Interactive Effects of Insulin-Like Growth Factor-1 and β-Estradiol on Endothelial Nitric Oxide Synthase Activity in Rat Aortic Endothelial Cells

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Insulin-like growth factor-1 (IGF-1) and β-estradiol (E2) have vasodilatory effects, in part, through stimulation of vascular nitric oxide (NO) production. However, their interactive effects on endothelial nitric oxide synthase (eNOS) and NO production have not been previously studied in endothelial cells (EC). Employing rat aortic EC (RAEC), the effects of acute (20 and 30 minutes) and prolonged (4 hours) stimulation with 100 nmol/L IGF-1 and 1 nmol/L E2 (alone or in combination) were assessed with respect to protein levels and enzymatic activities for phosphatidyl inositol 3-kinase (PI3K) and serine/threonine kinase Akt (Akt), enzymes involved in eNOS activation. Exposure to IGF-1 for 30 minutes or E2 for 20 minutes increased insulin receptor substrate-1 (IRS-1) association with the regulatory (p85) subunit of PI3K, enhanced tyrosine phosphorylation of p85, and increased PI3K activity. Combined treatment had a greater effect on p85 phosphorylation and PI3K activity then either agonist alone. Moreover, IGF-1 and E2 enhanced Akt Ser^{473} phosphorylation, with the effect of IGF-1 being much greater. Acute expose to both E2 (20 minutes) and IGF-1 (30 minutes) were associated with an increase in eNOS activity. Prolonged exposure (4 hours) to either IGF-1 or E2 increased expression of the p85 subunit as well as eNOS activity. Pretreatment with PI3K antagonist wortmannin (WT) prevented this increase in eNOS activity. The results suggest that IGF-1 and E2 may interact through PI3K/Akt-related pathways to increase eNOS activity.

INSULIN-LIKE growth factor-1 (IGF-1), a peptide growth factor, is synthesized by various cell types and acts as an autocrine/paracrine factor.[1,2] IGF-1 is known to mediate vasodilatation through phoshatidylinositol 3-kinase (PI3K)-mediated increases in vascular nitric oxide (NO) production.[1-4] β-Estradiol (E2), like IGF-1, also mediates vasodilation, by stimulating vascular NO production,[5-7] in part by activation of endothelial nitric oxide synthase eNOS[5,6] as well as eNOS gene expression.[5,7]

Both IGF-1 and E2 interact with their cognate receptors to initiate a signaling cascade that involves phosphorylation-dependent kinases, such as the serine/threonine kinase Akt (Akt)[8,9]. Activation of receptor tyrosine kinases leads to phosphorylation and binding of PI3K lipid products to the Akt plecstrin homology domain (PH) resulting in recruitment of Akt to the plasma membrane where it is phosphorylated at the threonine (Thr^{308}) and serine (Ser^{473}) residues and thus activated.[10,11]

There is accumulating evidence that IGF-1 can activate estrogen receptors (ER), even in the absence of E2.[10,12] It was recently reported that IGF-1 activation of the ER is mediated, in part, through a mechanism involving Akt signaling.[13] Thus, the membrane receptor-mediated IGF-1 signaling pathway may stimulate "ligand-independent" ER-mediated responses.[10] This raises the possibility that IGF-1 acting via the PI3K/Akt signaling pathway may be involved in E2-induced eNOS activation and visa versa.

Thus, we examined possible interactions of IGF-1 and E2 to stimulate NO production through activation of eNOS using primary cultures of rat aortic endothelial cells (RAEC). The aim of the present study was to examine the role of PI3K/Akt signaling in mediating individual/interactive effects of IGF-1 and E2 on eNOS activation.

Specifically, we addressed the hypothesis that in RAEC IGF-1 and E2 interact through PI3K/Akt related pathway to increase PI3K/Akt activity and eNOS level/activity.

MATERIALS AND METHODS

Cell Culture

RAEC were isolated from Spraque-Dawley rat thoracic aortic rings as described previously.[3] Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). Cells, grown to confluence, were transferred to serum-free DMEM for 24 hours before adding test compounds. The SUNY-Brooklyn Institutional Animal Care and Use Committee approved the animal experimentation described within this report.

Preparation of Cell Lysates

Cells were treated with 100 nmol/L IGF-1 (Genetech, South San Francisco, CA) and/or with 1 nmol/L E2 (Sigma, St Louis, MO) for 5, 10, 20, 30 minutes, and 4 hours. These doses of IGF-1[3] and E2[6,7] have been shown to maximally stimulate eNOS activity in ECs. Cell lysates were prepared according to previously published protocol.[3,4]

Immunoblot Analysis

Protein was isolated from RAEC using buffers and protease inhibitors as described previously[3,4] and subjected to sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE), transferred to a nitrocellulose membrane, and probed with the primary antibody (used at 1:5,000 dilution), against the p85 subunit of PI3K (Upstate Biotech-

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nology, Lake Placid, NY) or polyclonal antibodies (used at 1:1,000 dilution) to phosphorylated-Akt (p-Akt) and total Akt (Cell Signaling, Beverly, MA); this was followed by incubation with horseradish peroxidase-labeled anti-rabbit IgG antibody (used at 1:5,000 and 1:2,000 dilutions) and subsequent detection with enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Immunoprecipitation

RAEC lysates were normalized for protein content (500 μg), and incubated for 24 hours with 4 μg of antiphosphotyrosine or IRS-1 antibodies (Santa Cruz, Santa Cruz, CA). Immunocomplexes were absorbed with Protein A/G Agarose (Santa Cruz) for 2 hours at 4°C and then recovered by centrifugation, washed 4 times, and separated on a SDS-PAGE gel, transferred to a nitrocellulose membrane and probed with a monoclonal anti-p85 antibody. The secondary antibody, linked to horseradish peroxidase–labeled anti-rabbit IgG antibody (used at 1:5,000 dilution), was used for chemiluminiscent detection of the p85 regulatory subunit of PI3K.[4]

PI3K Activity

Cell lysates were immunoprecipitated from aliquots of the resulting supernatants by incubating them overnight at 4°C with the antiphosphotyrosine antibody. Immunocomplexes were absorbed onto protein A-agarose beads (Santa Cruz) and washed successively, and PI3K activity was determined according to the published protocol.[3] Lipids were extracted by chlorophorm:ethanol and analyzed by thin-layer chromatography (TLC); ^{32}P-labeled phoshatidylinositol 3-P products were measured using a PhosphorImager and calculated by IMAGEQUANT software (Molecular Dynamics, Sunnyvale, CA). Cold phoshoinositides were run as a standard and visualized by primulin staining.

NOS Activity Determination

Citrulline, a coproduct of the NO biosynthetic reaction, was determined using a colorimetric assay.[3] A 400-μL quantity of the culture medium was incubated with urease (45 U/mL) for 30 minutes at 37°C. The mixture was deproteinized by the addition of ice-cold trichloroacetic acid to a final concentration of 5%. After centrifugation, 400 μL of the supernatant was mixed with 3 mL of chromogenic solution:1 part of (0.5% diacetylmonoxime /0.01% thiosemicarbazide) and 2 parts of acid-ferric solution (0.025% of $FeCl_3$ in a solution containing 25% sulfuric acid and 20% phosphoric acid), and boiled at 96°C for 5 minutes. After cooling the mixture to room temperature, the absorbance was measured at 570 nm in a plate reader. L-Citrulline concentrations in the sample were calculated from a citrulline standard curve.[3]

Statistical Analysis

Values are mean ± SEM, with n values representing the number of experiments. Statistical significance was evaluated with Student's *t* test or analysis of variance (ANOVA) with the appropriate correction for multiple comparisons (Newman-Keuls method). $P < .05$ was considered significant (compared with control values unless otherwise specified).

RESULTS

IGF-1 and E2 Regulate Expression of the PI3K Regulatory Subunit (p85 kd)

RAEC were treated with IGF-1 (100 nmol/L) or E2 (1 nmol/L) for 4 hours, and membranes were probed with anti-p85 (85 kd) antibody (Fig 1). Densitometry quantitation of Western blots revealed that IGF-1, as well as E2, increased PI3K protein level after 4 hours of hormone treatment (Fig 1).

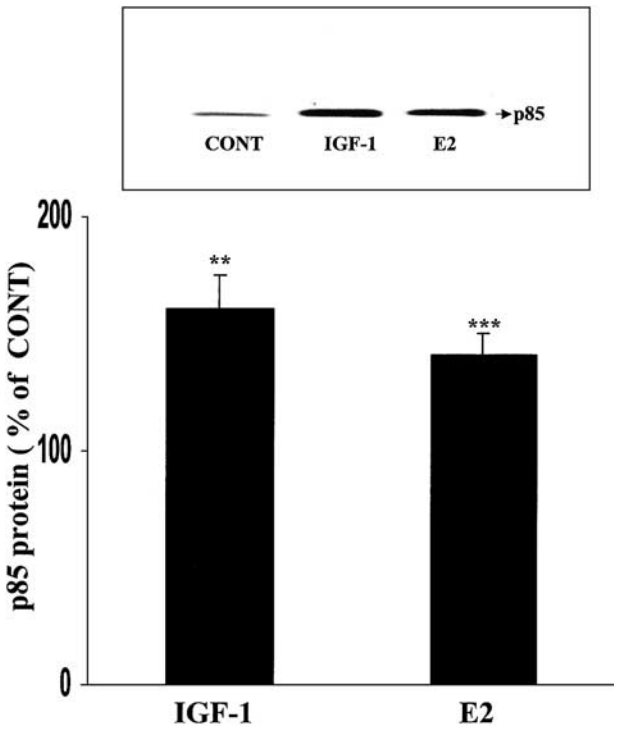

Fig 1. Effect of IGF-1 and E2 on regulation of p85 protein level. Cells were treated with IGF-1 (100 nmol/L) or E2 (1 nmol/L) for 4 hours. Untreated control samples were arbitrarily assigned a value of 100% and values for all treatments were normalized to 100% (% of CONT). (Insert) Representative Western blot. Mean ± SEM, n = 8. **$P < .01$, ***$P < .001$ *v* control values.

Effects of IGF-1 and E2 on IRS-1 Protein Association With p85

To evaluate the effects of IGF-1 and E2 on IRS-1 protein association with p85, RAEC were pretreated with 100 nmol/L IGF-1 or 1 nmol/L E2 for 5, 10, 20, and 30 minutes, and association between IRS-1/p85 was determined. Peak increases in association of p85 to IRS-1 were observed at 30 minutes of exposure to IGF-1 (Fig 2A) and 20 minutes exposure to E2 (Fig 2B). Since the maximum effect of IGF-1 and E2 induced IRS-1/p85 association was at 30 minutes and 20 minutes, respectively, these exposure periods were subsequently utilized. Pretreatment with E2 for 20 minutes and then with IGF-1 for an additional 30 minutes had no greater effect then either agonist alone (data not shown). These results suggest that both hormones increased IRS-1 association with p85 isoform for the tyrosine-phosphorylated protein.

Effects of IGF-1 and E2 on p85 Phosphorylation and PI3K Activity

To examine whether IGF-1 and E2 induce phosphorylation of p85, cells were exposed to IGF-1 for 30 minutes or E2 for 20 minutes, prior to assay of cell lysates for phosphorylation of the p85 regulatory subunit. Immunoprecipitation with an antiphosphotyrosine antibody and subsequent immunoblot with an anti-p85 antibody demonstrated that both hormones enhanced tyrosine phosphorylation of p85 (Fig 3A). Pretreatment with E2

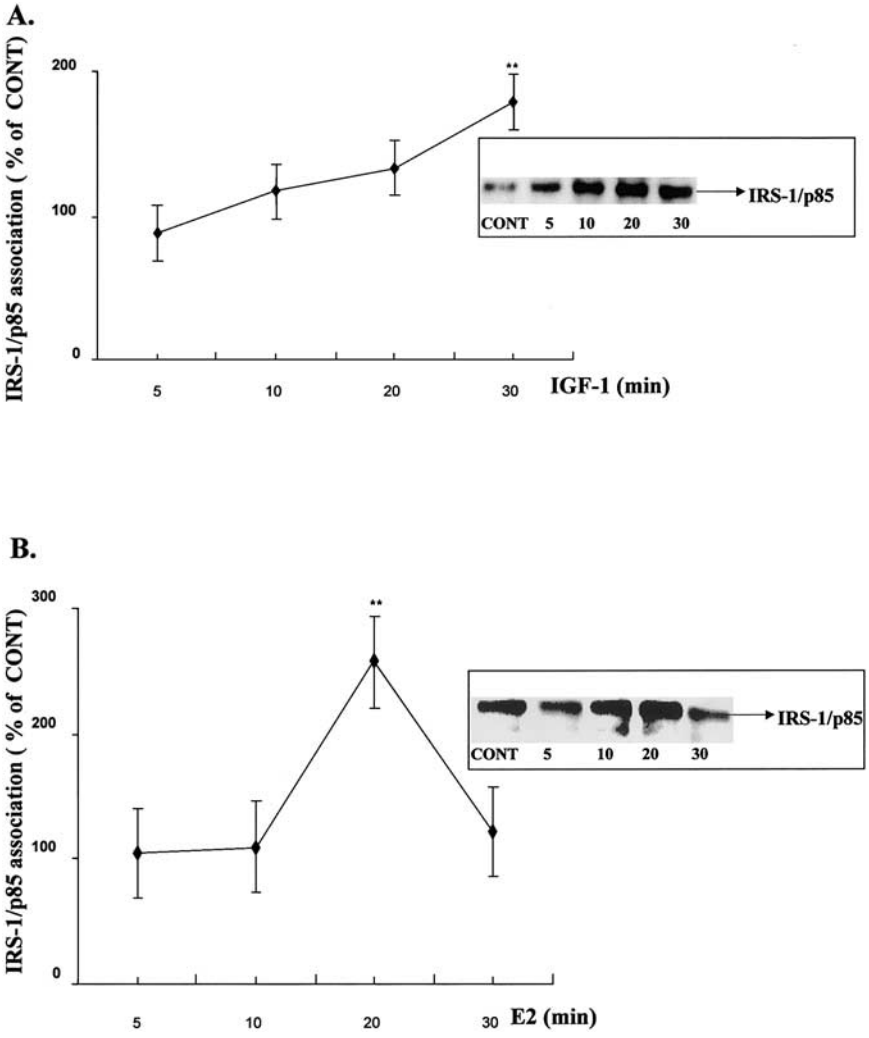

Fig 2. Time-dependent effect of IGF-1 and E2 on IRS-1/p85 association in RAEC. Cells were treated with (A) IGF-1 (100 nmol/L) and (B) E2 (1 nmol/L) for 5, 10, 20, and 30 minutes. (Upper and lower inserts) Representative immunoblots. Mean ± SEM, n = 5. **$P < .01$ *v* control values. Normalizations as in Fig 1.

for 20 minutes and then with IGF-1 for an additional 30 minutes resulted in an increase in p85 phosphorylation compared to control or to IGF-1 alone. Since we sought to determine whether PI3K is involved, we further investigated the mechanism of E2/IGF-1 induced activation of PI3K (Fig 3B). As shown in Fig 3B, exposure to IGF-1 for 30 minutes and/or E2 for 20 minutes increased PI3K activity, as evidenced by ^{32}-P incorporation into PIP3. Pretreatment with E2 for 20 minutes and then with IGF-1 for an additional 30 minutes resulted in a greater effect then either agonist alone. Thus, using 2 indices of activation (p85 regulatory subunit phosphorylation and PI3K activity), we determined that E2 enhances IGF-1–induced stimulation of PI3K as evidenced by enhanced enzyme activity in RAEC (Fig 3B).

Effects of IGF-1 and E2 on Akt Phosphorylation in RAEC

We further examined whether Akt, an important downstream target for PI3K,[9-11] is involved in IGF-1 and E2 interactions in RAEC. Therefore, we assessed the effect of these hormones on phosphorylation state of Akt at Ser^{473} by immunobloting with a phospho-specific Akt antibody that recognizes Akt only when phosphorylated at Ser^{473} (Fig 4). Stimulation with IGF-1 (30 minutes) or E2 (20 minutes) enhanced Akt Ser^{473} phosphorylation, with the effect of E2/IGF-1 being much greater then IGF-1 alone. However, the pretreatment with E2 for 20 minutes before IGF-1 for an additional 30 minutes produced no enhancement in an increase in Akt phosphorylation (Fig 4). These data indicate that PI3K is involved not only in IGF-1/E2-mediated Akt activation, but also in the maintenance of basal activity of Akt in RAEC.

IGF-1/E2-Stimulated eNOS Activity

We next examined whether IGF-1, E2, and combinations of both hormones were capable of increasing eNOS activity. Acute stimulation with either IGF-1 (30 minutes) or E2 (20 minutes) was associated with an increase in eNOS activity. However, combined treatment with both hormones resulted in no further increase in eNOS activity (Fig 5A). Because our previous results suggested that PI3K is involved in the E2/IGF-1 regulation of eNOS activity, we next tested the effect of PI3K inhibition on E2/IGF-1 regulation of citrulline production. The effect of acute stimulation (20 to 30 minutes) by the

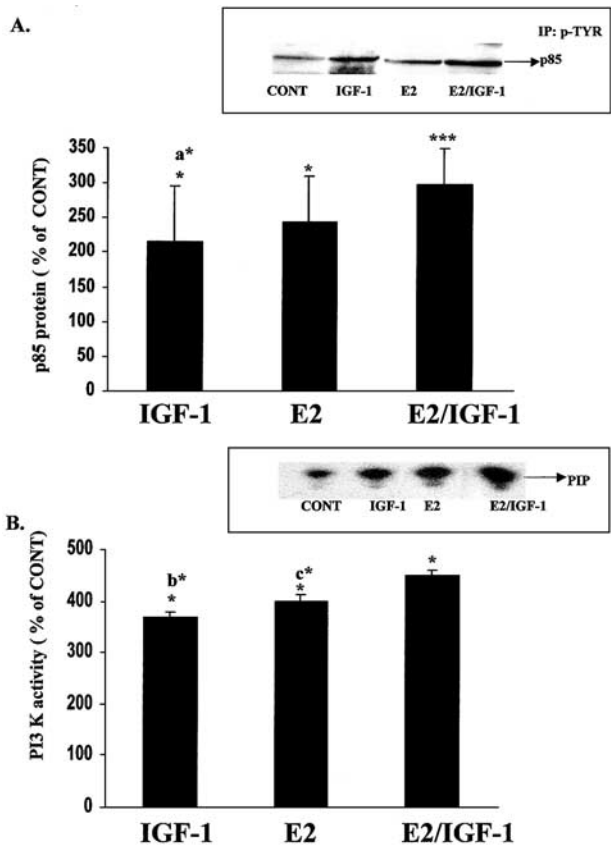

Fig 3. Effect of IGF-1 and E2 on (A) p85 phosphorylation and (B) PI3K activity. Cells were treated with IGF-1 (100 nmol/L) for 30 minutes and/or E2 (1 nmol/L) for 20 minutes. (Upper insert) Representative immunoblot. (Lower insert) Representative autoradiogram from TLC performed on PI3K product is shown. E2/IGF-1 indicates pretreatment with E2 for 20 minutes and then with IGF-1 for an additional 30 minutes. Mean ± SEM, n = 3. *$P < .05$ and ***$P < .001$ *v* control values. "a" indicates *$P < .05$, IGF-1 *v* E2/IGF-1; "b" indicates *$P < .05$, IGF-1 *v* E2/IGF-1; and "c" indicates *$P < .05$, E2 *v* E2/IGF-1. Normalizations as in Fig 1.

hormone combination was not prevented by PI3K blocker, wortmannin (WT) (Fig 5A). Prolonged stimulation (4 hours) to either IGF-1 or E2 resulted in significant increases in citrulline production (Fig 5B). Combined treatment with both hormones for 4 hours increased citrulline production compared to control and compared to IGF-1 alone (Fig 5B). Pretreatment for 30 minutes with WT decreased the E2/IGF-1–stimulated citrulline production (Fig 5B). The current results show that E2/IGF-1 significantly increases eNOS activity, as determined by citrulline production, in conjunction with PI3K /Akt activation. Thus, WT-sensitive p85 regulatory subunit of PI3K is involved in IGF-1, as well as E2-induced eNOS activity in EC.

DISCUSSION

The principal new finding of the present study is that the PI3K/Akt signaling pathway mediates, in part, the interactive effects of IGF-1 and E2 to stimulate NOS activity. These data extend our previously finding that PI3K is involved in regulation of eNOS in RAEC.[3,4] Exposure to either IGF-1 or E2 increased IRS-1 association with p85, enhanced tyrosine phosphorylation of p85 (a hallmark of PI3K activation), and increased PI3K activity. In the latter 2 cases, combined E2/IGF-1 treatment had a greater effect then either agonist alone. Current data indicate that E2 can induce expression of other downstream IGF-1 signaling molecules, including the p85 regulatory subunit of PI3K. Further, these results suggest that regulation of these molecules results in a potentiation of E2 on the IGF-1 stimulation of IRS-1 tyrosine phosphorylation and activation of PI3K.

Results from current study are in accord with prior studies in MCF-7 cells,[14,15] showing that both IGF-1 and E2 act synergistically to increase Akt enzyme activity. These data may be explained by a redistribution of IGF-1 receptors as well as increased IRS-1 expression, following E2 treatment of the cells. In ECs, IRS-1 is the main substrate for IGF-1R signaling,[2] and PI3K is thought to be the main pathway involved in the IGF-1 signaling pathway.[8]

Moreover, both IGF-1 and E2 enhanced Akt Ser^{473} phosphorylation, with the effect of IGF-1 being much greater. Interestingly, the E2/IGF-1 treatment produced no greater enhancement on Akt phosphorylation then either IGF-1 or E2 alone. In this investigation both IGF-1 and E2 were associated with an increase in eNOS activity, with no greater effect seen with the combined treatment. That PI3K blocker WT was able to prevent the increase in eNOS activity associated with 4 hours exposure to the agonists suggests that PI3K/Akt activation is a

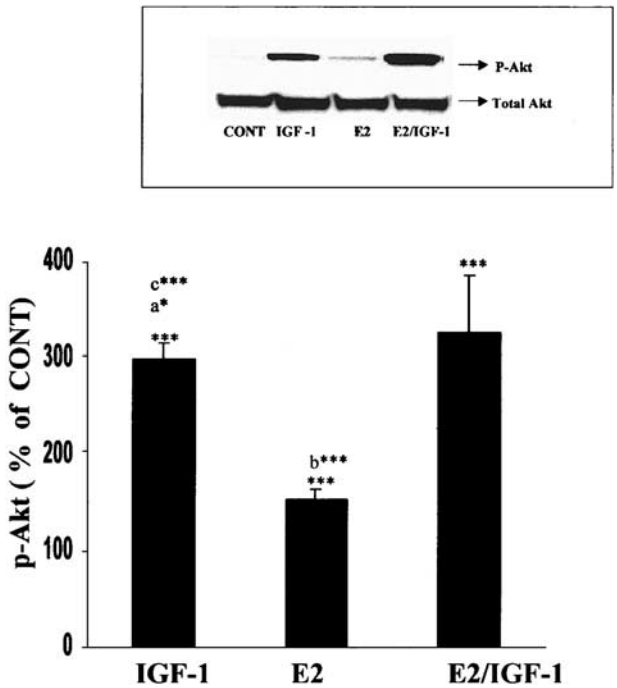

Fig 4. Effect of IGF- 1 and E2 on Ser^{473} phosphorylation in RAEC. Cells were treated with IGF-1 (100 nmol/L) for 30 minutes and/or with E2 (1 nmol/L) for 20 minutes or cells were pretreated with E2 for 20 minutes before IGF-1 treatment. (Insert0 Representative Western blots. Mean ± SEM, n = 4. ***$P < .001$ *v* control values. "a" indicates *$P < .05$, IGF-1 *v* E2/IGF-1; "b" indicates ***$P < .001$, E2 *v* E2/IGF-1; and "c" indicates $P < .001$, IGF-1 *v* E2. Normalizations as in Fig 2.

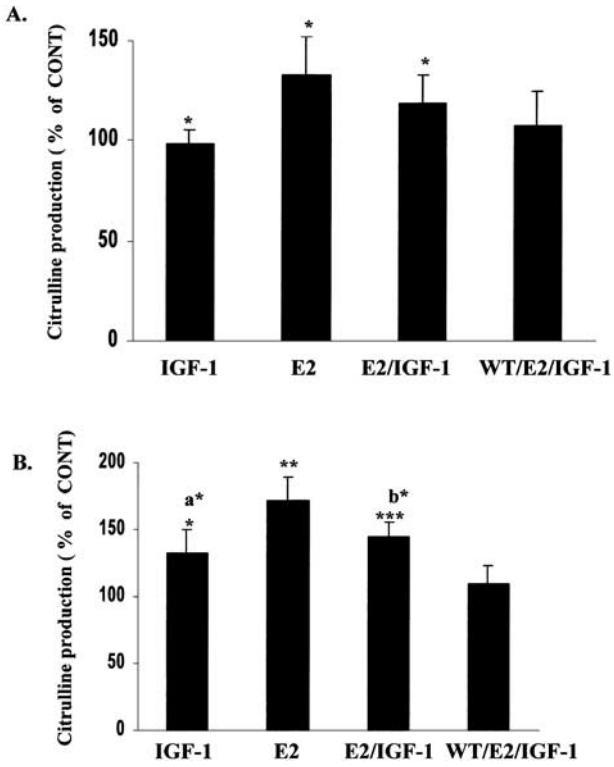

Fig 5. Effect on inhibition of PI3-kinase on IGF-1 and E2 stimulated eNOS activity. (A) Citrulline production were measured in medium of RAEC exposed to 100 nmol/L of IGF-1 for 30 minutes and/or to 1 nmol/L of E2 for 20 minutes or cells were pretreated with E2 for 20 minutes or 100 nmol/L wortmannin (WT) for 30 minutes before IGF-1 treatment for an additional 30 minutes. (B) Citrulline production was measured in RAEC exposed to IGF-1 or E2 for 4 hours, a combination of both for 4 hours, or pretreated with WT before hormone treatment for an additional 4 hours. Mean ± SEM, n = 7. ***$P < .001$, **$P < .01$, *$P < .05$ *v* control values; "a" indicates *$P < .05$, IGF *vs* E2/IGF-1 and "b" indicates *$P < .05$, E2/IGF-1 *v* WT/E2/IGF. Normalizations as in Fig 2.

critical signaling pathway for IGF-1/E2 stimulation of the eNOS. Recent evidence indicates that eNOS is regulated, in part, through the coordinated phosphorylation and dephosphorylation of the amino acid residues Ser-^{1177} and Thr-^{495} (human sequence).[16,17] Thus, IGF-1 and E2 may also activate PI3K/Akt signaling resulting in promotion of eNOS activity and NO production by increasing Ser-^{1177} phosphorylation.[16,18-20]

In the current study, potentiation of the effects of IGF-1 and E2 on Akt phosphorylation, as well as IRS-1/p85 protein association, was paralleled by eNOS activation. This is the first observation that IGF-1 and E2 interact via an IRS-1/p85 signaling pathway to regulate eNOS activity in RAEC. Since the PI3K blocker WT inhibits E2/IGF-1–stimulated NOS activity, we conclude that WT-sensitive p85 regulatory subunit of PI3K is involved in E2/IGF-1–induced eNOS activity Thus, E2/IGF-1 activation of PI3K/Akt pathway results in activation of the eNOS activity in RAEC.

There are several possibilities to explain the interactions of IGF-1 and E2 in modulation of eNOS. It is possible that E2 maintains the proper local level of expression of IGF-1 and its receptor on ECs to allow for optimal IGF-1 regulation of PI3K under normal conditions.[21] On the other hand, IGF-1 may also sensitize the E2 receptor on ECs into full function.[10,21,22] Indeed, ligand bound E2 receptor α is required for rapid activation of the IGF-1R signaling cascade in COS2 and HEK293 cells.[18] Current and prior data[8,19] indicate that both hormones enhance phosphorylation of p85 regulatory subunit, increase IRS-1/p85 association, and activate the PI3K/Akt pathway that is critical for NOS activation in ECs[8] and other cells.[19]

In summary, these data suggest that relative potency of IGF-1 versus E2 in stimulation of eNOS activity is related to the ability of these peptides to enhance PI3K/Akt activity in RAEC. Further, these data indicate that both hormones are necessary for optimal eNOS activation as regulated by the PI3K/Akt pathway signaling in RAEC. In this study, we have elucidated some of the signaling mechanisms involved in the cross talk between the E2 and the IGF-1 in primary cultured RAEC.

REFERENCES

1. Sowers JR: Insulin and insulin-like growth factor in normal and pathological cardiovascular physiology. Hypertension 29:691-699, 1997

2. LeRoith D: Insulin-like growth factors. N Engl J Med 336: 633-640, 1998

3. Isenovic E, Muniyappa R, Milivojevic N, et al: Role of PI3-kinase in isoproterenol and IGF-1 induced ecNOS activity. Biochem Biophys Res Commun 27:285:954-958, 2001

4. Isenovic E, Walsh MF, Muniyappa R, et al: Phosphatidylinositol 3-kinase may mediate isoproterenol-induced vascular relaxation in part through nitric oxide production. Metabolism 51:380-386, 2002

5. Chen IZ, Yuhanna IS, Galcheva-Gargova Z, et al: Estrogen receptor alpha mediates the nongenomic activation of endothelial nitric oxide synthase by estrogen. J Clin Invest 103:401-416, 1999

6. Haynes PM, Sinha SD, Rusell BK, et al: Membrane estrogen receptor engagement activates endothelial nitric oxide synthase via PI3-kinase-AKT pathway in human endothelial cells. Circ Res 87:677-682, 2000

7. Xu R, Sowers JR, Skafar DF, et al: Hydrocortisone modulates the effect of estradiol on endothelial nitric oxide synthase expression in human endothelial cells. Life Sci 69:2811-2817, 2001

8. Zeng G, Nystrom FH, Ravichandran LV, et al: Roles for insulin receptor, PI3-kinase, and Akt in insulin-signaling pathways related to production of nitric oxide in human vascular endothelial cells. Circulation 101:1539-1545, 2000

9. Murga C, Laguinge L, Wetzker R, et al: Activation of Akt/protein kinase B by G protein-coupled receptors. A role for alpha and beta gamma subunits of heterotrimeric G proteins acting through phosphatidylinositol-3-OH kinase gamma. J Biol Chem 273:19080-19085, 1998

10. Stoica A, Saceda M, Fakhro A, et al: Role of insulin-like growth factor-I in regulating estrogen receptor-alpha gene expression. J Cell Biochem 76:605-614, 2000

11. Downward J: Mechanisms and consequences of activation of protein kinase B/Akt. Curr Opin Cell Biol 10:262-267, 1998

12. Aronica SM, Katzenellenbogen BS: Stimulation of estrogen receptor-mediated transcription and alteration in the phosphorylation state of the rat uterine estrogen receptor by estrogen, cyclic adenosine

monophosphate, and insulin-like growth factor-I. Mol Endocrinol 7:743-752, 1993

13. Molloy CA, May FEB, Westley BR: Insulin receptor substrate-1 expression is regulated by estrogen in the MCF-7 human breast cancer cell line. J Biol Chem 275:12565-12571, 2000

14. Ahmad S, Singh N, Glazer RI: Role of AKT1 in 17beta-estradiol- and insulin-like growth factor I (IGF-I)-dependent proliferation and prevention of apoptosis in MCF-7 breast carcinoma cells. Biochem Pharmacol 58:425-430, 1999

15. Dupont, J, Karas M, LeRoith D: The potentiation of estrogen on insulin-like growth factor-1 action in MCF-7 cell human breast cancer cells includes cell cycle components. J Biol Chem 275:35893-35901, 2000

16. Fulton D, Gratton JP, McCabe TJ, et al: Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. Nature 399:597-601, 1999

17. Michell BJ, Chen ZP, Tiganis T, et al: Coordinated control of endothelial nitric-oxide synthase phosphorylation by protein kinase C and the cAMP-dependent protein kinase. J Biol Chem 276:17625-17628, 2001

18. Hisamoto K, Ohmichi M, Kurachi H, et al: Estrogen induces the Akt-dependent activation of endothelial nitric-oxide synthase in vascular endothelial cells. J Biol Chem 276:3459-35467, 2001

19. Montagnani M, Chen H, Barr VA, et al: Insulin-stimulated activation of eNOS is independent of Ca2+ but requires phosphorylation by Akt at Ser1^{179}. J Biol Chem 276:30392-30398, 2001

20. Michell BJ, Griffiths JE, Mitchelhill KI, et al: The Akt kinase signals directly to endothelial nitric oxide synthase. Curr Biol 9:845-848,1999

21. Simoncini T, Hafezi-Moghadam A, Brazil DP, et al: Interaction of estrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. Nature 6803:538-541, 2000

22. Kahlert S, Nuedling S, Eickels MV, et al: Estrogen receptor α rapidly activates the IGF-1 receptor pathway. J Biol Chem 275: 18447-18453, 2000