Down-regulation 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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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 2 (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) pathways. PYK2 antisense oligonucleotides were used to down-regulate PYK2 expression in cultured VSMC. An 80% down-regulation in PYK2 expression resulted in an 80% inhibition of ERK1/2 (3.8 ± 1.3 versus 16.6 ± 1.8), p70S6 kinase (1.03 ± 0.03 versus 3.8 ± 0.5), and Akt activation (3.0 ± 0.8 versus 16.0 ± 1.0) by Ang II. Furthermore, PYK2 down-regulation 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 phosphatidylinositol 3-kinase/Akt pathways that are involved in Ang II-induced VSMC protein synthesis.

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 2 (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 and PI3-kinase/Akt pathways control cell growth 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 protein S6 kinase, is 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 phosphorylated heat and acid-stable protein 1 (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. (7) have demonstrated a role for FAK in Ang II-mediated VSMC protein synthesis. 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 down-regulation 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). Oligonucleotides were annealed and purified by high-performance liquid chromatography or reverse phase HPLC. Oligonucleotides were custom-made by BIOGNOSTIK (Göttingen, Germany). Oligonucleotides were annealed and purified by high-performance liquid chromatography or reverse phase HPLC.
PYK2 Regulates VSMC Protein Synthesis

PYK2 Antisense Oligonucleotides Down-regulate PYK2 Expression—We first determined the effects of PYK2 antisense oligonucleotides on PYK2 expression. PYK2 antisense oligonucleotides decreased PYK2 total protein levels by 50% (0.20 ± 0.06 for antisense oligonucleotides versus 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 versus control) as measured by Western blot analysis with anti-total FAK antibodies (Fig. 1). Transfection efficiency, as assessed by fluorescence in isothiocyanate-labeled antisense oligonucleotides, was >85% (data not shown).

Results

PYK2 Antisense Oligonucleotides Inhibit ERK1/2 Activation in Response to Ang II—We have previously shown that PYK2 interacts with Src 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. Down-regulation of PYK2 resulted in a significant 77% (3.8 ± 1.3-fold increase versus control for antisense oligonucleotides compared with 16.6 ± 1.8-fold increase versus control) 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 affect ERK1/2 expression as determined by anti-total ERK1/2 antibodies (Fig. 2).

PYK2 Antisense Oligonucleotides Down-regulate Ang II-induced Akt and p70S6 Kinase Activation—We have previously demonstrated that PYK2 associates with the adaptor molecule p130Cas and with PI3-kinase in response to Ang II (10). Here we sought to determine whether PYK2 down-regulation by PYK2 antisense oligonucleotides would prevent Ang II-induced activation of Akt and p70S6 kinase, the major downstream effectors of the PI3-kinase signaling pathway. Here we show that Akt and p70S6 kinase are activated in response to Ang II. PYK2 down-regulation resulted in an ~80% inhibition (3.00 ± 0.82-fold increase versus control for antisense oligonucleotides compared with 16.0 ± 1.0-fold increase versus control for Ang II) in Ang II-induced Akt and a 76% inhibition of p70S6 kinase activation (1.03 ± 0.03-fold increase versus control for antisense oligonucleotides compared with a 3.8 ± 0.5-fold increase versus control for Ang II), as measured by Western blot analysis with anti-phospho Akt and anti-phospho p70S6 kinase antibodies, respectively. PYK2 antisense oligonucleotides did not affect Akt or p70S6 kinase expression as measured with anti-total Akt and anti-total p70S6 kinase antibodies (Figs. 3 and 4).

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 h of a 24-h Ang II treatment. Ang II induced a significant increase in VSMC protein synthesis (2.1-fold increase versus control). Treatment with PYK2 antisense oligonucleotides resulted in a complete inhibition of Ang II-induced protein synthesis (Fig. 5). Neither LipofectAMINE alone nor scrambled oligonucleotides had any significant effect on Ang II-induced protein synthesis.
PYK2 Antisense Oligonucleotides Down-regulate Ang II-induced FAK Phosphorylation at Tyr-397 and Tyr-861—Govindarajan et al. (7) have previously demonstrated a role for FAK in Ang II-induced protein synthesis. FAK activation results in its phosphorylation on multiple tyrosine residues including Tyr-397, the autophosphorylation site, and Tyr-861, a proposed docking site for the adapter molecules Grb2 and p130Cas. We sought to determine whether FAK phosphorylation on Tyr-397 and Tyr-861 in response to Ang II is dependent on PYK2. A decrease in PYK2 protein levels by PYK2 antisense oligonucleotide treatment resulted in a corresponding decrease in Tyr-397 and Tyr-861 phosphorylation of FAK, without any effect on FAK expression (Fig. 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 the 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 established PYK2 as a proximal signaling intermediate that links the AT1 receptor 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 down-regulation resulted in a complete inhibition of Ang II-induced VSMC protein synthesis.

We chose an antisense strategy to down-regulate PYK2 expression rather than overexpression of a dominant negative PYK2 mutant because 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 down-regulation of PYK2 in VSMC. This effect was specific, because the expression of FAK and downstream signaling molecules was unaffected.

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 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 showed that PYK2 is required for Ang II-induced ERK1/2 activation (Fig. 2).

Ang II has been shown to activate PI3-kinase (11), p70S6 kinase (19), and Akt (20) in VSMC. We have previously demonstrated a Ca2+-dependent complex formation between PYK2, the adaptor molecule p130Cas, and PI3-kinase in response to Ang II (10). These data are suggestive of PYK2-dependent activation of the PI3-kinase signaling pathway. We here demonstrated that PYK2 is required for activation of the PI3-kinase signaling pathway by Ang II, because PYK2 down-regulation significantly blocked Ang II-induced phosphorylation of both Akt and p70S6 kinase (Figs. 3 and 4). On the other hand, Eguchi et al. (19) reported that Ca2+-dependent transactivation of the epidermal growth factor receptor was involved in Ang II-induced Akt and p70S6 kinase. Therefore it is possible that the Ca2+-sensitive PYK2 may play a role in Ang II-induced epidermal growth factor receptor...
PYK2 Regulates VSMC Protein Synthesis

PYK2 antisense oligonucleotides decrease FAK phosphorylation on Tyr-397 and Tyr-861. Lysates from control VSMC (lanes 1 and 2) or from cells treated with LipofectAMINE alone (lanes 3 and 4) or PYK2 antisense oligonucleotides (AS ODN, lanes 5 and 6), for 8 h, were placed in 0.2% CS-DMEM overnight and then treated with 100 nM Ang II for 5 min as indicated. Representative Western blots using anti-total PYK2, anti-phospho Y397FAK, anti-phospho Y861FAK, and anti-total FAK antibodies are shown.

Because PYK2 is necessary for the activation of these pathways, we reasoned that PYK2 down-regulation would prevent Ang II-induced protein synthesis. As shown in Fig. 5, pretreatment with PYK2 antisense oligonucleotides completely blocked protein synthesis in response to Ang II. These results suggest that PYK2 links the AT1 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 eIF4E/PHAS-1 complex. eIF4E mediates the initiation phase of mRNA translation, the rate-limiting step for protein synthesis (23). The availability of eIF4E is regulated by PHAS-1; when phosphorylated, PHAS-1 dissociates from eIF4E, 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. (24) also reported that p70S6 kinase is activated independently of FAK by an upstream, Ca2+-sensitive tyrosine kinase. To link the inhibition of ERK1/2, Akt, and p70S6 kinase phosphorylation observed in this study to specific down-regulation of PYK2, we examined the effects of PYK2 antisense oligonucleotides on FAK expression. Under conditions where PYK2 was significantly down-regulated, PYK2 antisense oligonucleotides had no effect on ERK1/2 and PI3-kinase pathways (14, 23). Future studies will elucidate the exact mechanisms involved in the regulation of translation initiation by PYK2.

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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 resemble 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 speculate that this adaptor protein is a necessary link between PYK2 and FAK activation. In summary, 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.

REFERENCES
1. Huckle, W. R., and Earp, H. S. (1994) Prog. Growth Factor Res. 5, 177–194
2. Pratt, R. E. (1996) Blood Press. Suppl. 2, 6–9
3. Owens, G. K. (1995) Physiol. Rev. 75, 487–517
4. Ishida, M., Marrero, M. B., Schieffer, B., Ishida, T., Bernstein, K. E., and Berk, B. C. (1998) Circ. Res. 83, 841–851
5. Brinson, A., Harding, T., Diliberto, P., He, Y., Li, X., Hunter, D., Herman, B., Earp, H., and Graves, L. (1998) J. Biol. Chem. 273, 1711–1718
6. Sabri, A., Govindarajan, G., Griffin, T., Byron, K., Samarel, A., and Lucchesi, B. C. (1996) Circ. Res. 83, 710–716
7. Govindarajan, G., Ehle, D. M., Lucchesi, P. A., and Samarel, A. M. (2000) Circ. Res. 87, 710–716
8. Berk, B., and Corson, M. (1997) Circ. Res. 80, 687–616
9. Lucchesi, P. A., Bell, J. M., Willis, L. S., Byron, K. L., Corson, M. A., and Berk, B. C. (1996) Circ. Res. 78, 962–979
10. Rocic, P., Govindarajan, G., Sabri, A., and Lucchesi, P. A. (2001) Am. J. Physiol. 280, C90–C99
11. Saward, L., and Zahradka, P. (1997) Circ. Res. 81, 249–257
12. Servant, M., Giasson, E., and Meloche, S. (1996) J. Biol. Chem. 271, 16047–16052
13. Giasson, E., and Meloche, S. (1995) J. Biol. Chem. 270, 5225–5231
14. Lawrence, J. C., and Abraham, R. T. (1997) Trends Biochem. Sci. 22, 345–349
15. Eguchi, S., Iwasaki, H., Inagami, T., Numaguchi, K., Yamakawa, T., Metley, E., Owada, K., Marumo, F., and Hirata, Y. (1999) Hypertension 33, 201–206
16. Rocic, P., and Lucchesi, P. A. (2001) FASEB J. 15, 488 (abstr.)
17. Koller, E., Gaarde, W. A., and Mania, B. P. (2000) Trends Pharmacol. Sci. 21, 142–148
18. Avraham, H., Park, S. Y., Schinkmann, K., and Avraham, S. (2000) Cell. Signalling 12, 123–133
19. Eguchi, S., Iwasaki, H., Ueno, H., Frank, G. D., Motley, E. D., Eguchi, K., Marumo, F., Hirata, Y., and Inagami, T. (1999) J. Biol. Chem. 274, 36843–36851
20. Ushio-Fukai, M., Alexander, R. W., Akers, M., Yin, Q., Fujio, Y., Walsh, K., and Griendling, K. K. (1999) J. Biol. Chem. 274, 22099–22704
21. Hou, M., Pantev, E., Eble, D. M., Lucchesi, P. A., and Samarel, A. M. (2000) J. Biol. Chem. 275, 16018–16022
22. Sonenberg, N., and Gingras, A. C. (1998) Curr. Opin. Cell Biol. 10, 268–275
23. Graves, L. M., He, Y., Lambert, J., Hunter, D., Li, X., and Earp, H. S. (1997) J. Biol. Chem. 272, 1920–1926
24. Schlaepfer, D. D., Hauck, C. R., and Sieg, D. J. (1999) Prog. Biophys. Mol. Biol. 71, 435–478
25. Harte, M. T., Hildebrand, J. D., Burnham, M. R., Bouton, A. H., and Parsons, J. T. (1996) J. Biol. Chem. 271, 13649–13655
26. Litvak, V., Tuan, D., Shaul, Y. D., and Lev, S. (2000) J. Biol. Chem. 275, 32736–32746
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