Downregulation by Antisense Oligonucleotides Establishes a Role for the Proline-rich Tyrosine Kinase PYK2 in Angiotensin II-induced Signaling in Vascular Smooth Muscle

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Running Title:, PYK2 regulates VSMC protein synthesis
ABSTRACT

Abnormal vascular smooth muscle cell (VSMC) growth plays a key role in the pathogenesis of hypertension and atherosclerosis. Angiotensin II (Ang II) elicits a hypertrophic growth response characterized by an increase in protein synthesis in the absence of DNA synthesis and cell proliferation. Intracellular signaling mechanisms linking angiotensin type I receptor activation to protein synthesis in VSMC have not been fully characterized. The present study investigates the role of the nonreceptor proline-rich tyrosine kinase (PYK2) in Ang II-induced VSMC protein synthesis and in the regulation of two signaling pathways that have been implicated in the control of protein synthesis, the extracellular signal-regulated kinase (ERK1/2) and the phosphatidylinositol 3-kinase (PI3-kinase)/Akt pathways. PYK2 antisense oligonucleotides were used to downregulate PYK2 expression in cultured VSMC. An 80% downregulation in PYK2 expression resulted in an ~ 80% inhibition of ERK1/2 (3.8±1.3 vs. 16.6±1.8), p70S6 kinase (1.03±0.03 vs. 3.8±0.5) and Akt activation (3.0±0.8 vs. 16.0±1.0) by Ang II. Furthermore, PYK2 downregulation resulted in a complete inhibition of Ang II-induced VSMC protein synthesis. These data conclusively identify PYK2 as an upstream regulator of both the ERK1/2 and the PI3-kinase/Akt pathways that are involved in Ang II-induced VSMC protein synthesis.

Key words: ERK1/2, PI3-kinase, Akt, tyrosine kinases, FAK, p70S6 kinase
INTRODUCTION

Altered VSMC growth has been implicated in the pathogenesis of atherosclerosis, restenosis and hypertension. In response to different agonists, VSMC are capable of both a hyperplastic growth response, characterized by an increase in cell number, and a hypertrophic growth response characterized by an increase in cell size accompanied by an increase in protein synthesis in the absence of cell division (1,2). Ang II regulates blood pressure both acutely via potent vasoconstriction and chronically by its effects on vascular smooth muscle growth. Ang II induces hypertrophic growth in cultured VSMC as well as in intact aorta that is characterized by an increase in protein synthesis (3).

Numerous cellular signaling pathways have been implicated in Ang II-induced VSMC protein synthesis. These include the nonreceptor tyrosine kinases, c-Src (4), proline-rich tyrosine kinase (PYK2) (5,6) focal adhesion kinase (FAK) (7), the extracellular signal-regulated kinase 1/2 (ERK1/2) (8,9) and phosphatidylinositol 3-kinase (PI3-kinase) (10,11). Of these, the ERK1/2 and the PI3-kinase/Akt pathways are key regulators of cell growth in many cell types (11,12). We and others have shown that both pathways are activated in response to Ang II in VSMC (8,10,11). 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 the initiation of protein translation. The activation of PI3-kinase and its downstream targets, Akt and the ribosomal p70S6 kinase, are critical for protein synthesis in many cell types, including VSMC (11). 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 (13). In other cell types, both ERK1/2 and PI3-kinase have been shown to regulate the association of the phosphorylated, heat and acid-stable protein (PHAS-1) with the eukaryotic translation initiation...
factor 4E (eIF4E), a key regulator of translation initiation (14). We have previously shown that pharmacological inhibition of ERK1/2 and PI3-kinase reduce Ang II-induced protein synthesis (7).

The precise molecular mechanisms that couple AT₁ receptor activation to these distinct signaling pathways have not been fully established. We and others have shown that both PYK2 and the closely related FAK can form signaling complexes with the upstream regulators of the ERK1/2 pathway, Src, Shc and Grb2 and with p130Cas, an adapter protein implicated in PI3-kinase activation (6,10,15). Govindarajan et. al. have demonstrated a role for FAK in Ang II-mediated VSMC protein synthesis (7). Little is known about the interrelationship between PYK2 and FAK signaling in response to Ang II in VSMC.

In the present study, we examined the role of PYK2 in Ang II-induced VSMC protein synthesis. We show that downregulation of PYK2 expression by antisense oligonucleotides resulted in a significant inhibition of Ang II-induced protein synthesis that was correlated with inhibition of ERK1/2, Akt and p70S6 kinase activation. Moreover, PYK2 antisense treatment caused a remarkable reduction in Ang II-induced FAK phosphorylation without any effect on FAK expression. A preliminary report has appeared(16).

**EXPERIMENTAL PROCEDURES**

*Materials* - PYK2 antisense oligonucleotides and scrambled control oligonucleotides were custom-made by BIOGNOSTIK (Göttingen, Germany). Anti-total PYK2 and anti-total FAK antibodies were from Transduction Laboratories. Anti-total ERK1/2 antibodies were from Santa Cruz. Anti-phospho ERK1/2 antibodies were from Promega. Anti-phospho Akt (pSer473), anti-phospho p70S6 kinase (pThr389, pSer424), anti-total Akt and anti-total p70S6 kinase antibodies
were from New England Biolabs. Anti-phospho FAK (pY397 and pY861) was from Biosource. 

[3H]-phenylalanine was from Amersham. Ang II was from Sigma.

**Cell Culture** - VSMC were prepared and cultured as previously described (6).

**PYK2 Antisense Oligonucleotide Incorporation** - VSMC were grown in 10% CS-DMEM to approximately 60% confluency. Cells were washed three times in Opti-MEM media (Gibco-BRL) one hour before antisense treatment. VSMC were treated with PYK2 antisense oligonucleotides (0.75 µM) for 8 hours. Lipofectamine Plus (10 µg/ml, Gibco-BRL) was used as a transfection reagent. After 8 hours, the media was replaced with 0.2% CS-DMEM and left overnight. The next day, 0.2% CS DMEM was replaced with serum free-DMEM for at least 1 hour prior to treatment with Ang II.

**Protein synthesis measurements** - VSMC were treated with PYK2 oligonucleotides (antisense and scrambled controls) as described above. The next day, 0.2% CS DMEM was replaced with serum free-DMEM for at least 8 hours prior to stimulation with 100 nM Ang II for 24 hours. During the last 6 hours of Ang II incubation, 1 µCi/ml of [3H]-phenylalanine (30 Ci/mmol, Amersham) was added to each dish. Total protein measurements were assessed over the 24-hour incubation period. VSMC were rinsed with 1 ml ice-cold PBS and protein was precipitated by 10% TCA for 30 minutes on ice. The TCA-precipitable material was solubilized with 0.2 mM NaOH for 20 minutes at 60°C. A portion of the sample was used to determine total protein using a bicinchoninic acid (Pierce) protein assay, and [3H]-phenylalanine was determined by liquid scintillation counting. Triplicate dishes were used for each measurement.

**Immunoblotting** - Western blot analysis was performed as described (10).

**Data Analysis** - Data are expressed as mean ± SEM for at least n=3 experiments. One-way Repeated Measures Analysis of Variance (ANOVA) followed by Bonferroni’s test was used for
comparisons among multiple groups. Differences among means were considered significant at 
P<0.05. Data were analyzed using InStat Statistical Software (Graphpad).

RESULTS

PYK2 antisense oligonucleotides downregulate PYK2 Expression - We first determined the 
effects of PYK2 antisense oligonucleotides on PYK2 expression. PYK2 antisense 
oligonucleotides decreased PYK2 total protein levels by 80% (0.20 ± 0.06 for antisense 
oligonucleotides vs. control) as measured by western blot analysis. PYK2 antisense treatment 
had no effect on the expression of the closely related kinase FAK (1.04 ± 0.04 for antisense 
oligonucleotides vs. control) as measured by western blot analysis with anti-total FAK antibodies 
(Figure 1). Transfection efficiency, as assessed by FITC-labeled antisense oligonucleotides, was 
>85% (data not shown).

PYK2 antisense oligonucleotides inhibit ERK1/2 activation in response to Ang II - We have 
previously shown that PYK2 interacts with Shc and Grb2, upstream activators of the ERK1/2 
signaling pathway (10). To demonstrate that PYK2 is necessary for Ang II-induced ERK1/2 
activation we examined the effects of PYK2 antisense oligonucleotides on ERK1/2 
phosphorylation. Downregulation of PYK2 resulted in a significant 77% (3.8± 1.3 increase vs. 
control for antisense oligonucleotides compared to 16.6 ± 1.8 fold increase vs. control for Ang 
II) decrease in Ang II-induced ERK1/2 activation as detected by western blot analysis with anti- 
phospho ERK1/2 antibodies. PYK2 antisense oligonucleotides did not effect ERK1/2 expression 
as determined by anti-total ERK1/2 antibodies (Figure 2).

PYK2 antisense oligonucleotides downregulate Ang II-induced Akt and p70S6 kinase 
activation - We have previously demonstrated that PYK2 associates with the adaptor molecule
PYK2 antisense oligonucleotides block Ang II-induced VSMC protein synthesis- To determine whether PYK2 is required for Ang II-induced VSMC protein synthesis, we measured the effects of PYK2 antisense oligonucleotides on Ang II-induced protein synthesis. Protein synthesis was measured using [3H]-phenylalanine incorporation during the last 6 hours of a 24 hour Ang II treatment. Ang II induced a significant increase in VSMC protein synthesis (2.1 fold vs. control). Treatment with PYK2 antisense oligonucleotides resulted in a complete inhibition of Ang II-induced protein synthesis (Figure 5). Neither lipofectamine alone nor scrambled oligonucleotides had any significant affect on Ang-II induced protein synthesis.

PYK2 antisense oligonucleotides downregulate Ang II-induced FAK phosphorylation at Y397 and Y861- Govindarajan et. al. have previously demonstrated a role for FAK in Ang II-induced protein synthesis (7). FAK activation results in its phosphorylation on multiple tyrosine residues including Y397, the autophosphorylation site, and Y861, a proposed docking site for the adapter.
molecules Grb2 and p130Cas. We sought to determine whether FAK phosphorylation on Y397 and Y861 in response to Ang II is dependent on PYK2. A decrease in PYK2 protein levels by PYK2 antisense oligonucleotides treatment resulted in a corresponding decrease in Y397 and Y861 phosphorylation of FAK, without any effect on FAK expression (Figure 6).

**DISCUSSION**

Angiotensin II is a potent mediator of VSMC hypertrophy. The intracellular signaling components that link AT₁ receptors to VSMC growth involve a complex network of protein-protein interactions and kinase cascades, but little is known about the intracellular signaling intermediates that link AT₁ receptor to these pathways. There is increasing evidence that non-receptor tyrosine kinases are the upstream regulators of signaling pathways important in the regulation of cellular growth. Here, using antisense oligonucleotide strategies, we establish PYK2 as a proximal signaling intermediate that links the AT₁ receptor activation to the activation of ERK1/2 and the activation of Akt and p70S6 kinase, downstream effectors of PI3-kinase signaling. Inhibition of these pathways by PYK2 downregulation resulted in a complete inhibition of Ang II-induced VSMC protein synthesis.

We chose an antisense strategy to downregulate PYK2 expression rather than overexpression of a dominant negative PYK2 mutant since we were concerned that protein overexpression could alter protein-protein interactions that are largely governed by protein concentration and localization (17). Treatment with PYK2 antisense oligonucleotides, but not scrambled oligonucleotides, lead to a significant downregulation of PYK2 in VSMC. This effect was specific since the expression of FAK and downstream signaling molecules was unaltered.
PYK2 has been shown to be an upstream regulator of a variety of cellular signaling pathways, including Src and multiple members of the mitogen activated protein kinase (MAP kinase) family (18). We and others have previously shown that Ang II induced complex formation between PYK2 and the upstream regulators of the ERK1/2 pathway, Src, Shc and Grb2 in VSMC (6,10) suggesting a role for PYK2 in the regulation of ERK1/2 activation in response to Ang II. Using PYK2 antisense oligonucleotides in this study, we conclusively show that PYK2 is required for Ang II-induced ERK1/2 activation (Figure 2).

Ang II has been shown to activate PI3-kinase (11), p70S6 kinase (19) and Akt(20) in VSMC. We have previously demonstrated a Ca\(^{2+}\)-dependent complex formation between PYK2, the adaptor molecule p130Cas and PI3-kinase in response to Ang II in manner (10). These data are suggestive of PYK2-dependent activation of the PI3-kinase signaling pathway. We now demonstrate that PYK2 is required for activation of the PI3-kinase signaling pathway by Ang II, since PYK2 downregulation significantly blocked Ang II-induced phosphorylation of both Akt and p70S6 kinase (Figures 3 and 4). On the other hand, Eguchi et al reported that Ca\(^{2+}\)-dependent transactivation of the EGF receptor was involved in Ang II-induced Akt and p70S6 kinase (19). Therefore it is possible that the Ca\(^{2+}\) sensitive PYK2 may play a role in Ang II-induced EGFR receptor transactivation to mediate PI3-kinase signaling.

Recent evidence suggests that ERK1/2 and PI3-kinase play a crucial role in Ang II-induced VSMC hypertrophy (7,13,21,22). These pathways appear to be independent in smooth muscle since inhibitors of PI3-kinase do not block Ang II-induced ERK1/2 activation (10) and inhibitors of ERK1/2 signaling have no effect on Ang II-induced activation of Akt (19). Since PYK2 is necessary for the activation of these pathways, we reasoned that PYK2 downregulation would prevent Ang II-induced protein synthesis. As shown in Figure 5, pretreatment with PYK2
antisense oligonucleotides completely blocked protein synthesis in response to Ang II. These results suggest that PYK2 links the AT$_1$ receptor to divergent signaling pathways that control VSMC protein synthesis.

The regulation of translation initiation is the rate-limiting step for protein synthesis. Both Akt and ERK1/2 are thought to regulate this step via phosphorylation of the eukaryotic initiation factor eIF-4E/PHAS-1 complex. eIF-4E mediates the initiation phase of mRNA translation, the rate-limiting step for protein synthesis (23). The availability of eIF-4E is regulated by PHAS-1; when phosphorylated, PHAS-1 disassociates from eIF-4E allowing the factor to participate in translation initiation (14). Both proteins are regulated via phosphorylation by ERK1/2 and PI3-kinase pathways (14,23). Thus, the ability of PYK2 antisense oligonucleotides to prevent Ang II-induced protein synthesis may be due, in part, to decreased phosphorylation of the eIF4E/PHAS-1 complex by ERK1/2 and PI3-kinase pathways. Future studies will elucidate the exact mechanisms involved in the regulation of translation initiation by PYK2.

In the present study, we show that PYK2 is involved in Ang II-induced FAK activation. A recent report, using overexpression of the C-terminal domain of FAK (FRNK), demonstrated that FAK is necessary for ERK1/2 activation and the induction of protein synthesis by Ang II (7). FRNK, however, inhibited Ang II-induced protein synthesis only partially which may be related to its inability to inhibit Ang II-induced activation of p70S6 kinase (7). Graves et al. also reported that p70S6 kinase is activated independently of FAK by an upstream, Ca$^{2+}$-sensitive tyrosine kinase (24). To link the inhibition of ERK1/2, Akt and p70S6 kinase phosphorylation observed in this study to specific downregulation of PYK2, we examined the effects of PYK2 antisense oligonucleotides on FAK expression. Under conditions where PYK2 was significantly
downregulated, PYK2 antisense oligonucleotides had no effect on FAK expression (Figure 1). We then determined if PYK2 was involved in Ang II-induced FAK phosphorylation. Using phospho-specific antibodies, we show that PYK2 downregulation lead to a significant decrease in Ang II-induced FAK phosphorylation at Y397 and Y861 (Figure 6). Y397 is an autophosphorylation site that can also be phosphorylated by Src in response to integrin engagement (25,26). Y861 has recently been shown to be a major site that is phosphorylated by Src (27). These data suggest that PYK2 may be at least one of the upstream regulators of FAK activation by Ang II.

The mechanisms by which PYK2 links AT₁ receptor activation to FAK phosphorylation remain to be elucidated. One possible mechanism is that the adapter protein p130Cas assembles both PYK2 and FAK in response to Ang II. The proline rich motifs of both PYK2 and FAK are thought to bind to the SH3 domains of the large adapter protein p130Cas. We have previously shown that PYK2, p130Cas and PI3-kinase form a signaling complex in VSMC in response to Ang II (10), and others have shown an interaction between FAK and p130Cas in other cell types (28). Therefore, it is tempting to speculate that this adaptor protein is a necessary link between PYK2 and FAK activation. On the other hand, the PYK2-Src complex that forms in response to Ang II activation (6) may directly activate FAK. Furthermore, PYK2 can localize into focal adhesions in response to G-protein-coupled receptor and PKC activation, resulting in PYK2 binding to the focal adhesion proteins paxillin and p130Cas (29).

In summery, our data establish a requirement for PYK2 in the activation of two signaling pathways implicated in the regulation of VSMC growth, the ERK1/2 and the PI3-kinase/Akt pathways in response to Ang II. Furthermore, we establish a requirement for PYK2 in the initiation of Ang II-induced VSMC protein synthesis, the major hallmark of VSMC hypertrophy.
Thus, PYK2 may represent an important molecular target for molecular and pharmacological strategies to minimize VSMC growth in vivo.
FOOTNOTES

1 The abbreviations used are: VSMC, vascular smooth muscle; Ang II, angiotensin II. PYK2, proline rich tyrosine kinase 2; FAK, focal adhesion kinase; ERK1/2, extracellular signal recognition kinase 1/2; MAP kinase, mitogen activated protein kinase; PI3-kinase, phosphatidylinositol 3- kinase; p70S6 kinase, 70-kDa ribosomal S6 kinase; PHAS-1, phosphorylated heat and acid stable protein; eIF4E, eukaryotic initiation factor 4E; p130Cas, 130-kDa Crk-associated substrate; CS, calf serum; TCA, trichloroacetic acid; EGF, epidermal growth factor.

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REFERENCES


FIGURE LEGENDS.

**Figure 1.** PYK2 antisense oligonucleotides selectively reduce PYK2 expression. Lysates from control VSMC (lane 1), from cells treated with lipofectamine alone (lane 2), scrambled control oligonucleotides (S-ODN, lane3) or PYK2 antisense oligonucleotides (AS ODNs, lane 4), for 8 hours, were placed in 0.2% CS DMEM overnight. Top panel: representative western blots using anti-total PYK2 and FAK antibodies. Bottom panel: cumulative results of n=6 experiments. *p<0.05 vs. control; ‡p<0.05 vs. scrambled oligonucleotides.

**Figure 2.** PYK2 antisense oligonucleotides downregulate ERK1/2 activation by Ang II. Lysates from control VSMC (lanes 1 and 2), from cells treated with lipofectamine alone (lanes 3 and 4), scrambled control oligonucleotides (S-ODN, lanes 5 and 6) or PYK2 antisense oligonucleotides (AS ODNs, lanes 7 and 8), for 8 hours, were placed in 0.2% CS DMEM overnight then treated with 100 nM Ang II for 5 minutes as indicated. Top panel: representative western blots using anti-total PYK2, anti-phospho ERK1/2 and anti-total ERK1/2 antibodies. Bottom panel: cumulative results of n=6 experiments. *p<0.05 vs. control; #p<0.05 vs. Ang 5; ‡p<0.05 vs. scrambled oligonucleotides.

**Figure 3.** PYK2 antisense oligonucleotides downregulate Akt activation by Ang II. Lysates from control VSMC (lanes 1 and 2), from cells treated with lipofectamine alone (lanes 3 and 4), scrambled control oligonucleotides (S-ODN, lanes 5 and 6) or PYK2 antisense oligonucleotides (AS ODNs, lanes 7 and 8), for 8 hours, were placed in 0.2% CS DMEM overnight then treated with 100 nM Ang II for 5 minutes as indicated. Top panel: representative western blots using anti-total PYK2, anti-phospho Akt and anti-total Akt antibodies. Bottom panel: cumulative
results of n=6 experiments. *p<0.05 vs. control; #p<0.05 vs. Ang 5; ‡p<0.05 vs. scrambled oligonucleotides.

**Figure 4.** PYK2 antisense oligonucleotides downregulate p70S6 kinase activation by Ang II. Lysates from control VSMC (lanes 1 and 2), from cells treated with lipofectamine alone (lanes 3 and 4), scrambled control oligonucleotides (S-ODN, lanes 5 and 6) or PYK2 antisense oligonucleotides (AS ODNs, lanes 7 and 8), for 8 hours, were placed in 0.2% CS DMEM overnight then treated with 100 nM Ang II for 5 minutes as indicated. Top panel: representative western blots using anti-total PYK2, anti-phospho p70S6 kinase and anti-total p70S6 kinase antibodies. Bottom panel: cumulative results of n=6 experiments. *p<0.05 vs. control; #p<0.05 vs. Ang 5; ‡p<0.05 vs. scrambled oligonucleotides.

**Figure 5.** PYK2 antisense oligonucleotides block Ang II-induced protein synthesis. VSMC were treated with lipofectamine, scrambled control oligonucleotides (S-ODN) or PYK2 antisense oligonucleotides (AS-ODN) as indicated for 8 hours, placed in 0.5% CS DMEM overnight, transferred to 0.02% CS DMEM for at least 6 hours then treated with 100 nM Ang II for 24 hours. [3H]-phenylalanine was added for the last 6 hours of Ang II treatment. Cumulative data are presented as DPM [3H]-phenylalanine incorporated over total protein. *p<0.05 vs. control; #p<0.05 vs. Ang 5; ‡p<0.05 vs. scrambled oligonucleotides.

**Figure 6.** PYK2 antisense oligonucleotides decrease FAK phosphorylation on Y397 and Y861. Lysates from control VSMC (lanes 1 and 2), from cells treated with lipofectamine alone (lanes 3 and 4) or PYK2 antisense oligonucleotides (AS ODNs, lanes 5 and 6), for 8 hours, were placed
in 0.2% CS DMEM overnight then treated with 100 nM Ang II for 5 minutes as indicated.
Representative western blots using anti-total PYK2, anti-phospho Y397FAK, anti-phospho
Y861FAK and anti total FAK antibodies are shown.
Figure 1.
Figure 2.
Figure 3.
0.75 µM PYK2 AS ODN - - - - - - + +
0.75 µM scrambled control - - - - + + - -
10 µg lipofectamine - - + + + + + +
100 nM Ang II (5 min) - + - + - + - +

anti-PYK2

anti-pp70S6K

anti-p70S6K

CTRL Ang 5 LFN LFN + S-ODN S-ODN + Ang 5 AS-ODN AS ODN + Ang 5

pp70S6

Figure 4.
Figure 5.
0.75 μM PYK2 AS ODN
10 μg lipofectamine
100 nM Ang II (5 min)

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Figure 6.
Downregulation by antisense oligonucleotides establishes a role for the proline-rich tyrosine kinase PYK2 in angiotensin II-induced signaling in vascular smooth muscle
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