosphorylation and ROS requirement for Ser199/204 phosphorylation may be due to distinct downstream PAK activation mechanisms that are utilized by the AT\(_1\) and PDGF receptors in VSMCs. Studies also have demonstrated that tyrosine phosphorylation of PYK-2 by ANG II augments its association with Src (6, 30). Rybin et al. (29) found that H\(_2\)O\(_2\)-stimulated phosphorylation of PKC\(_\delta\) at Tyr311 was mediated by a Src family tyrosine kinase in cultured cardiac myocytes. Thus the role of Src and its possible involvement in PAK1 activation in response to ANG II requires further investigation.

PAK1 is a well-known effector of Rac (36), and Rac has been implicated in ANG II-induced PAK1 activation in VSMCs (33). Because Rac is associated with the activation of NADPH oxidase, which is a major source of ROS in ANG II-stimulated VSMCs (34), it would follow that Rac would also play a role in ANG II-induced activation of PAK1 via ROS production. In the present study, we verified the essential role of Rac in the ANG II-induced activation of PAK1 and PKC\(_\delta\) with dnRac. Because dnRac inhibited PAK phosphorylation by ANG II without affecting PKC\(_\delta\) phosphorylation, we submit a model in which Rac had no connection to ROS-sensitive components of PAK1’s upstreams involving PKC\(_\delta\) and PYK-2. Our findings may be partially in line with...
Fig. 6. Involvement of PYK-2, reactive oxygen species (ROS), and Rac in ANG II-induced PAK phosphorylation. A: VSMCs were infected with adenovirus encoding dnPYK-2 or control empty vector (MOI 100) for 48 h and stimulated with ANG II (100 nM) for 5 min. B: cells were pretreated with diphenyleneiodonium chloride (DPI; 10 μM) or N-acetyl-L-cysteine (NAC; 20 mM) for 30 min and stimulated with ANG II (100 nM) for 5 min. C: VSMCs were infected with adenovirus encoding dnRac1 or control empty vector (MOI 100) for 48 h and stimulated with ANG II (100 nM) for 5 min. D: VSMCs were infected with adenovirus encoding dnRac1 or control empty vector (MOI 100) for 48 h and stimulated with ANG II (100 nM) for 2 min. Cell lysates were analyzed by immunoblotting with antibodies as indicated. Arrows denote endogenous Rac and infected dnRac. Data are representative of at least 3 separate experiments giving similar results. *P < 0.05 vs. ANG II-stimulated control.
previous findings and proposals in VSMCs (34) that there are two distinct ROS production mechanisms by ANG II in which initial ROS production via NADPH oxidase is Rac independent but is essential for activation of tyrosine kinases by ANG II. Thus we propose an upstream mechanism of PAK1 activation involving Rac by ANG II, as illustrated in Fig. 8, which may need further detailed clarification in the future.

Previous studies revealed a functional role of PAK1 in mediating PDGF-induced migration in VSMCs (39) as well as in tracheal smooth muscle cells (2). In tracheal smooth muscle cells, it was further shown that PAK stimulates migration by signaling to p38 MAPK. Herein we have shown that PAK is involved in protein synthesis induced by ANG II as well. Although we have not investigated downstream of PAK1 in mediating protein synthesis, JNK could be the candidate. This is because JNK has been demonstrated as a downstream effector of PAK in VSMCs (32).

In conclusion, we have demonstrated that the signaling molecules Ca\(^{2+}\), PKC\(\alpha\), PYK-2, ROS, and Rac play critical roles in ANG II-induced activation of PAK1 and that PAK1 is involved in protein synthesis in VSMCs. These data may provide novel insight into the cellular mechanisms by which ANG II mediates vascular hypertrophy. Further elucidation of this signaling pathway will reveal new therapeutic targets that, when inhibited, attenuate actions of the AT\(_1\) receptor, thereby reducing cardiovascular remodeling associated with development and progression of cardiovascular diseases.

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