A role for PYK2 in regulation of ERK1/2 MAP kinases and PI 3-kinase by ANG II in vascular smooth muscle

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Received 11 May 2000; accepted in final form 7 August 2000

ANGIOTENSIN II (ANG II) regulates a variety of physiological responses including salt and water balance, blood pressure, and vascular tone. In addition to its vasoconstrictor effects, ANG II is also a potent growth factor for vascular tissue (19). These effects of ANG II have been linked to the development of various diseases associated with altered vascular smooth muscle cell (VSMC) growth such as hypertension, restenosis, and atherosclerosis (9, 20, 21).

ANG II induces hypertrophic growth in cultured VSMC as well as in intact aorta (reviewed in Ref. 19). In cultured VSMC, the hypertrophic growth response is defined as an increase in cell size and protein content without change in cell number and DNA replication (3, 7). These growth-promoting effects of ANG II include changes in gene expression, protein synthesis and turnover, and protein assembly into stress fibers. A variety of intracellular signaling cascades are involved in the control of ANG II-induced VSMC growth, including the extracellular recognition kinases (ERK1/2) family of mitogen-activated protein (MAP) kinases (28) and phosphatidylinositol 3-kinase (PI 3-kinase) (25). The mechanisms by which ERK1/2 mediate ANG II-induced-protein synthesis have not been fully identified but are thought to occur at the level of gene expression and/or the initiation of protein translation. The activation of PI 3-kinase and its downstream targets, Akt and the ribosomal p70S6 kinase, is critical for protein synthesis in many cell types, including VSMC (25). For example, p70S6 kinase is thought to be the major in vivo mediator of ribosomal S6 protein phosphorylation, a necessary step in ANG II-mediated protein synthesis in VSMC (8). In other cell types, both ERK1/2 and PI 3-kinase have been shown to regulate the phosphorylation of PHAS-1/eIF4E complex, a key regulator of translation initiation (16).

The precise molecular mechanisms that link AT1 receptor activation to the activation of the ERK1/2 and the PI 3-kinase signaling pathways have not been fully established. In other cell types, the proline-rich nonreceptor tyrosine kinase 2 (PYK2) has been shown to link G protein-coupled receptors to upstream regulators of ERK1/2 MAP kinases, such as Grb2, Shc, and the nucleotide exchange factor Sos (5). We have demonstrated that PYK2 is activated by ANG II in VSMC in a Ca2+- and protein kinase C (PKC)-dependent manner, resulting in its interaction with Src (23). More recent studies from Eguchi et. al. (6) suggest that interaction between Src and...
PYK2 leads to the recruitment of Grb2, a signaling intermediate in the ERK1/2 pathway. Thus PYK2 may be a key upstream regulator of ERK1/2 activation in VSMC.

Much less is known about the upstream signaling pathways that link AT1 receptor activation to the PI 3-kinase pathway. In other cell types, regulation of PI 3-kinase involves interactions of the Src homology 2 (SH2) domains of the regulatory p85 α-subunit with tyrosine phosphorylated proteins (14). One potential regulator of PI 3-kinase in VSMC is the adapter molecule p130Cas (a Crk-associated substrate). p130Cas, initially identified as a major tyrosine phosphorylated protein in v-Crk and v-Src transformed cells (10), also contains proline-rich sequences that may allow it to act as a docking protein for the SH3 and SH2 domains of PI 3-kinase. In addition, p130Cas is tyrosine phosphorylated in response to ANG II in VSMC in a PKC- and Ca2+-dependent manner (26). Because PYK2 activation by ANG II requires PKC and Ca2+, we hypothesized that this kinase could also mediate p130Cas phosphorylation, and thus PI 3-kinase activation.

The present study investigates the signaling pathways in VSMC that link the AT1 receptor activation to ERK1/2 and PI 3-kinase signaling. We have identified Shc as an additional component of a signaling complex formed among PYK2, Src, and Grb2 in VSMC. We have shown, for the first time, that PYK2 forms a complex with PI 3-kinase in response to ANG II. In addition, our data indicate that PYK2 is involved in p130Cas phosphorylation and its subsequent association with PI 3-kinase in VSMC. Finally, we have shown that the
activation of p70^S6_ kinase is dependent on PI 3-kinase, a tyrosine kinase, and Ca^{2+}.

**MATERIALS AND METHODS**

**Cell culture.** VSMC from thoracic aortas of 10- to 12-wk-old male Sprague-Dawley rats were isolated by enzymatic digestion as described previously (17). VSMC (passages 3–7) were maintained in culture at 37°C in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum, 100 U/ml penicillin, 10 μg/ml streptomycin, and 2 mM glutamine. VSMC were grown to ~75% confluency and then growth arrested for 48 h in serum-free DMEM supplemented with 0.1% BSA.

**Immunoblot analysis.** Cell lysates were prepared as described previously (17). Equal amounts of protein (30 μg) were resolved by 10% SDS-PAGE and transferred to nitrocellulose. Immunoblot analysis was performed using phosphorylation state-specific antibodies against ERK1/2 MAP kinases (1:5,000; Promega), PYK2 (pY881, 1:1,000; Biosource), or p70^{S6}_ kinase (1:1,000; New England Biolabs). Bands were visualized by enhanced chemiluminescence (ECL; Amersham) and quantified using NIH Image software.

**Immunoprecipitation.** Cell lysates were prepared for immunoprecipitation as described (23). Equal amounts of protein (600 μg) were immunoprecipitated with anti-pTyr, anti-Shc polyclonal antibodies, or anti-PYK2 monoclonal antibodies (Transduction Labs) overnight at 4°C. Immune complexes were collected by incubation with protein A-Sepharose or protein G-agarose beads. For immunoprecipitations using glutathione-S-transferase (GST)-fusion proteins conjugated to agarose beads, cell lysates were precleared with protein G for 30 min. Immunoprecipitates were separated by SDS-PAGE, and proteins were detected by immunoblotting as described above using anti-PYK2 (1:1,000), anti-p130Cas (1:1,000), anti-pTyr, or anti-Grb2 (1:2,000, Transduction Labs) monoclonal antibodies.

**Data analysis.** Blots shown are representative of at least n = 3 experiments. One-way repeated-measures analysis of variance (ANOVA) followed by Bonferroni’s t-test were used for comparisons among multiple groups. Differences among means were considered significant at P < 0.05. Data were analyzed using Instat statistical software (GraphPad).

**Materials.** PI 3-kinase p85 α-subunit NH$_2$- and COOH-SH2 domain GST-fusion proteins conjugated to agarose beads and polyclonal PI 3-kinase antibodies were from Up-
state Biotechnology. Anti-p130Cas, anti-PYK2, and anti-phosphotyrosine antibodies were from Transduction Laboratories. Anti-phosphoPYK2 was from Biosource. A monoclonal anti-PI 3-kinase antibody was from Panvera. Anti-phosphoERK1/2 were from Promega. Anti-p70S6 kinase antibodies were from New England Biolabs. 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA-AM) was from Alexis Laboratories. LY-294002 and genistein were purchased from Calbiochem.

RESULTS

Ca\(^{2+}\)-dependent p130Cas phosphorylation and PYK2-p130Cas complex formation in response to ANG II. In other cell types, PYK2 directly interacts with the docking protein p130Cas (15). Once tyrosine is phosphorylated, p130Cas can bind to SH2 domain-containing proteins. Therefore, we speculated that p130Cas might link PYK2 to PI 3-kinase in VSMC because the regulatory p85 \(\alpha\)-subunit of PI 3-kinase contains several SH2 domains. In Fig. 1A, we determined whether ANG II induces p130Cas phosphorylation. VSMC were treated with 100 nM ANG II for 0–60 min. VSMC were then lysed and immunoprecipitated with a polyclonal anti-pTyr antibody or monoclonal anti-p130Cas antibodies. Phosphorylated p130Cas in the immunoprecipitates was measured by immunoblot analysis with a monoclonal anti-p130Cas or anti-pTyr antibody. Little basal p130Cas phosphorylation occurred in the absence of ANG II. Upon treatment with ANG II, a rapid and sustained increase in p130Cas tyrosine phosphorylation was observed as early as 1 min, was maximal at 2–5 min, and returned toward control levels at 30 min (Fig. 1A). Pretreatment with BAPTA-AM inhibited ANG II-dependent p130Cas phosphorylation at all time points (Fig. 1B).

To establish PYK2 as a link between the AT\(_1\) receptor activation and p130Cas tyrosine phosphorylation, we immunoprecipitated lysates with an anti-PYK2 antibody followed by immunoblot analysis with anti-p130Cas antibodies. There was a slight association between PYK2 and p130Cas that was significantly increased after 1 min of treatment with ANG II (Fig. 1C, lanes 1 and 3–6) and returned to basal levels after 20 min (lane 7). Pretreatment with 50 \(\mu\)M BAPTA-AM inhibited ANG II-dependent PYK2-p130Cas complex formation at all time points.
examined (Fig. 1B, lanes 2 and 8–12). To confirm equal lane loading, blots were stripped and reprobed with anti-PYK2 antibodies.

**ANG II stimulates a Ca\(^{2+}\)-dependent interaction among PYK2, p130Cas, and PI 3-kinase in VSMC.** We next determined whether phosphorylated p130Cas could bind to the p85\(\alpha\) regulatory subunit of PI 3-kinase. GST-fusion proteins containing the NH\(^2\)- or COOH-terminal SH2 domains of PI 3-kinase p85\(\alpha\)-subunit were immobilized on agarose beads and incubated with lysates from control and ANG II-treated cells. After the beads were washed, affinity-purified proteins were subjected to SDS-PAGE and immunoblotted using anti-p130Cas or anti-PYK2 antibodies. Under basal conditions, little p130Cas and PYK2 were bound to both SH2 domains of PI 3-kinase p85\(\alpha\). ANG II stimulation caused a significant increase in the amount of p130Cas and PYK2 bound to the SH2 domains (Figs. 2A and 3A). The time course for this interaction was consistent with that for ANG II-mediated p130Cas tyrosine phosphorylation. Pretreatment with 50 \(\mu\)M BAPTA-AM prevented the association of the PI 3-kinase p85\(\alpha\)-subunit SH2 domains with p130Cas and PYK2 (Figs. 2B and 3B). To confirm results obtained with the use of the SH2 domains of PI 3-kinase, we also used coimmunoprecipitation analysis to demonstrate that ANG II stimulates an endogenous complex formation between PI 3-kinase and p130Cas and PYK2 (Figs. 2C and 3C). To confirm equal lane loading, blots shown in Figs. 2C and 3C were stripped and reprobed with anti-PI 3-kinase and anti-PYK2 antibodies.

**Effects of PI 3-kinase inhibition on PYK2 and p130Cas activation.** To further strengthen the idea that both PYK2 and p130Cas lie upstream of PI 3-kinase activation, we pretreated VSMC with the PI 3-kinase inhibitors LY-294002 and wortmannin. ANG II-induced phosphorylation of either PYK2 or p130Cas was not affected by these inhibitors (Fig. 4, A and B).

**ANG II activates p70\(^{S6}\) kinase, a downstream target of PI 3-kinase, in a Ca\(^{2+}\)- and tyrosine kinase-depen-

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**Fig. 4.** p130Cas and PYK2 activation are upstream of PI 3-kinase activation. Growth-arrested VSMC were pretreated with LY-294002 or wortmannin for 45 min before treatment with ANG II for 5 min. A: cell lysates were immunoprecipitated with pTyr antibodies, and Western blot analysis was performed using anti-p130Cas antibodies (top, LY-294002; bottom, wortmannin). B: Western blot analysis was performed with anti-active PYK2 antibodies (top, LY-294002; bottom, wortmannin).
dent manner. To determine whether ANG II activates p70S6 kinase in a PI 3-kinase- and tyrosine kinase-dependent manner, we pretreated growth-arrested VSMC with LY-294002, wortmannin, and genistein. Activation was assessed with the use of anti-p70S6 kinase antibodies that recognize the phosphorylated, active form of the kinase (pThr-421/Ser-424 and pThr-401). ANG II-activation of p70S6 kinase was completely blocked by each inhibitor, suggesting that both tyrosine kinase(s) and PI 3-kinase are required for activation (Fig. 5A). p70S6 kinase phosphorylation was also completely inhibited by pretreatment with 50 μM BAPTA-AM (Fig. 5B). Drug treatment had no effect on p70S6 kinase expression as detected by Western blotting with anti-total p70S6 kinase antibodies (Fig. 5C). The apparent higher molecular weight of the bands in lanes 3-5 was due to a reduced mobility of the fully phosphorylated form of p70S6 kinase. In fact, this band shift detected in the total p70S6 kinase blot provides an independent confirmation of the data in Fig. 5B.

PYK2 regulates upstream activators of ERK1/2. We next identified some of the tyrosine kinases involved in the regulation of the ERK1/2 signaling pathway. We previously showed that ANG II causes a rapid activation of PYK2 that is associated with the formation of a PYK2-Src complex (23). Because Src-induced tyrosine phosphorylation of the adapter molecule Shc leads to the formation of a Shc-Grb2 complex (30), we next determined whether PYK2 is associated with these signaling intermediates. To assay for PYK2-Shc-Grb2 complex formation, VSMC were treated with ANG II for 0–20 min and then lysed and immunoprecipitated with an anti-Shc antibody. The PYK2-Shc complex formation was determined by immunoblotting with a monoclonal anti-PYK2 antibody. PYK2-Shc complexes were detected as early as 1 min and were maintained at 30 min (Fig. 6A). The blots were then stripped and reprobed with anti-Grb2 antibodies and with anti-Shc antibodies to confirm equal lane loading. The association of Shc with Grb2 is also increased in ANG II-treated VSMC (Fig. 6A). These results, along with our previous data (23), indicate that treatment with ANG II stimulates the formation of a signaling complex that consists of at least PYK2, Shc, Src, and Grb2. These results were confirmed by immunoprecipitation with an anti-PYK2 antibody and blotting for Grb2 or Src (data not shown). The PYK2-Grb2 complex formation was associated with an increase in phosphorylation of Y881 of PYK2 (Fig. 6B), which is necessary for PYK2-Grb2 interaction in other cell types (4).

**Fig. 5. Phosphorylation p70S6 kinase in response to ANG II.** A: VSMC were pretreated with LY-294002, wortmannin, or genistein (45 min) before treatment with ANG II for 2 and 5 min. p70S6 kinase was detected by immunoblot analysis with anti-phospho p70S6 kinase antibodies. B: same as A except that VSMC were pretreated with BAPTA-AM for 30 min before treatment with ANG II for 0–60 min. C: VSMC were pretreated with BAPTA-AM for 30 min before treatment with ANG II for the times indicated. Western blot analysis was performed with anti-total p70S6 kinase antibodies.
PI 3-kinase inhibition has no effect on ANG II-induced ERK1/2 activation. Previous reports suggest that PI 3-kinase may regulate cell growth through the activation of ERK1/2 MAP kinases (11, 22). In these experiments, we pretreated VSMC with the specific PI 3-kinase inhibitor LY-294002 (1–20 μM). PI 3-kinase inhibition had no effect on ERK1/2 activation as measured by Western blotting with anti-active ERK1/2 antibodies (Fig. 7).

DISCUSSION

The intracellular signaling components that link AT1 receptors to VSMC growth involve a complex network of protein-protein interactions and kinase cascades. Recent evidence suggests that ERK1/2 MAP kinase (2) and PI 3-kinase (25) play a crucial role in ANG II-induced VSMC hypertrophy, but little is known about the intracellular signaling intermediates that link AT1 receptor to these pathways. Here, we have shown, for the first time, that the Ca2+-sensitive, nonreceptor tyrosine kinase PYK2 links the AT1 receptor to the PI 3-kinase signaling pathway. Using coimmunoprecipitation analysis and the SH2 domains of PI 3-kinase in “pull-down” assays, we have demonstrated that PYK2 activation is associated with the tyrosine phosphorylation of the adapter molecule p130Cas and complex formation with the p85α regulatory subunit of PI 3-kinase.

In other cell types, the adapter molecule p130Cas is an important mediator of both PYK2 (15) and focal adhesion kinase (FAK) (10) signaling. Because of its combination of proline-rich sequences, an SH3 domain, and multiple tyrosine phosphorylation sites, p130Cas serves as a docking protein for SH2 and SH3 domain-containing proteins (24). Moreover, p130Cas can constitutively interact with proline-rich sequences in PYK2 via its SH3 domains (1). The present data provide the first evidence that ANG II stimulates the association of PYK2 with p130Cas in VSMC. There was a modest PYK2-p130Cas interaction in control cells that was dramatically increased with ANG II activation and tyrosine phosphorylation of PYK2 (Fig. 1).

Fig. 6. ANG II stimulates PYK2-Shc-Grb2 complex formation. A: lysates were immunoprecipitated with anti-Shc antibodies. Immunoblot analysis was performed with anti-PYK2 antibodies (top). The blot was stripped and reprobed with anti-Grb2 (middle) and anti-Shc antibodies (bottom). B: VSMC were pretreated with BAPTA-AM followed by ANG II. Immunoblot analysis was performed with anti-phosphoPYK2 (pY881) antibody (1:2,000).

Fig. 7. PI 3-kinase inhibition has no effect on ANG II-induced extracellular recognition kinase (ERK) 1/2 activation. VSMC were pretreated with 1–20 μM LY-294002 for 45 min before treatment with ANG II for 5 min. Immunoblot analysis was performed with anti-phospho-specific ERK1/2 antibodies (1:5,000).
Further support for an interaction between PYK2 and p130Cas comes from our finding that p130Cas tyrosine phosphorylation in response to ANG II in VSMC is Ca\(^{2+}\)-dependent. These results are confirmed by Sayeski et al. (26), who demonstrated PKC- and Ca\(^{2+}\)-dependent p130Cas tyrosine phosphorylation by ANG II in cultured VSMC. However, Takahashi et al. (29) observed not only that the tyrosine phosphorylation of p130Cas and its association with c-Crk II was Ca\(^{2+}\) and PKC independent but also that there was a lack of PYK2-p130Cas interaction in response to ANG II. The reasons for these discrepancies are unclear but may reflect differences in the passages of VSMC used or in the differences in the detergents used in the lysis buffer.

To gain further insight into the functional consequences of p130Cas-PYK2 interaction in VSMC, we examined their interaction with PI 3-kinase. Our results provide the first demonstration of an interaction among PYK2, p130Cas, and PI 3-kinase. Using the NH\(_2\)- and COOH-terminal SH2 domains of the p85 \(\alpha\)-subunit of PI 3-kinase coupled to agarose beads, we were able to detect binding to both p130Cas and PYK2 in a Ca\(^{2+}\)-dependent manner (Figs. 2 and 3). This interaction seems to be associated with the activation of PI 3-kinase as determined by the LY-294002- and BAPTA-sensitive phosphorylation of the downstream effector p70\(^{56}\) kinase (Fig. 5). Furthermore, the ANG II-dependent activation of p70\(^{56}\) kinase was also prevented by tyrosine kinase inhibition. However, neither LY-294002 nor wortmannin had any effect on the tyrosine phosphorylation of either PYK2 or p130Cas (Fig. 4). These findings, along with the time course for ANG II-induced phosphorylation of these molecules, are consistent with our hypothesis that PYK2 lies upstream of the PI 3-kinase pathway; however, studies with dominant negative constructs of PYK2 are necessary to conclusively confirm these findings.

In addition to establishing a link between PYK2 and PI 3-kinase, the role of PYK2 in ANG II-dependent ERK1/2 MAP kinase activation was also investigated. Previous data had shown that PYK2 autophosphorylates on Y402 upon activation, creating a binding site for the SH2 domain of Src (5). We have shown that Src forms a complex with PYK2 in response to ANG II in VSMC (23). In other cell types, recruitment and activation of Src leads to tyrosine phosphorylation of PYK2 at Y881 to create a binding site for the SH2 domain of Grb2 (4). In addition, Src may also tyrosine phosphorylate the adapter molecule Shc, leading to its interaction with Grb2 (30). Grb2 could then recruit the guanine nucleotide exchange factor Sos, leading to Ras activation and, ultimately, ERK1/2 stimulation (2). In the present study, we have demonstrated that PYK2 forms a complex with both Grb2 and Shc in VSMC (Fig. 6A) and that this association is Ca\(^{2+}\) and tyrosine kinase dependent (Rocic P and Lucchesi PA, unpublished observations). We have concluded that the association of PYK2 with Src is necessary for Shc-Grb2 complex formation and ERK1/2 stimulation. This conclusion is supported by recent work from Berk’s laboratory (12), which demonstrated a role of Src in mediating ANG II-induced ERK1/2 activation, and from the work of Schieffer et al. (27), which defined a role for...
both Src and p21 in ANG II-induced VSMC growth. Eguchi et al. (6) also reported that a Grb2-GST fusion protein could immunoprecipitate PYK2 in VSMC but did not show an interaction with either endogenous Grb2 or Shc.

It is not clear whether PYK2 can directly interact with Shc because we were not consistently able to communoprecipitate Shc with an anti-PYK2 antibody (data not shown). It may be that PYK2 interaction with Shc is indirect, mediated by Src. For example, Blaukat et al. (4) showed that Src is required not only for PYK2 regulation of Shc-Grb2 complex formation but also for the direct interaction of PYK2 and Grb2 via phosphorylation of Y881 of PYK2. In our experiments, the association of Shc, PYK2, and Grb2 correlated with an increase in the tyrosine phosphorylation of Y881 (Fig. 6B).

There is some evidence that PI 3-kinase is involved in the activation of the ERK1/2 MAP kinase pathway (11). This modulation is proposed to involve the activity of Ras by the p110y isoform of PI 3-kinase (22). In this study, LY-294002 as high as 20 μM failed to block ANG II-induced ERK1/2 activation at all time points examined (Fig. 7). Moreover, we were consistently unable to detect the p110 γ-isofrom in VSMC cell lysates by immunoblot analysis (data not shown). Thus it appears that there is no cross talk between these pathways in response to ANG II in VSMC.

Our data suggest that PYK2 is an upstream regulator of two parallel signaling pathways, the ERK1/2 and the PI 3-kinase pathways, and thus represents a bifurcation point for the ANG II signal (Fig. 8). The formation of a PYK2-Grb2 and/or the Src-Shc-Grb2 complex leads to ERK1/2 regulation indirectly via activation of Ras. PYK2-dependent tyrosine phosphorylation and interaction with the adapter molecule p130Cas lead to their association with the p85 α-subunit of PI 3-kinase. We speculate that this complex formation leads to the activation of PI 3-kinase and its downstream targets Akt and p70S6 kinase. Our results do not rule out the involvement of other upstream tyrosine kinases or signaling molecules. For example, p125 FAK is activated in involved of other upstream tyrosine kinases or signaling molecules. For example, p125 FAK is activated by ANG II in VSMC (18) and has been shown to regulate Src and p130Cas (13).

In summary, this study establishes a role for PYK2 in linking AT1 receptor activation to two distinct signaling pathways in VSMC. The precise role this kinase plays in mediating ANG II-induced VSMC growth remains to be elucidated. Future studies with dominant negative constructs of PYK2 are needed to determine whether this kinase regulates rate-limiting steps necessary for VSMC hypertrophy in response to AT1 receptor activation.

This work was supported by National Heart, Lung, and Blood Institute Grant HL-56046, American Heart Association National Grant-in-Aid 96008970 (P. A. Lucchesi), and the John and Marian Falk Trust for Medical Research (A. Sabri).

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