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Role of Phosphatidylinositol 3-kinase/Akt Pathway in Angiotensin II and Insulin-like Growth Factor-1 Modulation of Nitric Oxide Synthase in Vascular Smooth Muscle Cells

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The aim of this study was to examine the role of the phosphatidylinositol 3-kinase (PI3K)/serine/threonine kinase Akt signaling pathway in mediating interactions between angiotensin II (Ang II) and insulin-like growth factor-1 (IGF-1) in regulation of inducible nitric oxide synthase (iNOS) in vascular smooth muscle cells (VSMCs). Exposure to 100 n*M* IGF-1 for 10 min resulted in increased insulin-receptor substrate-1 associated PI3K activity and Akt kinase activity, whereas 100 n*M* Ang II pretreatment for 5 min strikingly decreased these IGF-1 effects. NOS activity was also increased in VSMCs following exposure to IGF-1 (10 min up to 24 h). Pretreatment with Ang II for 5 min reduced IGF-1-induced NOS activity. IGF-1 treatment for 24 hr increased iNOS gene transcription, and Ang II pretreatment reduced this stimulation of iNOS gene expression by attenuating PI3K/Akt signaling. These results implicate PI3K/Akt pathways in Ang II/IGF-1 regulation of iNOS in VSMCs.

Key Words: Angiotensin II; nitric oxide synthase; insulin-like growth factor-1; phosphatidylinositol 3-kinase; Akt; vascular smooth muscle cells.

Introduction

Insulin-like growth factor-1 (IGF-1) is locally generated in vascular smooth muscle cells (VSMCs), where it plays an important role in the regulation of vascular tone *(1,2)*. IGF-1 is synthesized by vasculature under the control of angiotensin II (Ang II) and other growth factors, and thus acts as an autocrine/paracrine peptide *(1–3)*. One of the major pathways of IGF-1 signal transduction is activation of phosphatidylinositol 3-kinase (PI3K), which binds to insulin-receptor substrate-1 (IRS-1) through its regulatory subunit (p85) SH2 domain, thus activating its catalytic subunit (p110) *(4–6)*.

Serine/threonine protein kinase B (Akt) is an important downstream target of PI3K *(7,8)*. Akt has received special attention because of its central role in the regulation of VSMC metabolism including nitric oxide (NO) production *(9)*. This kinase can be activated by growth factor receptors of the tyrosine kinase class, a process that involves the tyrosine phosphorylation–dependent activation of PI3K *(7,10)*.

Ang II is a peptide that is also produced by the vasculature and acts in an autocrine/paracrine fashion, to cause vasoconstriction through a VSMC AT_1 receptor *(11,12)*. Recently, in vitro studies in cardiomyocytes and VSMCs have suggested that Ang II can inhibit insulin and IGF-1-induced increases in PI3K activity/association with IRS proteins *(13, 14)*. Thus, excess Ang II may produce a vascular resistance to insulin/IGF-1 via attenuation of the ability of IGF-1 to stimulate the PI3K/Akt signaling pathways.

NO is a potent endogenous vasodilator whose vascular synthesis is regulated by two of the three major types of NOS: a constitutive form (cNOS, NOS III) normally expressed in endothelial cells and an inducible form (iNOS, NOS II) mostly expressed in VSMCs after exposure to different stimuli *(15,16)*. iNOS is believed to be the critical isoform in VSMCs, although all three of the NOS isoforms have been detected in these cells *(16)*. NO produced by the vasculature decreases vascular tone, inhibits VSMC proliferation, and reduces cell adhesion to vascular walls *(17,18)*.

The aim of the present study was to investigate possible interactions between Ang II and IGF-1 in the regulation of iNOS activity/transcription via the PI3K/Akt signaling pathway in VSMCs. Specifically, we addressed the hypothesis that Ang II attenuates the ability of IGF-1 to increase iNOS activity by interfering with signaling through the PI3K/Akt pathway in VSMCs.

Results

Effect of IGF-1 and Ang II on IRS-1-Associated PI3K Activity

To evaluate interactions between Ang II and IGF-1 on the IRS-1 signaling pathway in VSMCs, cells were stimulated

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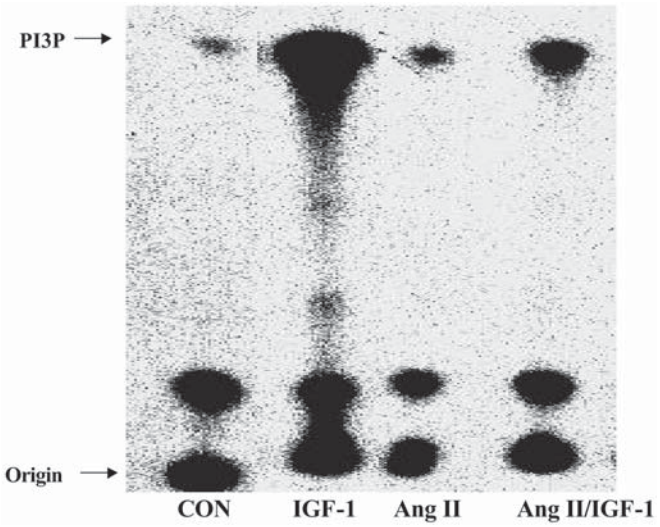

Fig. 1. Effect of Ang II and IGF-1 on IRS-1-associated PI3K activity. VSMCs were treated with 100 n*M* IGF-1 (10 min), 100 n*M* Ang II (5 min), or pretreated with Ang II for 5 min and then with IGF-1 for an additional 10 min. A representative autoradiogram from thin-layer chromatography (TLC) performed on PI3K product is shown.

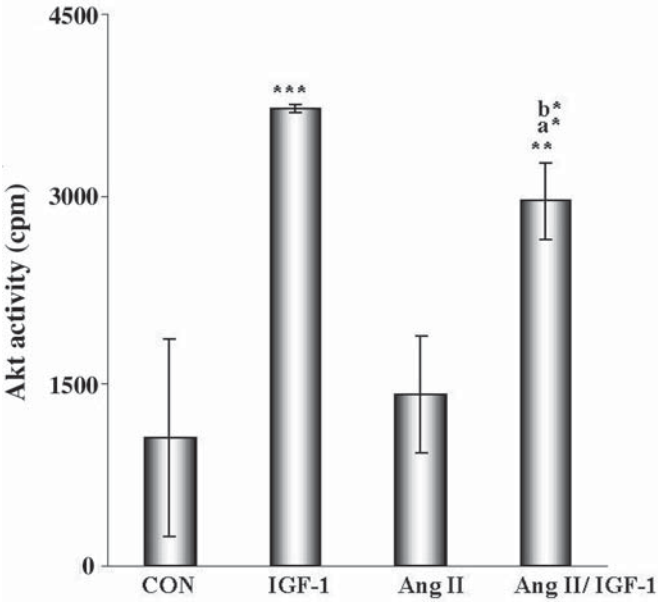

Fig. 2. Effect of Ang II and IGF-1 on Akt kinase activity in VSMCs. Cells were treated with 100 n*M* IGF-1 (10 min), 100 n*M* Ang II (5 min), or pretreated with Ang II for 5 min before IGF-1 treatment for an additional 10 min. Akt kinase activity was assayed in anti-Akt/immunoprecipitates using Akt/SKG as a substrate. Data are the mean ± SEM of up to four experiments: $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ vs control values; [a]indicates $*p < 0.05$ IGF-1 vs Ang II/IGF-1 and [b]indicates $*p < 0.05$ Ang II vs Ang II/IGF-1.

for 10 min with 100 n*M* IGF-1 in the absence or presence of 100 n*M* Ang II preincubation for 5 min. PI3K activity associated with IRS-1 precipitates was measured using an in vitro assay, and results are shown in Fig. 1. IRS-1-associated PI3K activity was present in the basal state, and this was stimulated 240% by IGF-1. Pretreatment with Ang II for 5 min decreased IGF-1-stimulated IRS-1-associated PI3K activity by 66% (Fig. 1). Thus, these results indicate that Ang II attenuates IGF-1-induced stimulation of PI3K as evidenced by attenuated enzyme activity in VSMCs.

Effect of IGF-1 and Ang II on Akt Kinase Activity in VSMCs

We next investigated whether Akt, an important downstream target for PI3K *(7,8)*, is involved in IGF-1 and Ang II interactions in VSMCs. It has been shown that a maximal effect on Akt phosphorylation is achieved 5 min after Ang II (100 n*M*) treatment in VSMCs *(19)*. Since pretreatment with Ang II for 5 min decreased IGF-1-induced IRS-1/PI3K activation, we assessed the effect of Ang II on IGF-1-induced Akt kinase activity at that time point. Stimulation of VSMCs with IGF-1 (10 min) caused an increase in Akt activity (Fig. 2) whereas stimulation with Ang II for 5 min did not increase Akt activity. As shown in Fig. 2, IGF-1 treatment for 10 min caused a 353% increase in Akt kinase activity when compared to control values. Interestingly, pretreatment with Ang II for 5 min and then with IGF-1 for an additional 10 min attenuated IGF-1-stimulated Akt kinase activity (Fig. 2). Thus, activation of PI3K/Akt pathways is involved in IGF-1 and Ang II signaling interactions.

Role of PI3K in IGF-1- and Ang II–Regulated NOS Activity

We examined whether IGF-1, Ang II, or combinations of both hormones were capable of increasing NOS activity. IGF-1 given for 10 min and 24 h increased NOS activity, whereas no changes were detected after Ang II treatment for 5 min (Fig. 3A) and 24 h (Fig. 3B), as measured by citrulline production. In addition, Ang II pretreatment for 5 min attenuated IGF-1-stimulated NOS activity (Fig. 3A). Furthermore, when IGF-1 and ANG II were given together for 24 h, Ang II attenuated IGF-1-stimulated NOS activity (Fig. 3B).

Effect of IGF-1 and Ang II on iNOS Gene Transcription

To delineate further the molecular mechanisms that underlie the Ang II/IGF-1 effect on iNOS gene transcription, VSMCs were transiently transfected with a luciferase reporter plasmid containing a portion of the rat iNOS promoter between −1026 and +16 bp. Exposure of cells to 100 n*M* IGF-1 for 48 h resulted in a 188 ± 31% increase in luciferase activity compared to control, whereas the −1206 to +16 portion of the iNOS promoter was unresponsive to Ang II (Fig. 4). Ang II abolished IGF-1-stimulated iNOS luciferase activity. These data indicate that IGF-1 signaling through the

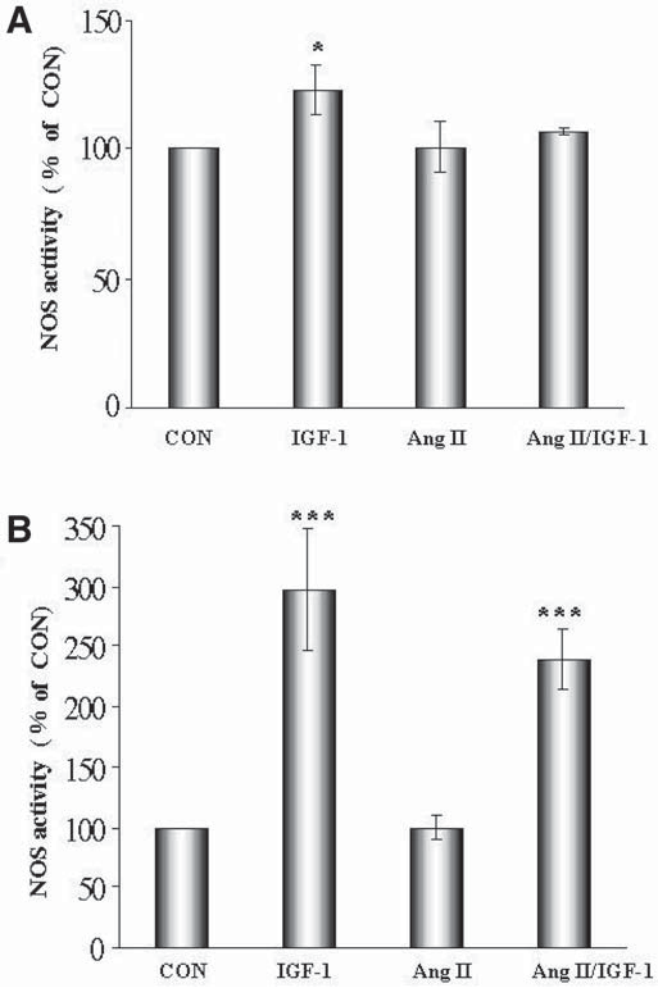

Fig. 3. Role of PI3K in IGF-1 and Ang II regulation of NOS activity. Cells were (**A**) exposed to acute (10-min) stimulation to 100 n*M* IGF-1 or cells were pretreated with Ang II (100 n*M*) for 5 min before treatment with IGF-1 for an additional 10 min or (**B**) cells were treated with both hormones for 24 h. Citrulline production was expressed as percentage of control (arbitrarily set at 100%). Data are the mean ± SEM of up to six experiments. $*p < 0.05$ and $***p < 0.001$ vs control values.

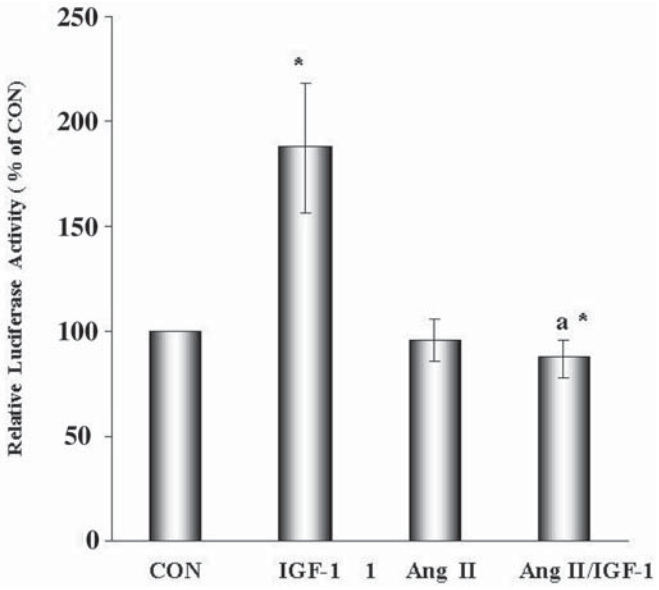

Fig. 4. IGF-1 and Ang II regulation of iNOS gene transcription. A luciferase reporter plasmid containing a portion of the iNOS promoter between −1026 and +16 bp was transiently transfected into VSMCs and control values were assessed as a relative value of 100%. Cells were treated with IGF-1 or Ang II or both hormones for 48 h. Data are the mean ± SEM of up to three experiments performed in duplicate. $*p < 0.05$ vs control values; [a]indicates $*p < 0.05$ IGF-1 vs Ang/IGF-1.

PI3K pathway is necessary for iNOS gene transcription, and that Ang II attenuates this process.

Discussion

Important findings of our study included the observation that Ang II inhibits IGF-1 inductions of iNOS activity/gene transcription and that the PI3K/Akt signaling pathway mediates, in part, the interactive effects of IGF-1 and Ang II in VSMCs.

Previous studies of Ang II/insulin interactions in cardiac tissue and VSMCs *(14)* have shown that Ang II can decrease insulin activation of PI3K, even in the presence of an increased amount of p85 associated with IRS-1. Since our initial results also suggested that the inhibitory effects of Ang II on IGF-1 involve signaling via the PI3K system, the effect of Ang II on IGF-1-induced IRS-1-associated PI3K activity was evaluated. Ang II inhibited IGF-1-stimulated PI3K activity assayed in the IRS-1 immunoprecipitates. This inhibitory effect occurred rapidly, with only a 5-min preincubation with Ang II as previously observed with insulin signaling. Thus, excess Ang II may produce a vascular resistance to IGF-1 and insulin via attenuation of the ability of these peptides to stimulate the PI3K pathway and, thus, NOS activity in VSMCs.

To determine further the mechanism of IGF-1 and Ang II interactions, we measured Akt activity by using an Akt in vitro kinase assay. In this investigation, IGF-1 stimulated Akt, the downstream signaling kinase from PI3K *(8)*. This observation is in accordance with several recent reports that IGF-1, insulin, and Ang II activate Akt in rat aortic VSMCs *(20)* as well as in endothelial cells *(19)*. In addition, results from our study demonstrate that Ang II inhibited IGF-1-mediated Akt activation in VSMCs. Akt activation appears to be a critical signaling pathway for IGF-1 stimulation of the NOS activity in VSMCs. Thus, current data indicate an important role of Akt as well as the upstream PI3K pathway in mediating interactions between Ang II and IGF-1 in the regulation of iNOS in VSMCs.

Locally produced IGF-1, through autocrine/paracrine actions, has vasodilating activity in vivo *(1,21–23)*. Prior investigation has shown that this vasodilating effect is related

to vascular NO production *(21–24)*. The current investigation showed that IGF-1 increased iNOS gene expression/activity in primary cultured aortic VSMCs. Further, Ang II inhibited IGF-1-stimulated NOS activity as well as IRS-1/p85 docking. While the specific role of IGF-1-stimulated PI3K in the vasculature has not yet been established, current results suggest that this pathway is important for IGF-1-stimulated NOS activity in VSMCs, as has been previously shown for endothelial cells *(9,19)*.

Many transcription factors that bind to the promoter region of the iNOS gene may stimulate its transcription *(17)*. We used transient transfection of the iNOS promoter to show that IGF-1 regulates iNOS in part through a transcriptional mechanism. After transfection of a 1026iNOS Luc construct into VSMCs, IGF-1 was observed to stimulate the luciferase reporter gene 1.8-fold. IGF-1 increased promoter activity of the iNOS gene in transfected VSMCs, suggesting a direct action of transcription machinery. These results are commensurate with a prior report that IGF-1 increases interleukin-1β induced iNOS protein expression and NO production *(24)*. In our investigation it was observed, for the first time, that Ang II inhibits the iNOS promoter activity induced by IGF-1. These data also agree with reports demonstrating that Ang II inhibits cytokine-stimulated iNOS gene expression in astroglial cells *(25)*. Unlike the constitutively expressed eNOS, the regulation of iNOS principally occurs at the level of transcription. Because inhibition of the PI3K/Akt pathway decreased iNOS gene expression, the predominant inhibitory effect of Ang II on IGF-1-induced iNOS expression quite likely occurs at the level of PI3K/Akt signaling. Results from the current investigation suggest that Ang II can contribute to IGF-1 regulation of iNOS in the vasculature, perhaps by inducing serine phoshorylation and/or tyrosine dephosphorylation of IRS-1. Because p85 represents the main regulatory component of IRS-1-mediated PI3K activation, multiple defects in PI3K activation involving p85 may contribute to IGF-1 resistance.

To conclude, the current study demonstrates, for the first time, that Ang II–mediated inactivation of the PI3K/Akt pathway reduces IGF-1-stimulated iNOS in VSMCs. The data indicate that there is a close interaction of Ang II and IGF-1 in VSMCs through common signaling mechanisms. Further, the data suggest that in vivo, where both peptides are produced by VSMCs *(26)*, relative effects on PI3K/Akt signaling and NOS activation may be dependent on the relative ambient levels of each of them, autocrine/paracrine peptides.

Materials and Methods

Cell Culture

VSMCs were isolated from Sprague-Dawley rat thoracic aortic rings by enzymatic dissociation as described previously *(27)*. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum. At 1 d postconfluence, cells were made quiescent by 19- to 20-h incubation in serum-free DMEM, before adding test compounds. The Animal Research Committee of the State University of New York HSC at Brooklyn approved the animal experimentation.

Preparation of Cell Lysates

VSMCs were treated with agonists, 100 n*M* IGF-1 (Genetech, San Francisco, CA) or 100 n*M* Ang II (Sigma, St. Louis, MO), for 10 and 5 min, respectively. Protein was isolated from VSMCs using buffers and protease inhibitors as described previously *(19,28)*. Supernatants were used for immunoprecipitation with anti-IRS-1 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Akt/PKB (Upstate Biotechnology, Lake Placid, NY).

Assay of IRS-1-Associated PI3K Activity

Cell lysates were immunoprecipitated from aliquots of the resulting supernatants by incubating them overnight at 4ºC with 4 µg of IRS-1 antibody as previously described *(28)*. Immunocomplexes were absorbed onto protein A–agarose beads and washed successively, and PI3K activity was determined according to methods previously described *(19,28)*. Briefly, lipids were extracted by chloroform:ethanol and separated by TLC on oxalate-pretreated Silica Gel 60 plates. Cold PI, PIP, and PIP_2 were run as standards and visualized by primulin staining. ^{32}P-labeled phoshatidylinositol products were measured using a Phosphor Imager and calculated by the IMAGEQUANT software (Molecular Dynamics, Sunnyvale, CA).

In Vitro Akt Kinase Assay

Akt kinase activity was assayed using an Akt kinase assay previously described in detail *(29)*. Briefly, an antibody to Akt was used to selectively immunoprecipitate Akt from cell lysates. The resulting immunoprecipitates were prepared from lysates containing 200 µg of proteins and then incubated with an Akt/SGK substrate in the presence of 1 µCi/µL of [γ-^{32}P] adenosine triphosphate (ATP). Supernatant fractions were transferred onto the center of a 2 × 2 cm P81 phosphocellulose paper. The assay squares were washed and the bound radioactivity was quantitated on the paper in a scintillation counter for 1 min. A suitable blank was performed to correct for nonspecific binding of [γ-^{32}P] ATP and its breakdown products to the phosphocellulose paper. Mouse monoclonal IgG and/or sample extracts plus/minus treatment was used to subtract the nonspecific binding and phosphorylation of the specific substrate from the experimental test sample *(29)*.

Determination of NOS Activity

Citrulline, a coproduct of the NO biosynthetic reaction, was measured using a colorimetric assay as described previously *(19)*. L-Citrulline concentrations in the sample were calculated from a citrulline standard curve. Data from duplicate wells were averaged and expressed as percentage of control (arbitrarily set to 100%).

Transfection and Luciferase Assay

VSMCs were transfected with cytofectene reagents (Bio-Rad, Hercules, CA) as previously described *(30)*. In brief, each plate containing a subconfluent culture was transfected with 1 μg of iNOS (−1026 to +16)-firefly luciferase promoter reporter and 20 ng of Renilla Lucifearse-pRL-SV40 (Promega, Madison, WI). Four hours after transfection, cells were cultured in serum-free medium and then treated with both hormones for 48 h. VSMCs were then lysed with passive lysis buffer (Promega), and lysates were analyzed for both firefly and Renilla luciferase activity using a Promega Dual-luciferase reporter assay kit and a Monolight 2010 model luminometer (Analytical Luminescence Laboratory). Activity of the iNOS promoter-reporter construct was normalized to the activity of the Renilla reporter *(30)*.

Statistical Analyses

Values are expressed as mean ± SEM. Statistical significance was evaluated with nonparametric test (Mann-Whitney rank sum test) or analysis of variance with the appropriate correction for multiple comparisons (Newman-Keuls method). A value of $p < 0.05$ was considered significant (compared with control values otherwise specified).

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