Phosphatidylinositol 3-Kinase May Mediate Isoproterenol-Induced Vascular Relaxation in Part Through Nitric Oxide Production

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Phosphatidylinositol 3-kinase (PI3-K) has been shown to mediate insulin and insulin-like growth factor-1 (IGF-1)–induced nitric oxide (NO) generation and, thus, vascular tone. A role for PI3-K in G-protein–coupled receptor signal transduction has also been reported. As β_2 -adrenergic vascular actions are partly dependent on NO, this study the role of PI3-K on in vitro isoproterenol (Iso)-induced endothelial cell (EC) nitric oxide synthase (NOS) activation and rat aortic vascular relaxation. Cell lysates of rat aortic EC (RAEC), exposed to Iso (10 μmol/L) for 5 minutes, were immunoprecipitated with an antiphosphotyrosine antibody prior to assay for Western blot for the p85-kd regulatory subunit of PI3-K. Endothelial NOS activity was determined by measuring nitrite production. Endothelium-intact aortic rings from male Wistar rats were preincubated with the PI3-K inhibitors, wortmannin (WT), or LY294002 (LY), precontracted with phenylepinephrine (PE), and relaxation to graded doses of Iso was measured. NO contribution to vascular relaxation was assessed by L-N^G-nitroarginine methyl ester (L-NAME), a NOS inhibitor. Both Iso and IGF-1 induced an increase in p85 subunit phosphorylation as demonstrated by Western analysis, effects inhibited by preincubation with WT. Iso also enhanced association of p85 with the Triton X-100–insoluble fraction of RAEC, reflecting translocation of this enzyme to a cytoskeletal fraction. In addition, Iso as well as IGF-1 significantly increased eNOS activity measured by nitrite production. Both WT and LY markedly inhibited relaxation to Iso, while L-NAME nearly abolished this β-adrenergic–mediated vasorelaxation. These data indicate that both Iso and IGF-1 activate the EC PI3-K pathway which mediates, in part, the release of NO and subsequent vasorelaxation in response to this β-agonist Iso as well as to IGF-1.

PHOSPHATIDYLINOSITOL 3-kinase (PI3-K), a heterodimeric protein composed of an 85-kd regulatory subunit and a 110-kd catalytic subunit, catalyzes the synthesis of 3-phosphorylated phosphoinositides that serve as intermediates for various specific downstream signal transduction events, including that of glucose transport.[1] Recent work indicates that PI3-K is activated by adrenergic receptor[2] and angiotensin II[3] signaling in vascular smooth muscle cells (VSMC) and by G-protein $\beta\gamma$ subunits in myeloid-derived cells.[4] In endothelial cells (EC), PI3-K is involved in insulin and insulin-like growth factor 1 (IGF-1)–mediated NO generation.[1,5]

All 3 known isoforms of nitric oxide synthase (NOS) have been identified in vascular tissue.[6] Endothelial NOS (eNOS) has been localized to caveolae and the golgi in the EC,[6] and the enzymatic machinery generating nitric oxide (NO) is localized to these small hydrophobic pockets, which act as storage reservoirs for NO.[7] Caveolae appear to play a role in the compartmentation of a number of plasma membrane-linked signal transduction pathways, including those mediated by muscarinic cholinergic[8] and adrenergic agonists.[9] Mobilization of eNOS appears to be necessary for its activation,[6] and the PI3-K pathway appears to mediate this process.

Isoproterenol (Iso), a β_2-adrenergic agonist, also relaxes arteries by stimulating endothelium-derived NO,[10,11] but the signal transduction pathways responsible for this action are poorly understood. In vitro evidence suggests a role of endothelial NO in the modulation of Iso[10-12] and insulin/IGF-1[5]–induced vasorelaxation.[11] Recent studies have showed that Iso-induced vascular relaxation,[12] as well as that mediated by IGF-1/insulin, may be impaired in diabetic rat models.[13] A better understanding of the mechanisms of β-adrenergic–mediated vasorelaxation is needed to elucidate pathophysiologic mechanisms involved in states of impaired vasorelaxation, such as diabetes.[1,5,13]

The multifactorial control of eNOS activation, which includes calcium-calmodulin activation, transient covalent modification, protein-protein interaction, and subcellular localization,[1,6] makes PI3-K a plausible intermediary in agonist-induced subcellular targeting of the enzyme. Since β-adrenergic stimulation causes vasorelaxation,[10-12] we have investigated the role of the PI3-K signaling pathway in mediating this process.

MATERIALS AND METHODS

Rat Aortic Endothelial Cells Isolation and Cell Culture

Rat aortic endothelial cells (RAECs) were isolated by explant outgrowth from Wistar rats aortic rings as described previously by one of the current investigators.[14] Briefly, 2-mm rings were incubated in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Grand Island, NY) supplemented with 20% fetal bovine serum (FBS) stripped with dextran-coated charcoal, and endothelial cell outgrowths passage in DMEM with 10% FBS, glutamine, and antibiotics. Cells, grown to confluence, were transferred to serum-free DMEM for 24 hours before each experiment.

Cell Lysis and Sample Preparation

Quiescent RAECs in 100-mm culture dishes were incubated with agonists, 10 μmol/L Iso (Sigma Chemicals, St Louis, MO) or 100

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nmol/L IGF-1[15] (Genetech Inc, San Francisco, CA) in balanced solution for 5 minutes and 10 minutes, respectively, and agonist-induced responses terminated by addition of ice-cold phosphate-buffered saline (PBS). The cells were rinsed again with PBS and lysed by the addition of 1 mL of lysis buffer (20 mmol/L Tris HCl [pH 7.4], 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 10 mmol/L $Na_4P_2O_7$, 2 mmol/L Na_3O_4, 1 mmol/L β-glycerolphosphate, 1% Triton X-100, and the following protease inhibitors: 1 mmol/L phenylmethylsulfonylfluoride, 10 μg/mL leupeptin, and 5 μg/mL aprotinin [Sigma Chemicals]). After 30 minutes on ice, total cell lysates were then obtained by sonicating the samples. To prepare Triton X-100–insoluble fractions,[16] EC were lysed as above and then homogenized in a glass homogenizer (20 strokes). The lysate was then centrifuged at 10,000 $\times$ *g* at 4°C, the supernatant (Triton-soluble) was transferred, and the pellets (triton-insoluble) were resuspended in 0.2 mL of modified RIPA buffer (20 mmol/L Tris.HCl [ph 7.4], 1 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 50 mmol/L NaF, 2 mmol/L Na_3O_4, 10 mmol/L $Na_4P_2O_7$), sonicated, and then sheared through a 21-G needle 10 times.

Immunoprecipitation and Western Blot Analysis

Equal amounts of cell lysate protein were mixed with 8 μg of antiphosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) and rotated overnight at 4°C. A 20-μL quantity of protein A/G (50% slurry) was added and samples were further incubated for an additional 4 hours. The immune complexes were then recovered by centrifugation; beads were washed 4 times and then suspended in SDS-sample buffer. Equal amounts of protein (60 μg) were separated on a 7.5% or 12% SDS-polyacrylamide gel and electrophoretically transferred to a nitrocellulose membrane (Schleicher & Schuell, Keene, NH) in Tris-glycine transfer buffer with 20% methanol in a Trans-Blot Cell (BioRad, Hercules, CA). Membranes were blocked overnight at 4°C with 9% instant nonfat dry milk in Tris-buffered saline (TBS [in mmol/L]: 20 Tris, 137 NaCl, pH 7.6 containing 0.3% Tween 20), washed in TBS, and incubated with the appropriate primary antibody, against the p85 subunit of PI3-K (1:750, Santa Cruz Biotech, Santa Cruz, CA) or polyclonal antibodies to caveolin-1 (1:500, Santa Cruz Biotech, CA). The membranes were washed thoroughly and incubated with horseradish peroxidase–coupled anti-rabbit or anti-mouse IgG antibody (1:5,000 for mouse and 1:2,500 for rabbit; Amersham, Arlington Heights, IL) for 1 hour. The bound antibodies were visualized by enhanced chemiluminescence using the Enhanced Chemiluminisence (ECL) system (Amersham) and exposure to Kodak X-OMAT film (Eastman Kodak, Rochester, NY). Signals were quantitated using the Ambis densitometry system (version 4.31, San Diego, CA). Multiple exposure of each blot was performed to ensure that signals were within the linear range of the film.

Nitrite Production

Nitrite production was measured in medium samples by the Griess reaction.[17] Briefly, a 1-mL aliquot of medium from each well was dried down and resuspended in 0.12-mL ultraPure water (Cayman Chemicals, Ann Arbor, MI). Duplicate (100 μL of culture supernatant) was allowed to react with an equal volume of Greiss reagent (1 part 0.1% napthylethlenediamine dihydrochloride and 1 part 1% sulfanilamide in 0.1N HCl), at room temperature for 15 minutes before colorimetric quantitation at 550 nm. Nitrite concentrations were calculated from a sodium nitrite standard curve.[17] Values from duplicate wells (in μmol/L) were averaged for each experiment.

Vascular Reactivity

Male Wistar rats (200 to 300 g) (Charles River Laboratories, Wilmington, MA) housed 2 per cage, given food and water ad libitum, were anesthetized with sodium pentobarbital (35 mg/kg intraperitoneally) and the thoracic aorta carefully dissected (institutional approval was obtained for all procedures and animal handling). The aorta was immediately placed in ice-chilled buffer containing (mmol/L): 130 NaCl, 15 $NaHCO_3$, 4.7 KCl, 1.2 KH_2PO_4, 1.6 $CaCl_2$, 1.2 $MgSO_4$, 0.03 EDTA, and 5.6 glucose). Studies were performed in 3-mm aortic rings mounted on stirrups and suspended from isometric force transducers (Gould Instruments, Cleveland, OH) as previously described[3,13,18], in muscle baths containing the buffer at 37°C aerated with 95%O_2/5% CO_2 to maintain pH at 7.35 to 7.40. All rings were stretched to 1.5 g of tension and allowed to equilibrate for 30 minutes before adding any drug. Contraction to graded doses of KCl (10 to 60 mmol/L) and phenylephrine (PE, 10^{-9} to 10^{-5} mol/L; Sigma Chemicals) was assessed in rings preincubated with wortmannin (WT, 300 nmol/L; Calbiochem, La Jolla, CA) for 40 minutes. We have previously demonstrated that IGF-1 produces relaxation in aortic rings through a PI3-K–mediated mechanism.[13,18] Thus, only Iso was evaluated in this component of the study. Relaxation responses to graded doses (10^{-9} to 10^{-5} mol/L) of Iso and acetylcholine (Ach), in PE-contracted rings, were repeated after preincubation with WT and L-N^G-nitroarginine methyl ester (L-NAME) (a NOS inhibitor, 300 μmol/L, Cayman Chemical)[19] and with LY294002 (LY; 50 μmol/L; Biomol, Plymouth Meeting, PA).[20] Both PI3-K inhibitors, WT and LY, have been demonstrated to have a high degree of specificity.[19,20] The presence of endothelium was confirmed by appropriate relaxation in response to Ach (10^{-5} mol/L) at the end of each experiment. Preincubation times and concentration of various agents used were similar to other studies or previously optimized in our laboratory.[13,16,18]

Statistical Analysis

Two-way (with Fisher's protected least significant difference) and repeated measures analyses of variance (ANOVAs) were used to compare contractility and relaxation changes between different groups. Effective dose 50 (ED_{50}) values were calculated with the Pharm C Program (Springer-Verlag, New York, NY); statistical differences between paired experiments were assessed by Students's *t* test. Values in the text are given as the mean $\pm$ SEM, and *P* values less than .05 were considered significant (compared with control unless otherwise specified). N represents the number of experiments.

RESULTS

Effect of Iso on p85 Phosphorylation and p85 Association With Cytoskeleton

To examine Iso-induced phosphorylation of PI3-K, RAEC were exposed to Iso (10 μmol/L) for 5 minutes or IGF-1 (100 nmol/L) for 10 minutes, prior to assay of cell lysates for phosphorylation of the p85kd regulatory subunit. Immunoprecipitation with an antiphosphotyrosine antibody and subsequent immunoblot with an anti-p85 antibody demonstrated that Iso, like IGF-1, induces an increase in p85 phosphorylation (control 474 $\pm$ 5, Iso 534 $\pm$ 41, IGF-1 647 $\pm$ 44 density units [DU]; $P < .05$) (Fig 1A). Reversal of the procedure, precipitation with anti- p85kd, and detection with antiphosphotyrosine gave similar results and demonstrated inhibition with WT (Fig 1B). As shown in Fig 2A, Iso increased association of PI3K (as shown by antibody binding to the p85 subunit) with the EC Triton-insoluble fraction. The slightly different migration pattern in the insoluble fraction likely reflects post-translational modification of the activated protein. Additionally, caveolin, a resi-

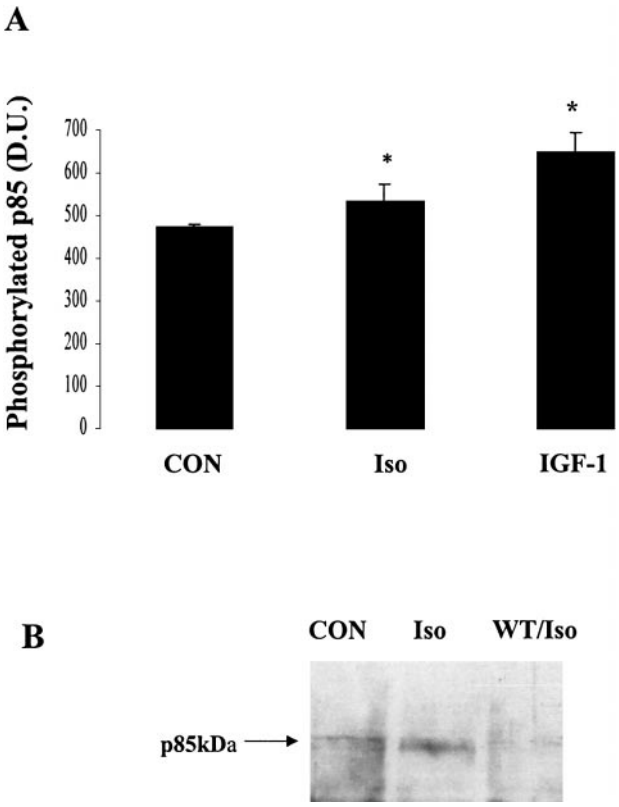

Fig 1. Effect of Iso on PI3-K in RAEC: Iso-induced phosphorylation of the p85 regulatory subunit after immunoprecipitation with anti p85 antibody and detection with an antityrosine antibody following 5 minutes exposure to Iso and 10 minutes to IGF-1 (A). Representative immunoblot (B). Each bar represents the mean ± SE of 3 experiments. *$P < .05$. CON, control; Iso, isoproterenol (10 μmol/L); IGF-1, insulin-like growth factor-1 (100 nmol/L); WT, wortmannin (30 nmol/L); D.U., density units. Position of the p85kd subunit is indicated on the left.

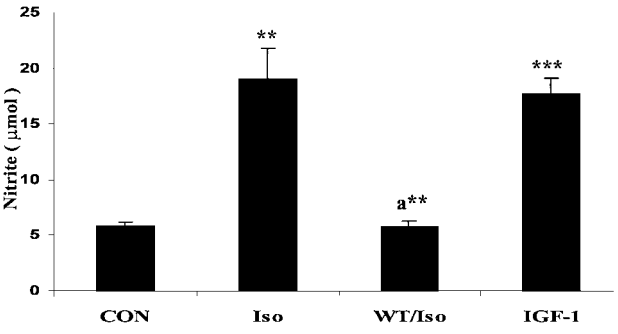

Fig 2. Association of the p85 subunit of PI3-K with the RAEC Triton X-100–insoluble fraction after Iso stimulation for 5 minutes (A). Caveolin, a specific protein of caveolae, is also enriched in this fraction (B). Results are representative of 3 similar experiments each. Positions of the p85kd subunit and calveolin-1 are indicated on the left.

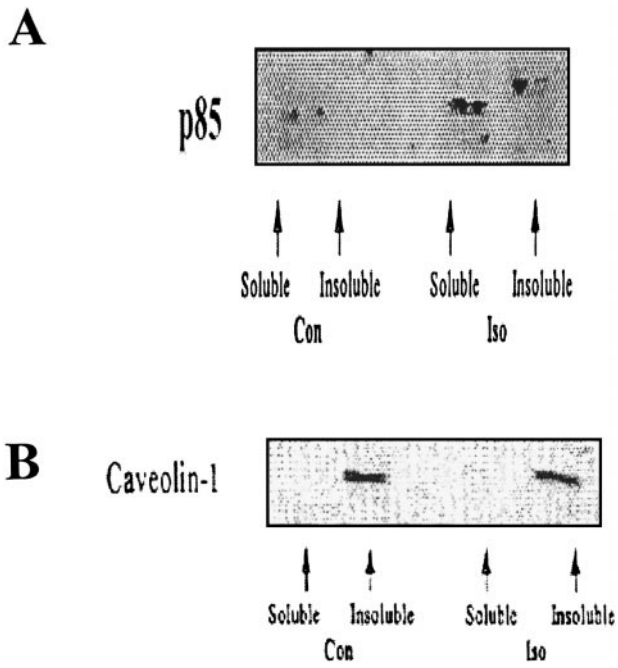

Fig 3. Effect on inhibition of PI3-K on Iso-stimulated nitrite production. Nitrite production was measured in RAEC exposed to Iso for 5 minutes or IGF-1 for 10 minutes, or pretreated with of WT for 15 minutes followed by the addition of Iso for 5 minutes or IGF-1 for 10 minutes. Nitrite production is expressed as μmol/L. Each bar represents the mean ± SE of 4 or 6 experiments performed in duplicate. ***$P < .0001$, **$P > .001$, [a]*$P < .001$ Iso *v* WT/Iso.**

dent protein of caveolae, was enriched in this RAEC fraction (Fig 2B).

Effect of Inhibition of PI3-K on eNOS Activity

We further examined whether PI3-K inhibition effected on eNOS activity. For these experiments, we treated cells with Iso (10 μmol/L) for 5 minutes, and nitrite production was measured by the Griess reaction.[17] Figure 3 shows that exposure of RAEC to 10 μmol/L Iso for 5 minutes significantly increased nitrite production by 226% (Iso, 18.99 ±2.77 μmol/L; control, 5.82 ± 0.354 μmol/L, $P < .001$) compared to control values (Fig 3). Under stimulated conditions, preincubation of cells for 15 minutes with 30 nmol/L WT before exposure to Iso for an additional 5 minutes (Fig 3) reduced Iso-stimulated nitrite production by 70% compared to the effect in the absence of PI3-K inhibition (WT/Iso, 5.73 ± 0.554 μmol/L, $P < .001$). On the other hand, the IGF-1 effect on nitrite production was also seen after 10 minutes (Fig 3). IGF-1 increased nitrite production by 200% compared to control values (IGF-1, 17.63 ± 1.51 μmol/L; control, 5.82 ± 0.354 μmol/L, $P < .0001$).

Role of PI3-K in Modulation of Vascular Contractility

Since the aim of this study was to examine the role of the PI3-K signaling pathway in endothelium-dependent relaxation in vitro, we next examined the effect of the PI3-K inhibitor,

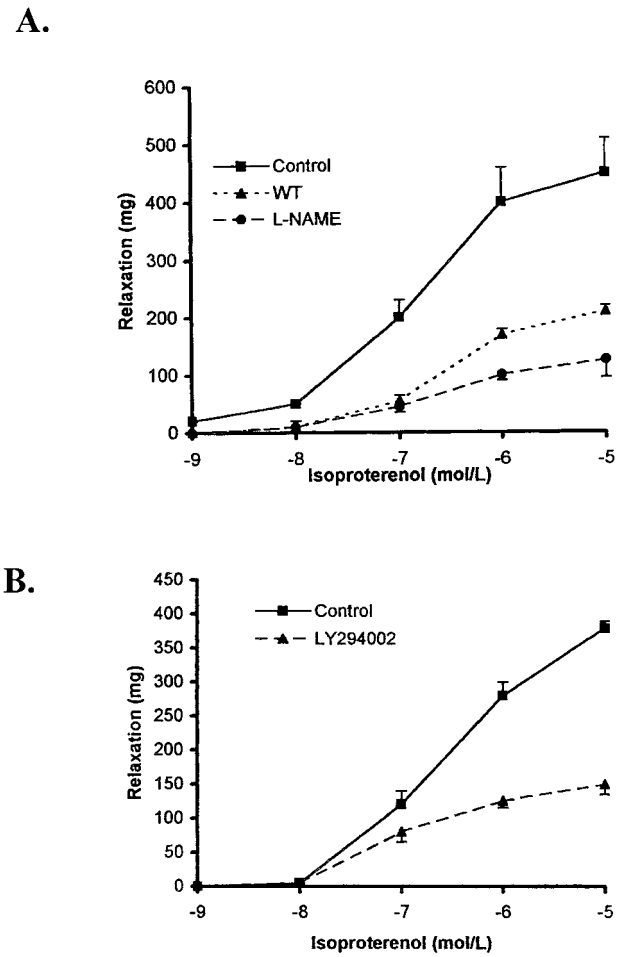

Fig 4. Iso dose-responses in PE-precontracted aortic rings. Both WT treatment (for 40 minutes) and L-NAME preincubation (for 30 minutes) markedly attenuated relaxation (A). Similarly, LY (50 μmol/L for 30 minutes) markedly inhibited the Iso relaxation response (B). *P* values were calculated over the entire dose-response by multiple ANOVA. Values are mean ± SEM (n = 5). **P* > .05. L-NAME, NOS inhibitor (300 μmol/L).

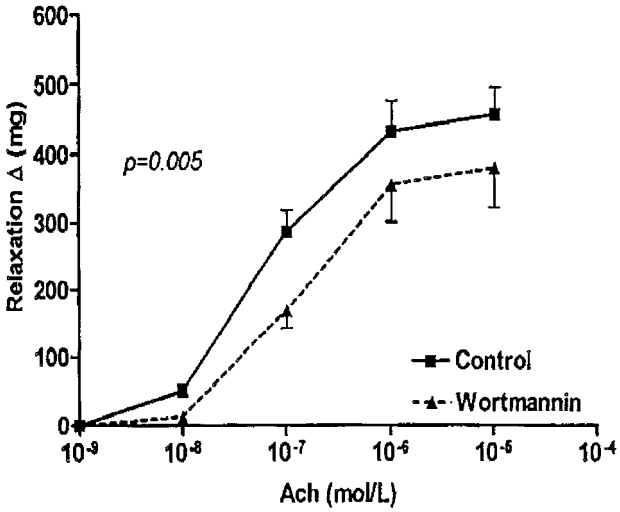

Fig 5. Effect of WT (300 nmol/L) preincubation on Ach-induced relaxation in PE-precontracted aortic rings. Relaxation was lowered by the WT, PI3-K inhibitor. Values are mean ± SEM (n = 5). **P* > .05. Ach, acetylcholine.

WT, on agonist-induced contractility in endothelium-intact aortic rings. Since WT incubation decreased contractility to KCl (maximal tension in mg: controls 910 ± 40 *v* WT 577 ± 88, $P < .0001$), but not to PE (maximal tension in mg: controls 925 ± 27 *v* WT 866 ± 48), PE was used as the precontractile agent for the dose-related vasorelaxation responses. PI3-K inhibition via 2 chemically distinct compounds, WT (Fig 4A) and LY (Fig 4B), attenuated Iso-induced relaxation (53% and 61%, respectively); preincubation of the rings with L-NAME nearly abolished relaxation to Iso (Fig 4A), confirming the involvement of NO in this relaxation (Figs 3 and 4). Thus, the effect of WT on Iso-stimulated vasorelaxation (Fig 4) was associated with increased activity of eNOS as evidenced by increased nitrite production (Fig 3). On the other hand, WT/LY and L-NAME significantly decreased maximal relaxation to Iso. Both WT and L-NAME caused a significant decrease in sensitivity (increased ED_{50}). In similar experiments, WT at the same dose, slightly (17%) but significantly attenuated relaxation to acetylcholine (Ach) (Fig 5 and Table 1). In studies carried out to explore the involvement of PI3-K in cyclic adenosine monophosphate (cAMP)-mediated vasorelaxation, WT caused only a slight decrease in sensitivity and no significant difference in maximal lowering of tension to forskolin (,1232 ± 110 mg *v* 1,032 ± 61 mg in controls), suggesting that WT inhibitory action does not reside at the level of cAMP generation and/or action.

Thus, using 2 indices of activation (p85kd regulatory subunit phosphorylation and cytoskeletal translocation), our results show for the first time that Iso stimulates PI3-K in RAEC and that in RAEC the PI3-K pathway mediates, in part, the release of NO and subsequent vasorelaxation in response to this β-agonist, Iso, as well as to IGF-1.

DISCUSSION

This is the first report of a role for EC PI3-kinase signaling pathway in Iso-mediated vasorelaxation. Results of this inves-

Table 1. Maximal Relaxation in Rat Aortic Rings Exposed to WT or L-NAME and ED_{50} for Iso- and Ach-Induced Relaxation in Rat Aortic Rings

		% (inhibition)	*P**	ED_{50} (nmol/L)	*P*†
Iso (mg)					
Control	450 ± 59				
WT	210 ± 23	53	<.0001	159 ± 26	.045
L-NAME	120 ± 37	73	<.0001‡	178 ± 30	.020
Ach (mg)					
Control	456 ± 38			78 ± 8	
WT	379 ± 57	17	<.005	131 ± 9	.014

*Two-way ANOVA.
†*t* test.
‡*v* control.

tigation indicate that: (1) Iso induces tyrosine phoshorylation of the p85kd subunit in EC; (2) Iso also induces translocation of tyrosine phosphorylated p85 to the caveolin-rich Triton-insoluble fraction of rat aortic EC reflecting its association with the cytoskeleton[21]; and (3) the PI3-K inhibitors, WT and LY, as well as the NOS inhibitor, L-NAME, all reduced relaxation to Iso in rat aortic rings. L-NAME was more potent in this regard than either WT or LY, indicating that the PI3-K inhibitors probably did not affect cAMP-related mechanisms. Our studies with L-NAME confirm the strong dependence on EC-derived NO of Iso-elicited vasorelaxation.

In vascular EC, increases in cytosolic calcium and consequent calmodulin binding to eNOS stimulate NO production.[16] In the current investigation, Iso mobilized "phosphorylated" PI3-K to the cytoskeleton containing fraction of the cell lysates, further implicating the EC PI3-K signaling pathway in the post-translational regulation of eNOS. Iso has been shown to increase PI3-K activity in parotid acinar cells[22] and several studies have reported that the p85 subunit of PI3-K is phosphorylated on tyrosine residues in response to various growth factors, including insulin/IGF-1[1,13] and angiotensin II.[3] In prior[7,23] and our current study, translocation of the enzyme from the cytosol to the cytoskeleton was documented. Also, in the current investigation, pretreatment with WT attenuated p85 subunit phosphorylation in RAEC, suggesting a novel role for this enzyme in β-receptor–mediated vascular relaxation. This observation suggests that Iso and peptides such as IGF-1 may activate the enzyme through a common mechanism, perhaps G-protein–mediated.[3,5,13,15]

Triton X-100–insoluble cytoskeletal membranes contain many key proteins that participate in cellular signal transduction, including eNOS, G-α, $\beta\gamma$-subunits, muscarinic receptors, and tyrosine kinases among them.[21-24] Caveolins are 20- to 24-kd oligomeric membrane proteins, and the known caveolin isoforms have distinct tissue-specific patterns of expression.[23] Additionally, there appear to be important differences between the various caveolin isoforms in the nature of their direct interactions with and regulation of G-protein subunits.[21-24] In the current investigation, caveolin-1, a resident protein of caveolae, was enriched in the RAEC Triton-insoluble membrane fraction, as was the "phosphorylated" PI3-K after treatment with Iso. Since agonist-induced movement between these sites has been speculated to involve specific vesicular shuttles, PI3-Ks and their 3-phosphoinositide products, known modulators of vesicular traffic, are likely to participate.[26-28] We observed that Iso increased phosphorylation and translocation of the p85 subunit to the cytoskeleton. This observation is in concordance with reports demonstrating eNOS translocation to the cytoskeleton by various NO-stimulating agonists[26-28] and raises the interesting possibility of cross-talk between these signal transduction pathways in mediating both IGF-1– and β-adrenergic–induced NO generation in EC. Since several proteins involved in Ca^{2+}-dependent signaling processes (including phosphatidylinosistol-3-phosphate (IP_3)-dependent calcium release channels and Ca^{2+}-adenosine triphosphatase) were localized to caveolae,[27] it seems plausible that the targeting of these signaling molecules to caveolae may facilitate the calcium-calmodulin–dependent activation of eNOS.

Data from the current investigation provide evidence that activity of eNOS is increased after stimulation with Iso. It has recently been reported that caveolins can be isolated as multimeric complexes comprising 14 to 16 caveolin monomers, and that it is possible that these caveolin complexes might directly interact with G-protein subunits.[27,28] G-$\beta\gamma$ subunit activates PI3-K,[4] and a recent report indicates that these subunits stimulate PI3-K by direct interaction with domains of the catalytic p110 subunit.[29] Thus, it is possible that b -receptor activation by Iso results in the release of G $\beta\gamma$ subunits, which then activate PI3-K. A multitude of downstream effectors of PI3-K could subsequently mediate NOS activation, including protein kinase B (PKB/Akt/Rac), shown to mediate insulin-stimulated glucose uptake and to be translocated to GLUT4-containing vesicles.[30] The Iso- as well as insulin/IGF-1–mediated relaxation may be defective in hypertensive[31,32] and diabetic states.[33,34] In accordance with this notion, diminished PI3-K activity could be a common defect that manifests itself in impaired glucose uptake and NO synthesis and links hypertension and insulin-resistant states.[1,5,35]

Removal of the endothelium[34] and inhibition of the NO-cyclic guanosine monophosphate (cGMP) pathway[36,37] have been reported to decrease Iso-induced relaxation. Our studies using the NOS inhibitor, L-NAME, confirm the strong dependence of Iso-elicited vasorelaxation on EC-derived NO. Results of the current investigation also showed that pretreatment of aortic rings with the PI3-K inhibitors, WT and LY, significantly attenuated the endothelium-dependent relaxation response to Iso, further confirming a role for the PI3-K signaling pathway in β-receptor signal transduction. While WT did inhibit KCl-induced contractile responses, it had no effect on PE-initiated contraction, findings consistent with previous reports suggesting greater sensitivity of inhibition of KCl-induced myosin light chain kinase (MLCK) phosphorylation by WT at the dose used (300 nmol/L).[25] Another novel finding in this investigation was attenuated relaxation to Ach in WT-treated aortic rings. Although our in vitro studies suggest that WT inhibits EC eNOS, the vascular relaxation studies with rings could reflect PI3-kinase–mediated effects in VSMC as well as EC. This may be related to nonspecific inhibition of phospholipase C or MLCK by WT.[24,25] However, WT did not affect PE-induced contractions, suggesting that WT may exert actions not previously described. Further, all relaxation experiments were done after a similar degree of precontraction where inhibition of MLCK would not result in decreased relaxation. The data suggest potentially important interactions between muscarinic receptor–G-protein–NO pathways and PI3-K exist in vascular tissue, as they do in neuroblastoma cells, where muscarinic cholinergic phosphorylation of cytoskeletal proteins can be prevented with either WT or LY.[18]

The limited degree of attenuation seen with PI3-K inhibitors suggests that other mechanisms may be involved in Ach activation of NOS, the major mechanism for relaxation in response to this agonist.[27] On the other hand, VSMC relaxation mediated by stimulation of β-adrenergic receptors involves both an increase in cAMP and a rise in cGMP, a result of EC release of NO, which facilitates cAMP action in VSMC.[16,24] Also, the current results show that Iso significantly increases eNOS cy-

toskeletal translocation/activation as determined by increased nitrite production. Potential abnormalities of these interactive mechanisms may explain the diminished β-adrenergic–mediated vasorelaxation observed in individuals predisposed to hypertension[15,38] and diabetic rodent models.[5,13,33-35]

In conclusion, the current data suggest that the WT-sensitive p85 regulatory subunit of PI3-K is involved in Iso-mediated vasorelaxation via stimulation of eNOS activity in EC. Also, the current investigation demonstrates in vitro that Iso, like IGF-1, results in an increase in eNOS translocation/activation. These events may have relevance for eNOS regulation and trafficking within vascular endothelium and mediation of the vascular actions of Iso. Thus, regulation of eNOS activity and consequent NO production is one mechanism through which β-adrenergic receptors exert control of vascular contractility and relaxation. In summary, the current data suggest the critical role of PI3-K signaling pathway in EC-derived vasorelaxation mediated via β-adrenergic or IGF-1 receptors.

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