Endothelin-1 Modulates Insulin Signaling Through Phosphatidylinositol 3-Kinase Pathway in Vascular Smooth Muscle Cells

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Diminished insulin action in the vasculature may contribute to the development of cardiovascular diseases in diabetes. We have studied insulin's effects on the phosphatidylinositol (PI) 3-kinase pathway in arterial smooth muscle cells (SMCs) and its inhibition by endothelin (ET)-1, a potent vasoactive hormone reported to be elevated in insulin resistance and other vascular diseases. ET-1 increased the level of serine phosphorylation of insulin receptor β subunit but increased both tyrosine and serine phosphorylation of insulin receptor substrate (IRS)-2. Pretreatment of cells with ET-1 (10 nmol/l) inhibited insulin-stimulated PI 3-kinase activity associated with IRS-2 by 50–60% and inhibited the association of p85 subunit of PI 3-kinase to IRS-2. The inhibition of insulin-stimulated PI 3-kinase activity by ET-1 was prevented by BQ-123, a selective ET$_A$ receptor antagonist, but was not affected by pertussis toxin. Treatment of cells with phorbol 12-myristate 13-acetate, an activator of protein kinase C (PKC), reduced both insulin-stimulated PI 3-kinase activity by 57% and the association of IRS-2 to the p85 subunit of PI 3-kinase by 40%, whereas GF109203X, a specific inhibitor of PKC, partially prevented the inhibitory effect of ET-1 on insulin-induced PI 3-kinase activity. These results suggested that ET-1 could interfere with insulin signaling in SMCs by both PKC-dependent and -independent pathways. *Diabetes* 48:1120–1130, 1999

Insulin action is initiated by binding to cell surface insulin receptors (IRs), which trigger a cascade of signaling events beginning with the activation of the intrinsic tyrosine kinase (1), autophosphorylation of insulin receptor β subunit (IRβ) (2), and phosphorylation of intracellular proteins, including insulin receptor substrate (IRS)-1 and -2 (3,4). Tyrosine phosphorylated IRS-1 and -2 bind to multiple src-homology 2 domain (SH2)-containing molecules, including the p85 subunit of phosphatidylinositol (PI) 3-kinase (5). The interaction of IRS proteins and the p85 regulatory subunit of PI 3-kinase results in the activation of the p110 catalytic subunit of the kinase, which then phosphorylates cellular phosphatidylinositides at position D-3. A number of factors, such as activation of protein kinase C (PKC) (6–8) and inhibition of serine/threonine phosphatase (9,10), have been shown to modify phosphorylation states of IR and/or IRS proteins, resulting in impaired insulin signaling on the PI 3-kinase pathway.

In the vascular cells, insulin and IGF-1 have multiple important biological actions. These actions include metabolic and mitogenic effects, such as increases in amino acid transport, and glycogen and DNA synthesis, in both cultured vascular smooth muscle cells (SMCs) and microvascular endothelial cells (11–13). In addition, insulin has vascular-specific actions, such as increasing the expression of endothelin (ET)-1 mRNA (14) and the production of nitric oxide in cultured endothelial cells (15). Insulin administration also causes vasodilatation locally, which appears to be mediated through the enhancement of nitric oxide's action (16).

Impairment of insulin's actions in the vascular system has been reported in insulin-resistant states. One example is that insulin's vasodilatory effect is blunted in insulin-resistant nondiabetic individuals and type 2 diabetic patients (17,18). The biochemical mechanism underlying the development of insulin resistance in the vasculature remains elusive. Vasotropic hormones, such as angiotensin II and ET-1, have been suggested as possible inhibitors of insulin's effects on vascular cells because of their association with vascular diseases in diabetic patients (19). In this study, we have explored the interactions between insulin and ET-1 at the signal transduction levels.

ET-1, a vascular active polypeptide secreted by endothelial cells (20,21), induces vasoconstriction through ET$_A$ receptors (22,23). Biochemically, ET-1 binds to pertussis toxin (PTX)-insensitive Gαq-coupled ET$_A$ receptors on vascular SMCs and activates phospholipase C$_\beta$ (24,25), which increases the

formation of inositol triphosphate, and diacylglycerol, resulting in an increase of cytosolic Ca^{2+} and activating PKC (20,26,27). Moreover, ET-1 also activates receptor and non-receptor tyrosine kinases (28–30), stimulates serine kinases (27,31), and induces tyrosine or serine phosphorylation of intracellular proteins (32–34) in various types of cells, suggesting ET-1 can signal through a diverse array of pathways. Elevated ET-1 levels in the plasma have been reported in diseases such as diabetes (35), obesity (36), hypertension (37), and atherosclerosis (38), all of which are associated with insulin resistance (39,40). Interestingly, ET-1 inhibits insulin-stimulated glucose uptake in cultured rat adipocytes (41), and exogenous ET-1 can induce insulin resistance in conscious rats and healthy people (42,43). However, the biochemical mechanism by which ET-1 induces insulin resistance in the vascular cells is unknown.

To examine the possible interactions between insulin and ET-1, we have characterized insulin signal transduction on the PI 3-kinase pathway in cultured rat arterial SMCs derived from blood vessels isolated from rat epididymal fat pads. These studies have provided supportive evidence that insulin activated PI 3-kinase predominantly through its association with IRS-2 in vascular SMCs. ET-1 inhibited insulin-stimulated PI 3-kinase activity possibly by altering the phosphorylation of IR and IRS-2 in vascular SMCs.

RESEARCH DESIGN AND METHODS

Materials. Human recombinant insulin (Humulin R) was purchased from Eli Lilly Research Laboratories (Indianapolis, IN). IGF-1, platelet-derived growth factor (PDGF)-BB, monoclonal antibody against phosphotyrosine (αPY), and poly-clonal antibodies against the p85 subunit of PI 3-kinase were purchased from UBI (Lake Placid, NY). Polyclonal antibodies against rat IRS-1 (JD229) and IRS-2 (JD110) were raised in rabbits using glutathione S-transferase–fusion proteins containing amino acids 735–900 of rat IRS-1 and amino acids 976–1,094 of mouse IRS-2, respectively (3,4). Polyclonal antibody against the human IRβ was provided by Dr. B. Cheatham (Boston, MA). Rabbit anti-mouse antibody was purchased from Dako (Carpinteria, CA). Donkey anti-rabbit immunoglobulin (Ig) horseradish peroxidase–linked antibody, an enhanced chemiluminescence (ECL) kit, was from Amersham (Arlington Heights, IL). [γ-^{32}P]ATP was from Du Pont-NEN (Wilmington, DE). Reagents for SDS, polyacrylamide, and immunoblotting were obtained from Bio-Rad (Richmond, CA). NP-40 was from Calbiochem (La Jolla, CA). PI was from Avanti (Alabaster, AL), silica gel thin-layer chromatography (TLC) plates were from Merck (Gibbstown, NJ), protein A Sepharose 6MB was from Pharmacia (Uppsala, Sweden), and nitrocellulose paper (BA 85, 0.2 mm) was from Schleicher & Schuell (Keene, NH). All other reagents were from Sigma (St. Louis, MO).

Cell culture. Arterial SMCs were obtained from the outgrowth of blood vessels isolated from epididymal fat pads of male Zucker lean rats purchased from Harlan Sprague Dawley (Indianapolis, IN). Cells between passages 6 and 12 were used for the experiments described herein. Cells were grown to confluence in Dulbecco's minimal essential medium (DMEM) containing 10% calf serum at 37°C and 5% CO_2. Cells were then placed in serum-free DMEM containing 0.2% bovine serum albumin (BSA) for 24 h. On the day of the experiment, reagents were added at the appropriate concentrations and times as described herein. Lysed cells were then subjected to different assays depending upon experimental design.

Immunoprecipitation of IR, IRS-1, and IRS-2. After the addition of insulin or other reagents, cells were washed with ice-cold phosphate-buffered saline (PBS) and broken with 1 ml lysis buffer (50 mmol/l HEPES, pH 7.4, containing 10 mmol/l Na pyrophosphate, 100 mmol/l NaF, 2 mmol/l EDTA, 2 mmol/l Na_3VO_4, 10% [vol/vol] glycerol, 1% [vol/vol] Triton X-100, 2 mmol/l phenylmethylsulfonyl fluoride [PMSF], 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The lysate (1–2 mg protein, as indicated) was then precipitated with antibodies (αIRS-1 or αIRS-2 [10 μl/ml] and αIRβ [5 μl/ml]) in lysis buffer for 2 h, followed by incubation with 60 μl of protein A Sepharose 6MB (Pharmacia, Piscataway, NJ) for 2 h at 4°C. Beads were washed three times with lysis buffer before being treated with Laemmli buffer
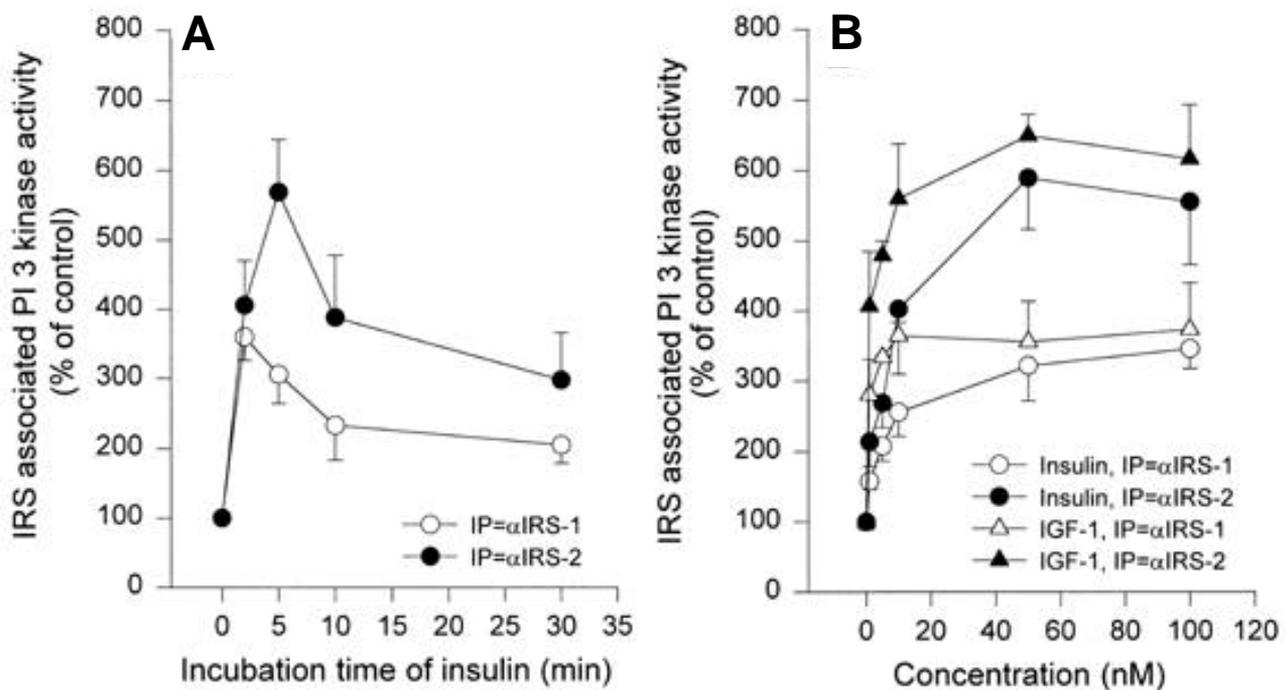
FIG. 1. Insulin's effect on the activation of IRS-associated PI 3-kinase in rat vascular SMCs. *A*: Time course. Confluent cells were starved for 24 h in serum-free DMEM containing 0.2% BSA before being treated with or without 100 nmol/l insulin for 2, 5, 10, and 30 min at 37°C. Cell lysates (1 mg protein) were immunoprecipitated with αIRS-1 or αIRS-2 antibodies, and the precipitates were assayed for PI 3-kinase activity (as described in METHODS). *B*: Concentration response curve. Starved cells were treated with or without insulin (1, 2, 5, 10, 50, and 100 nmol/l) or IGF-1 (1, 2, 5, 10, 50, and 100 nmol/l) for 5 min at 37°C. Cell lysates (1 mg protein) were immunoprecipitated with αIRS-1 or αIRS-2 antibodies, and the precipitates were assayed for PI 3-kinase activity. Data were means ± SD of three independent triplicate experiments.

and heated in boiling water for 4 min. Protein extracts were used for electrophoresis and immunoblotting studies as described below.

Immunoblotting studies for protein tyrosine phosphorylation and the p85 subunit of PI 3-kinase. Immunoprecipitated fractions (as described above) were separated with SDS-PAGE and electrotransferred to a nitrocellulose filter that was incubated with blocking buffer (PBS containing 0.2% Tween-20 [PBST]) and 3% BSA overnight at 4°C. To detect protein tyrosine phosphorylation, the blot was first incubated with monoclonal antibody αPY (UBI; 1 µg/ml) for 1 h, then with rabbit anti-mouse antibody (Dako; 1:1,000 dilution) for 2 h, and finally with ^{125}I-protein A (Amersham) for 1 h at room temperature. Blots were then washed with PBST for 1 h at room temperature after each incubation, and ^{125}I-protein A–bound proteins were detected and quantified using ImageQuant software on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). To detect the association of the p85 subunit of PI 3-kinase with IRS proteins that were immunoprecipitated by antibodies against IRS-1 or IRS-2, the same membrane was stripped with buffer (62.5 mmol/l Tris-HCl, pH 6.7, 100 mmol/l 2-mercapotoethanol, and 2% SDS) for 30 min at 37°C. The membrane was then reblotted with antibody against the p85 subunit of PI 3-kinase (UBI; 1:1,000 dilution in PBST [0.1% Tween-20] and 3% BSA) for 1 h at room temperature and washed for 1 h with PBST. The membrane was then incubated with the second antibody containing horseradish peroxidase–linked anti-rabbit Ig antibody (Amersham; 1:2,000 dilution) for 1 h, and the amount of the p85 subunit was detected with an ECL detection kit (Amersham).

In vivo phosphorylation. As described previously (44), vascular SMCs in 15-cm plates were serum-starved for 20 h and then incubated for 4 h in phosphate-free Eagle's minimum essential medium (Gibco, Grand Island, NY) containing 0.5 mCi/ml [32P]orthophosphate. Cells were then stimulated with 10 nmol/l of ET-1 or 100 nmol/l of insulin for 5 min and then lysed with 50 mmol/l HEPES (pH 7.4) containing 10 mmol/l Na pyrophosphate, 100 mmol/l NaF, 2 mmol/l EDTA, 2 mmol/l Na_3VO_4, 2 mmol/l PMSF, 10% (vol/vol) glycerol, 1% Triton X-100, 10 µg/ml leupeptin,

and 1 µg/ml aprotinin. Cell lysates were centrifuged for 15 min at 12,000 rpm at 4°C. Supernatants were incubated overnight at 4°C with αIRS-2, and the immunocomplexes were precipitated with a 50% solution of protein A Sepharose 6MB. For in vivo phosphorylation studies of IRs, cell lysates were centrifuged at 100,000g for 20 min and the supernatant was applied to a wheat germ agglutinin (WGA)-Sepharose column equilibrated with lysis buffer and rotated for 30 min at 4°C. After washing three times with lysis buffer, IRs were eluted from the WGA column with 0.3 mol/l N-acetyl-D-glucosamine (Sigma). Partially purified IRs were immunoprecipitated with polyclonal anti-IRβ antibody, and the immunocomplexes were precipitated with protein A Sepharose 6MB. The washed immunocomplexes were eluted with Laemmli sample buffer and separated on 6% SDS-PAGE for IRS-2 protein and 7.5% SDS-PAGE for IR. [32P]-labeled proteins were transferred to nitrocellulose and visualized by autoradiograph.

Phosphoamino acid analysis. Nitrocellulose containing [32P]-labeled IRβ or IRS-2 protein was excised and treated with 0.5% (wt/vol) polyvinylpyrrolidone-40 (Sigma) in 100 mmol/l acetic acid for 30 min at 37°C. Paper was washed extensively with water, cut into 1- to 2-mm squares, and digested with 100 µl of 0.1 mg/ml L-1-tosylamido-2-phenylethyl choloromethyl ketone (TPCK)-trypsin (Worthington, Millville, NJ) in $NaHCO_3$ (pH 8.2) containing 5% acetonitrile at 37°C for 24 h. Incubation was carried out for another 24 h with the addition of 10 µl of freshly dissolved 1 mg/ml TPCK-trypsin. The sample was then dried by speed vacuum. The [32P]-labeled protein was hydrolyzed in 100 µl of 6 N HCl at 110°C for 2 h, diluted with 1 ml of H_2O, evaporated again by speed vacuum, and redissolved in a 10-µl solution containing 1 mg/ml standard phosphoserine, phosphothreonine, and phosphotyrosine. Phosphoamino acid analysis was performed by electrophoresis on cellulose thin-layer plates and quantified with the PhosphoImager.

PI 3-kinase activity assay. PI 3-kinase activities were measured by the in vitro phosphorylation of PI (5). Briefly, cells were lysed with buffer containing 20 mmol/l Tris-HCl (pH 7.5), 137 mmol/l NaCl, 1 mmol/l $CaCl_2$, 1 mmol/l $MgCl_2$, 10
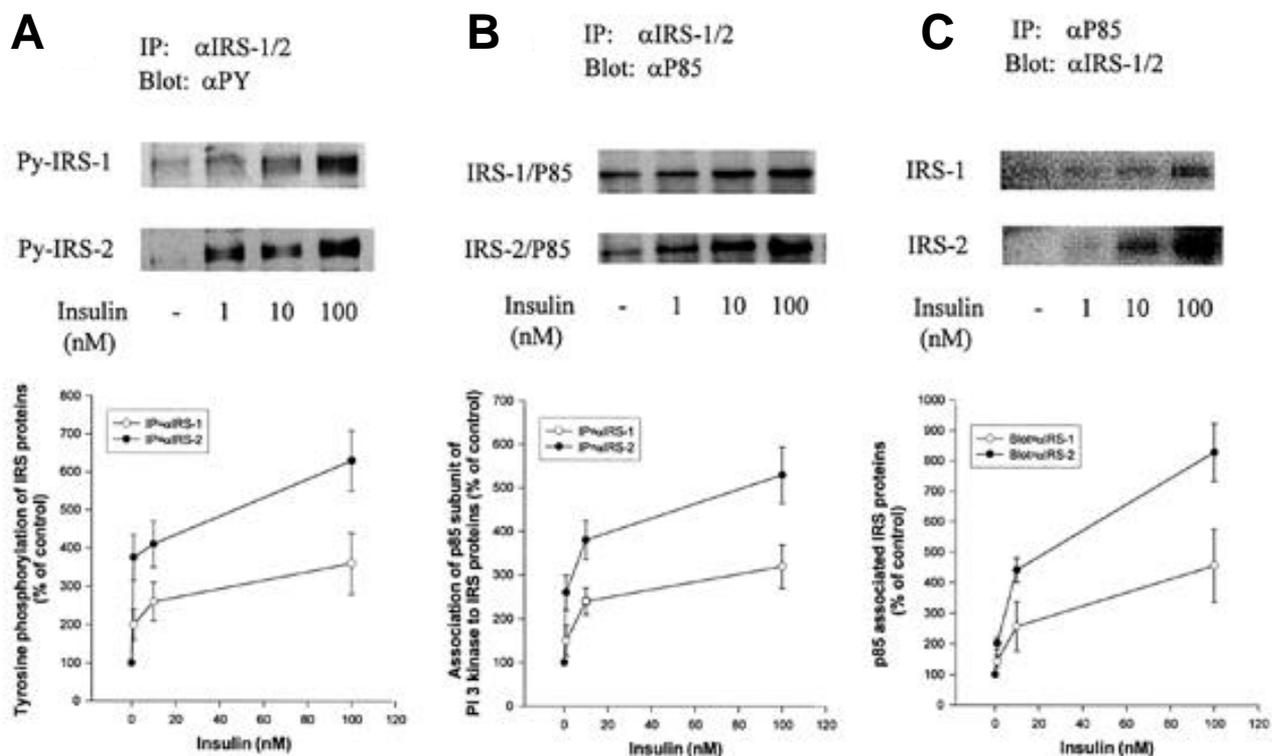

FIG. 2. Insulin-stimulated increases in tyrosine phosphorylation of IRS proteins and their association to PI 3-kinase in rat vascular SMCs. Cells were stimulated with 0, 1, 10, and 100 nmol/l insulin for 3 min. *A*: Cell lysates (1 mg protein) were immunoprecipitated with αIRS-1 or αIRS-2 antibodies, dissolved in 6% SDS-PAGE, and transferred to nitrocellulose membranes. To detect tyrosine phosphorylation of IRS proteins, membranes were blotted with αPY antibody, rabbit anti-mouse Ig antibody, and ^{125}I-protein A. Upper and middle panels show the representative phosphoimages of tyrosine-phosphorylated IRS-1 and IRS-2, respectively. Lower panel shows the concentration response curve of tyrosine phosphorylation of IRS-1 and IRS-2 stimulated by insulin. *B*: Same membranes were stripped and blotted with antibody against the p85 subunit of PI 3-kinase and anti-rabbit Ig antibody, and the amount of p85 associated with IRS proteins was detected with the ECL system. Upper and middle panels show the representative images of the ECL-detectable p85 subunit of PI 3-kinase associated with IRS-1 and IRS-2, respectively. Lower panel shows the amount of p85 associated with IRS-1 and IRS-2. *C*: Cell lysates (1 mg protein) were immunoprecipitated with αp85 antibody. Precipitates were dissolved in 6% SDS-PAGE and transferred to nitrocellulose membranes. To detect IRS protein levels, membranes were blotted with αIRS-1 (JD229) or IRS-2 (JD110) antibodies, respectively, followed by incubation with ^{125}I-protein A. Upper and middle panels show representative phosphoimages of IRS-1 and IRS-2, respectively. Lower panel shows insulin concentration–dependent stimulation of αp85 immunoprecipitable IRS-1 and IRS-2. Data are means ± SD of three independent triplicate experiments.

mmol/l Na pyrophosphate, 10 mmol/l NaF, 2 mmol/l Na_3VO_4, 2 mmol/l PMSF, 2 mmol/l EDTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 mmol/l benzamidine, 1% NP-40, and 10% glycerol. Cells were then immunoprecipitated with αIRS-1 or αIRS-2 (10 µl/ml) antibodies for more than 2 h, followed by incubation with 60 µl protein A Sepharose 6MB for 2 h at 4°C. Beads were washed three times with PBS containing 1% Nonidet P-40 and 100 µmol/l Na_3VO_4, three times with 100 mmol/l Tris (pH 7.5) containing 500 mmol/l LiCl and 100 µmol/l Na_3VO_4, and three times with 10 mmol/l Tris (pH 7.5) containing 0.1 mol/l NaCl, 1 mmol/l EDTA, and 100 µmol/l Na_3VO_4. The pellet was resuspended in 50 µl Tris-NaCl buffer, and 12 mmol/l $MgCl$ and 20 µg PI were added. PI 3-kinase activity was measured at room temperature and initiated by adding 5 µl 880 µmol/l ATP containing 30 µCi [γ-^{32}P]ATP. After 10 min of vigorous vortexing, the reaction was stopped by adding 20 µl of HCl (10 mol/l) and 160 µl of $CHCl_3/CH_3OH$ (1:1), and 50 µl of lower organic phase was spotted on a TLC plate pretreated with oxalate. Lipids were resolved by chromatography in $CH_3OH/CHCl_3/H_2O/NH_4OH$ (120:94:22.6:4) and quantified with the PhosphorImager.

Measurement of intracellular free calcium. Cells were cultured on glass microscopic slides under normal culture conditions. Confluent cells were treated with 5 µmol/l fura-2 acetoxymethyl ester at 37°C for 20 min before stimulation with lysophosphatidic acid (5 µg/ml). Changes in Ca^{2+}–fura-2 fluorescence were monitored at 37°C in an inverted epifluorescence microscope (Photon Technology International Deltascan 4000, South Brunswick, NJ) at excitation wavelengths of 340 and 380 nm and at an emission wavelength of 510 nm. Intracellular calcium levels were calibrated according to Grynkiewicz et al. (45).

Statistics. Unless indicated otherwise, comparison of data presented as means ± SD was performed using the Kruskall-Wallis one-way analysis of variance. Values of $P < 0.05$ were considered significantly different.

RESULTS

Concentration- and time-dependent activation of PI 3-kinase by insulin and IGF-1. Insulin and IGF-1 stimulated IRS-associated PI 3-kinase activities in vascular SMCs in a time- and concentration-dependent manner. Insulin increased PI 3-kinase activity associated with IRS-1 and IRS-2 by three- and sixfold, respectively, after 2–5 min of incubation. A decline from the maximum was noted after 10 min, but PI 3-kinase activity still remained significantly higher in insulin-stimulated cells than in untreated cells after 30 min (Fig. 1A). Interestingly, IRS-2–associated PI 3-kinase activities were consistently higher than those associated with IRS-1. Both insulin and IGF-1 activated αIRS-1 and αIRS-2 immunoprecipitable PI 3-kinase in a concentration-dependent manner starting at 1 nmol/l (Fig. 1B). The maximal levels of PI 3-kinase activities associated with IRS-1 (~3.4-fold increase) and IRS-2 (~5.8-fold increase) were achieved at 50 nmol/l of insulin and 10 nmol/l of IGF-1. For both insulin and IGF-1, IRS-2–associated PI 3-kinase activities were always about twofold greater than IRS-1–associated activities.

The effect of insulin on IRS-1 and -2 tyrosine phosphorylation and association with PI 3-kinase. The mechanism of insulin-stimulated activation of PI 3-kinase in vascular SMCs was characterized by determining the effects of 1, 10, and 100 nmol/l of insulin on IRS-1 and -2 tyrosine phosphorylation and their association to the p85 subunit of PI 3-kinase. Cell lysates were immunoprecipitated by αIRS-1 and αIRS-2 antibodies, fractionated by SDS-PAGE, and immunoblotted by using antibodies to phosphotyrosine. As shown in Fig. 2A and B, insulin stimulated tyrosine phosphorylation of IRS-1 and IRS-2 in a concentration-dependent manner with significant increases from 1 to 100 nmol/l of insulin. Similarly, insulin increased the amount of p85 protein associated with IRS-1 and IRS-2 significantly between 1 and 100 nmol/l. The amount of tyrosine phosphorylation on IRS-1 and IRS-2 correlated directly to their association with the p85 subunit of PI 3-kinase (Fig. 2A and B). The levels of IRS-2 tyrosine phosphorylation

and the amount of IRS-2/p85 complex stimulated by insulin were consistently twofold greater than those observed in IRS-1 immunoprecipitated proteins.

The observation that there was a greater association between the p85 subunit of PI 3-kinase and IRS-2 than IRS-1 suggested that insulin-induced PI 3-kinase activation could be mediated preferentially through IRS-2 rather than IRS-1 in vascular SMCs. It is also possible that this difference could be due to a higher affinity for immunoprecipitation of the αIRS-2 antibody rather than the αIRS-1 antibody. To differentiate between these possibilities, insulin-stimulated vascular SMCs were immunoprecipitated with the same antibody against the p85 subunit of PI 3-kinase, and the samples were blotted with specific antibodies to IRS-1 (JD229) and IRS-2 (JD110) that have been calibrated to have equal efficiency for identifying IRS-1 and IRS-2 fusion proteins on immunoblotting studies, respectively. As shown in Fig. 2C, insulin-stimulated formation of the IRS-2/PI 3-kinase complex was about twofold higher than that of the IRS-1/PI 3-kinase complex, indicating that insulin activated PI 3-kinase predominantly by the IRS-2 pathway in vascular SMCs.

Effects of ET-1 on the phosphorylation of amino acid residues in IRβ and IRS-2. Modulation of the phosphorylation states of both IRs and IRS proteins by protein kinases, such as serine/threonine kinases, are known to affect insulin
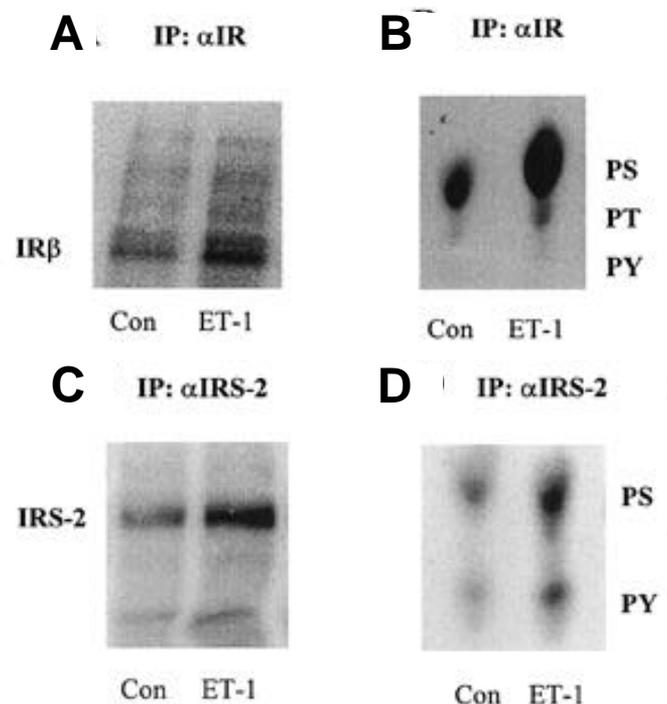

FIG. 3. Effects of ET-1 on the in vivo phosphorylation of IRβ and IRS-2. Serum-starved vascular SMCs were incubated for 4 h in phosphate-free Eagle's minimum essential medium containing 0.5 mCi/ml [^{32}P]orthophosphate. Cells were then incubated with or without ET-1 (10 nmol/l) for 7 min. Cell lysates were immunoprecipitated with αIRβ or αIRS-2 antibodies, and ^{32}P-labeled proteins were separated by SDS-PAGE and were transferred to nitrocellulose. The nitrocellulose-containing ^{32}P-labeled IRβ and IRS-2 proteins were excised, and phospho-amino acid analysis was performed. Representative autoradiographs of total phosphorylation and phosphoamino acids from two independent experiments are shown. A: Effects of ET-1 on IR phosphorylation in vivo. B: Phosphoamino acid analysis of IR. C: Effects of ET-1 on IRS-2 phosphorylation in vivo. D: Phosphoamino acid analysis of IRS-2. PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine.

A

IP: αIRβ
Blot: αPY

Py-IRβ

B

IP: αIRS-2
Blot: αPY

Py-IRS-2

C

IP: αIRS-2
Blot: αP85

P85

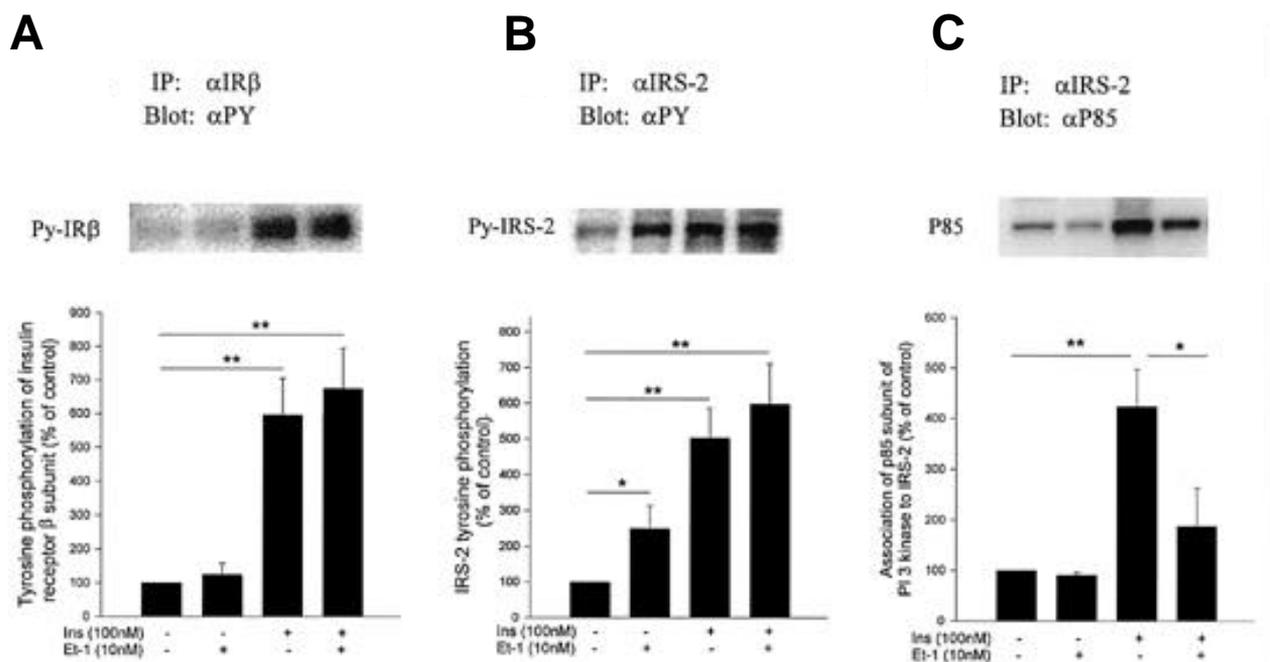FIG. 4. Effects of ET-1 on tyrosine phosphorylation of IR and IRS-2, and the formation of IRS-2/PI 3-kinase complex. *A*: Effect of ET-1 on basal and insulin-stimulated tyrosine phosphorylation of IR. Rat vascular SMCs were incubated with or without ET-1 (10 nmol/l) for 2 min before the addition of insulin (100 nmol/l) or solvent for another 3 min. Cell lysates (2 mg protein) were immunoprecipitated with αIRβ antibody. Tyrosine phosphorylation of αIRβ was detected with αPY antibody. *B*: Effect of ET-1 on basal and insulin-stimulated tyrosine phosphorylation of IRS-2. SMCs were incubated with or without ET-1 (10 nmol/l) for 2 min before the addition of insulin (100 nmol/l) or solvent for another 3 min. Cell lysates (1 mg protein) were immunoprecipitated with αIRS-2 antibody, and IRS-2 tyrosine phosphorylation was detected with αPY antibody. *C*: The same nitrocellulose membranes were stripped and reblotted with αp85 antibody to examine the effect of ET-1 on basal and insulin-stimulated association of the p85 subunit of PI 3-kinase to IRS-2. Upper panels show representative phosphoimages. Lower panels show the data (means ± SD) of four independent triplicate experiments. *$P < 0.05$, **$P < 0.01$.

signaling (6,7,9,46–50). Because ET-1 has been shown to induce insulin resistance (41–43) and can affect diverse arrays of intracellular signaling events, including the activation of growth factor receptor kinases (51), nonreceptor tyrosine kinases (28–30), and serine/threonine kinases (27,31), its effects on the insulin signaling pathway were investigated. The effect of ET-1 on the phosphorylation of IRβ was studied. After vascular SMCs were labeled with [^{32}P]orthophosphate, cells were stimulated with ET-1 (10 nmol/l), and cell lysates were partially purified with WGA and immunoprecipitated with IRβ antibody. As shown in Fig. 3*A*, ET-1 induced a 2.5-fold increase in the total phosphorylation of the 95-kDa IRβ. Phosphoamino acid analysis of the 95-kDa bands of IRβ revealed that ET-1 increased serine and threonine but not tyrosine phosphorylation of IRβ (Fig. 3*B*).

The effect of ET-1 on phosphorylation of IRS-2 was also characterized, since IRS-2 appeared to be preferentially involved in the activation of the PI 3-kinase pathway in vascular SMCs by insulin. As shown in Fig. 3*C*, ET-1 increased IRS-2 phosphorylation by 3.3-fold. Phosphoamino acid analysis demonstrated that ET-1 increased the phosphorylation of both tyrosine and serine residues of IRS-2 (Fig. 3*C* and *D*).

Characterization of ET-1's effect on insulin-stimulated tyrosine phosphorylation of IR and IRS-2 and its association with the p85 subunit of PI 3-kinase. It is possible that the increases in serine phosphorylation of the IR induced by ET-1 may interfere with the kinase activity and autophosphorylation of the IR. To test this possibility, vascular SMCs were incubated with ET-1 (10 nmol/l) for 2 min before the addition of insulin (100 nmol/l) for 3 min, followed by

immunoprecipitation with αIR antibody. Tyrosine phosphorylation of IRβ, as estimated by Western blotting with αPY antibody in vascular SMCs, was increased by sevenfold (Fig. 4*A*) by the addition of insulin (100 nmol/l) in comparison with the control. ET-1 alone did not stimulate tyrosine phosphorylation of the IR and had no inhibitory effect on insulin-stimulated tyrosine phosphorylation of the IR (Fig. 4*A*).

Several reports have shown that increases in tyrosine or serine phosphorylation of IRS proteins can affect their docking with SH2-containing proteins such as the p85 subunit of PI 3-kinase (5,48). Because ET-1 alone increased both serine and tyrosine phosphorylation of IRS-2, we examined whether ET-1 could alter insulin-stimulated tyrosine phosphorylation of IRS-2 and its association with the p85 subunit of PI 3-kinase in vascular SMCs. As expected, insulin (100 nmol/l) increased the tyrosine phosphorylation level of IRS-2 in vascular SMCs by sixfold (Fig. 4*B*). ET-1 (10 nmol/l) alone also increased by twofold the tyrosine phosphorylation on IRS-2, consistent with the in vivo ^{32}P labeling studies shown in Fig. 3. Pretreatment of SMCs with ET-1 had no significant effect on insulin-stimulated tyrosine phosphorylation of IRS-2. Using the same nitrocellulose membranes that were blotted with antibody to the p85 subunit of PI 3-kinase co-immunoprecipitated with IRS-2 (Fig. 4*C*), insulin increased IRS-2/p85 subunit association by about fourfold. ET-1 (10 nmol/l) alone did not significantly increase the amount of IRS-2–associated p85 subunit, although ET-1 increased tyrosine phosphorylation of IRS-2. When insulin (100 nmol/l) was added to vascular SMCs pretreated with ET-1 (10 nmol/l), the amount of the p85 subunit of PI 3-kinase associated with IRS-2 was decreased by 55% (Fig. 4*C*).

Inhibition of insulin-stimulated PI 3-kinase activity by ET-1. Because ET-1 inhibited insulin-induced association of IRS-2 to the p85 subunit of PI 3-kinase, as detected by immunoblotting, the effect of ET-1 on insulin-stimulated PI 3-kinase activity was examined in vascular SMCs using the in vitro kinase assay. SMCs were pretreated with ET-1 (10 nmol/l) for 2, 6, 15, 30, and 60 min before the addition of 100 nmol/l insulin for 5 min. Insulin induced an approximately five-fold increase in IRS-2–associated PI 3-kinase activity, which was inhibited by 60% when cells were pretreated with ET-1 between 2 and 15 min. However, the inhibitory effect of ET-1 decreased to 28 and 12% when the preincubation time of ET-1 was extended to 30 and 60 min, respectively (Fig. 5).

ET-1 inhibited insulin- or IGF-1–stimulated PI 3-kinase activity through the ET_A receptor. The inhibitory effect of ET-1 on insulin-induced PI 3-kinase activity could be either specific to insulin or applicable to other growth factors. To address this question, we studied the effect of ET-1 on PDGF-induced PI 3-kinase activity in vascular SMCs. As shown in Fig. 6A, PDGF-BB (5 ng/ml) induced a 50-fold increase in the activity of PI 3-kinase immunoprecipitated by αPY antibody that was not affected by ET-1. Cells preincubated with 10 nmol/l ET-1 for 2 min did not alter PDGF-induced activation of PI 3-kinase, suggesting ET-1's inhibitory effect may be limited to IRS-associated PI 3-kinase activities.

ET_A receptors are the predominant ET-1 receptors on vascular SMCs (52). To determine whether the inhibitory effect of ET-1 on insulin-stimulated PI 3-kinase activity was mediated through ET_A receptors, the effect of BQ123, a selective antagonist to ET_A receptors, was examined (53). When cells were pretreated with ET-1 (10 nmol/l) for 2 min before the addition of insulin (100 nmol/l) for another 5 min, the activity of PI 3-kinase associated with IRS-2 was significantly reduced by 62% as compared with cells treated with insulin alone (Fig. 6B). Preincubation with BQ123 (500 nmol/l) for 20 min before the addition of ET-1 and then insulin totally blocked ET-1's inhibitory effect on insulin-stimulated PI 3-kinase activity. Again, ET-1 alone did not alter the activity of PI 3-kinase associated with IRS-2 (Fig. 6B).

The effect of ET-1 on IGF-1–stimulated PI 3-kinase activity was also determined. Figure 6C showed that IGF-1 (10 nmol/l) stimulated an eightfold increase in IRS-2–associated PI 3-kinase activity. The effect of IGF-1 on PI 3-kinase activity was inhibited by 50% in cells pretreated with ET-1 (10 nmol/l) for 2 min. When cells were pretreated with BQ123 (500 nmol/l) for 20 min before the addition of ET-1, the inhibitory effect of ET-1 on IGF-1–stimulated PI 3-kinase activity was no longer observed. Both ET-1 and BQ123 alone did not alter IRS-2–associated PI 3-kinase activity.

ET-1's inhibitory effect is independent of PTX-sensitive G-protein but partially dependent on PKC activation. ET_A receptors have been reported to be coupled to either $G\alpha i$ (54) or $G\alpha q$ (24,25). The effect of preincubation of PTX, a $G\alpha i$-selective inhibitor, was studied in vascular SMCs. Preincubation of cells with PTX (1 µg/ml) for 2 h did not prevent ET-1's inhibitory effect on insulin-activated PI 3-kinase (Fig. 7A). The effect of PTX on intracellular Ca^{2+} levels was examined in vascular SMCs stimulated by lysophosphatidic acid (LPA), which activates $G\alpha i$ protein. LPA (5 µg/ml) elevated the intracellular Ca^{2+} level from a basal of 95 ± 6 nmol/l to a peak level of 680 ± 44 nmol/l. However, when cells were pretreated with PTX (1 µg/ml) for 2 h, the intracellular Ca^{2+} level was
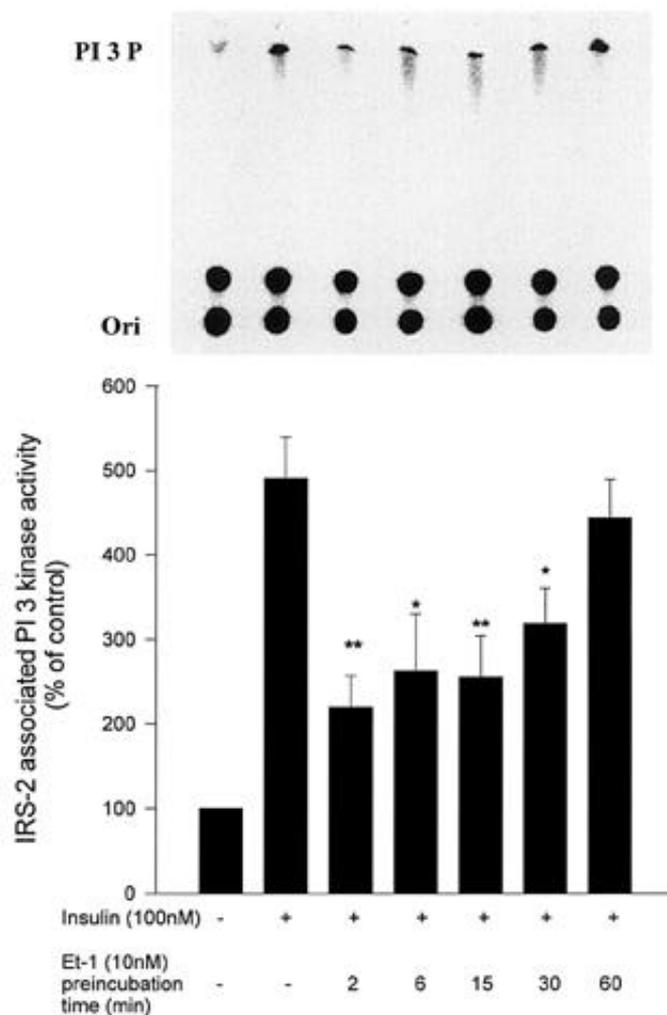

FIG. 5. ET-1 inhibited insulin-stimulated PI 3-kinase activity in a time-dependent manner. Rat vascular SMCs were incubated with ET-1 (10 nmol/l) for 2, 6, 15, 30, and 60 min before the addition of insulin (100 nmol/l) for 5 min. Cell lysates (1 mg protein) were immunoprecipitated with αIRS-2 antibody. IRS-2–associated PI 3-kinase activity was assessed as described in METHODS. Upper panel shows a representative phosphoimage. Lower panel shows the data (means ± SD) of three independent triplicate experiments. *P < 0.05, **P < 0.01.

increased only to 192 ± 10 nmol/l from the basal by LPA, suggesting PTX did inhibit LPA-$G\alpha i$ protein–mediated actions.

$G\alpha q$-coupled ET_A receptors mediate their effects by activation of phospholipase C and PKC, as described previously (27). Activation of PKC has been shown to modulate insulin signaling via increases in serine phosphorylation of IRS protein (8). In this experiment, we examined whether activation of PKC is essential for ET-1's inhibitory effect on insulin-stimulated PI 3-kinase in the vascular SMCs. The effects of phorbol ester–sensitive PKC on basal and insulin-stimulated PI 3-kinase activity were studied. Pretreatment of cells with 100 nmol/l phorbol 12-myristate 13-acetate (PMA) for 20 min inhibited insulin-stimulated PI 3-kinase activity by 57% (Fig. 7B), suggesting activation of PKC could negatively modulate insulin's signaling on the PI 3-kinase pathway. In contrast, pretreatment of SMCs with the specific PKC inhibitor bisindolylmaleimide I (GF109203X) (55) (5 µmol/l) for 20 min increased insulin-stimulated PI 3-kinase activity by ~30% but
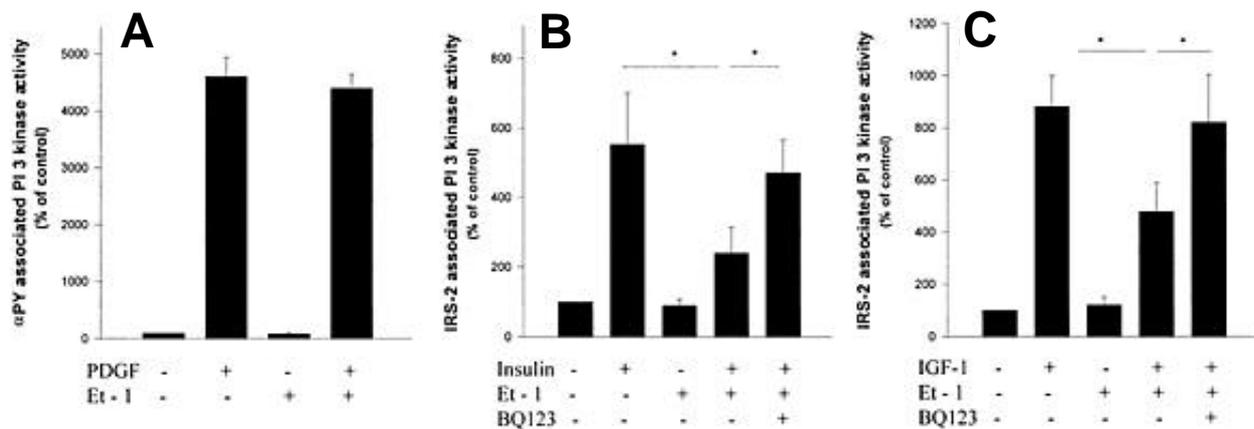
FIG. 6. A: Effect of ET-1 on PDGF-stimulated PI 3-kinase activity. Serum-starved vascular SMCs were preincubated with or without ET-1 (10 nmol/l) for 2 min before the addition of PDGF-BB (5 ng/ml) for 5 min. Cell lysates were immunoprecipitated with αPY, and αPY-associated PI 3-kinase activity was determined. B and C: BQ123 blocks the inhibitory effect of ET-1 on insulin- and IGF-1–induced PI 3-kinase activity. Serum-starved vascular SMCs were preincubated with or without BQ123 (500 nmol/l) for 20 min before the addition of ET-1 (10 nmol/l) for 2 min and then 100 nmol/l insulin (B) or 10 nmol/l IGF-1 (C) for 5 min. Cell lysates were immunoprecipitated with αIRS-2 antibody. IRS-2–associated PI 3-kinase activity was measured as described in METHODS. Data are means ± SD of four independent triplicate experiments. *P < 0.05.

did not affect basal PI 3-kinase activity. Interestingly, preincubation with GF109203X also partially prevented the inhibitory effect of ET-1 on insulin-stimulated PI 3-kinase activity (Fig. 7C).

The effects of PKC activation on the tyrosine phosphorylation of IRβ and IRS-2 and the formation of the IRS-2/p85 subunit of the PI 3-kinase complex were also determined. Incubation with 100 nmol/l of PMA did not change the basal and insulin-stimulated tyrosine phosphorylation of IRβ (Fig. 8A). However, PMA inhibited insulin-stimulated IRS-2 tyrosine phosphorylation by 45% (Fig. 8B) and decreased the insulin-induced association of IRS-2 to the p85 subunit of PI 3-kinase by 40% (Fig. 8C). Pretreatment of vascular SMCs with GF109203X, a PKC inhibitor, did not alter the basal or ET-1–stimulated tyrosine phosphorylation of IRS-2 (Fig. 9A) but did partially reverse ET-1's inhibitory effect on the

insulin-induced association of the p85 subunit of PI 3-kinase and IRS-2 (Fig. 9B).

DISCUSSION

Intracellular cross talks between different signaling systems, such as G-protein–coupled receptors and tyrosine kinase–coupled receptors, are important mechanisms by which hormones and cytokines regulate cellular functions. Interactions between insulin and G-protein–coupled receptor agonists have been reported recently. Rao et al. (56) have shown that thrombin can stimulate IRS-1 protein phosphorylation. Angiotensin II has also been reported to alter phosphorylation of IRS proteins and insulin-stimulated PI 3-kinase activity in the heart and aortic SMCs (19,57,58). We have previously reported that insulin at physiological levels increased the transcription rate of ET-1 in vas-
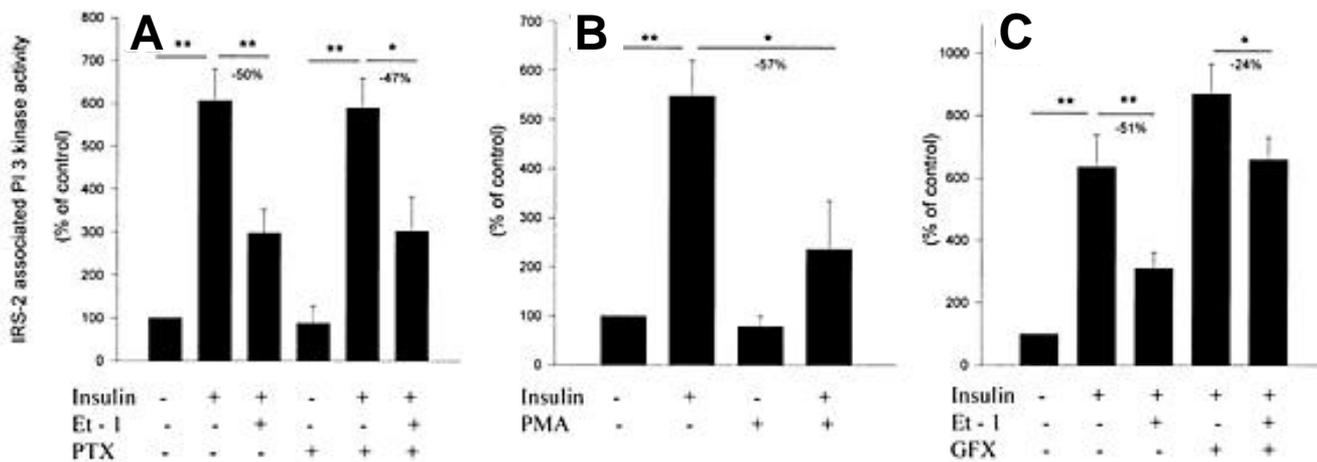

FIG. 7. Effects of PTX, PMA, and the PKC inhibitor GF10923X on insulin, and ET-1's effects on PI 3-kinase activity. A: Quiescent vascular SMCs were incubated for 2 h in the presence or absence of PTX (1 µg/ml) then for 5 min with or without insulin (100 nmol/l). B: Quiescent rat vascular SMCs were incubated in DMEM with or without 100 nmol/l PMA for 20 min then for 5 min in the presence or absence of 100 nmol/l insulin. C: Cells were treated with or without GF10923X (5 µmol/l) for 20 min before addition of ET-1 (10 nmol/l) for 2 min and then 100 nmol/l of insulin for 5 min. Cell lysates were immunoprecipitated with αIRS-2 antibody. IRS-2–associated PI 3-kinase activity was then measured. Data are means ± SD of four (C) and three (A and B) independent triplicate experiments. *P < 0.05, **P < 0.01.
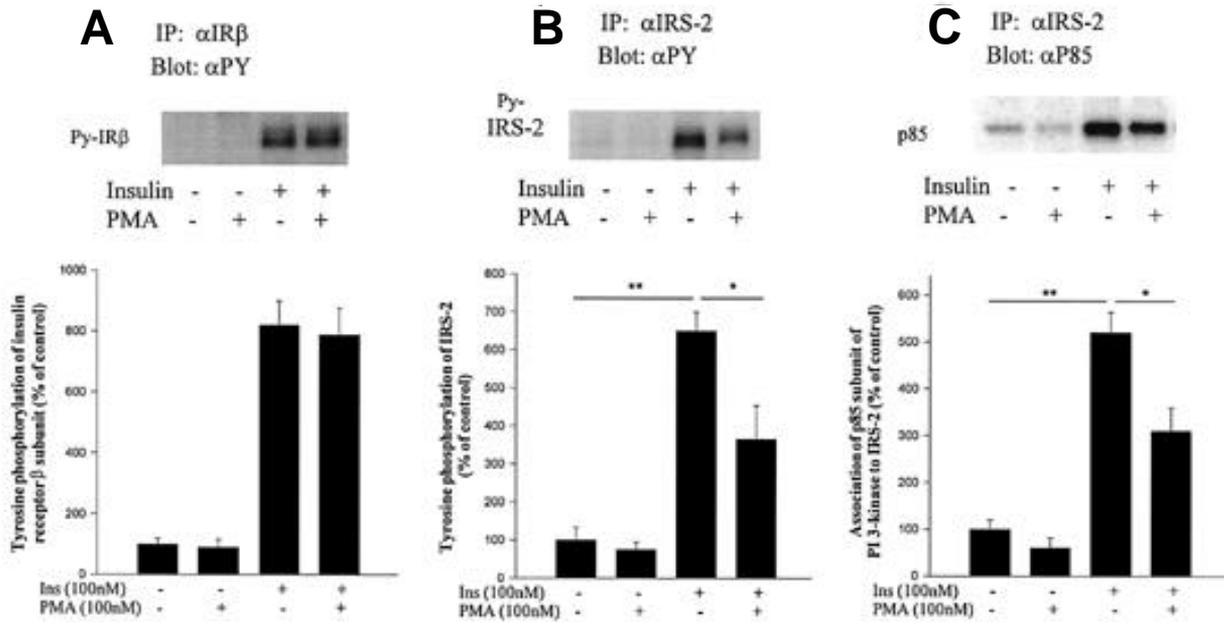
FIG. 8. Effects of PMA on tyrosine phosphorylation of IR and IRS-2, and the formation of IRS-2/PI 3-kinase complex. *A*: Effect of PMA on basal and insulin-stimulated tyrosine phosphorylation of IR. Vascular SMCs were incubated with or without PMA (100 nmol/l) for 20 min before the addition of insulin (100 nmol/l) or solvent for another 3 min. Cell lysates (2 mg protein) were immunoprecipitated with αIRβ antibody. Tyrosine phosphorylation of αIRβ was detected with αPY antibody. *B*: Effect of PMA on basal and insulin-stimulated tyrosine phosphorylation of IRS-2. SMCs were incubated with or without PMA (100 nmol/l) for 20 min before the addition of insulin (100 nmol/l) or solvent for another 3 min. Cell lysates (2 mg protein) were immunoprecipitated with αIRS-2 antibody, and IRS-2 tyrosine phosphorylation was detected with αPY. *C*: Same nitrocellulose membranes were stripped and reblotted with αp85 antibody to examine the effect of PMA on basal and insulin-stimulated association of the p85 subunit of PI 3-kinase to IRS-2. Upper panels show representative phosphoimages. Lower panels show the data (means ± SD) of three independent duplicate experiments. $*P < 0.05$, $**P < 0.01$.

cular endothelial cells (14). Elevated plasma levels of ET-1 are negatively correlated with total glucose uptake during euglycemic-hyperinsulinemic clamp in patients with type 2 diabetes (59). Therefore, interactions between ET-1 and insulin in the vascular wall are likely under hyperinsulinemic and insulin-resistant states. Accordingly, it has been reported that ET-1 inhibits glucose metabolism in vivo (42,43) and insulin-stimulated glucose transport in cultured adipocytes (41), suggesting that ET-1 might exert negative effects on insulin's actions. In this report, we have characterized the interactions in the signaling pathways between ET-1 and insulin in cultured rat vascular SMCs.

We have shown that ET-1 can interfere with insulin's signal transduction pathways at multiple sites. First, ET-1 induces serine phosphorylation but not tyrosine phosphorylation at either basal or insulin-stimulated states in IRβ of vascular SMCs. Second, ET-1 stimulates both serine and tyrosine phosphorylation of IRS-2. Interestingly, ET-1 decreases the insulin-stimulated association between IRS-2 and the p85 subunit of PI 3-kinase, although ET-1 increases IRS-2 tyrosine phosphorylation. Third, ET-1 inhibits both insulin- and IGF-1–induced activation of PI 3-kinase associated with IRS-2. Because ET-1 does not affect PDGF-BB–induced activation of PI 3-kinase, ET-1 may only regulate the interaction between PI 3-kinase and IRS proteins after insulin and/or IGF-1 stimulation. ET-1–mediated inhibition of insulin-activated PI 3-kinase is a rapid, reversible, and regulated event, since the inhibitory effect reaches the maximal level when the cells are pretreated for 2 min, starts to decline after 10 min, and is completely reversed in 60 min.

The mechanism of ET-1's effect on insulin-stimulated PI 3-kinase activity appears to be mediated via ET$_A$ receptors, since vascular SMCs pretreated with BQ123, an ET$_A$ receptor–selective antagonist, prevents ET-1's effect. This is in agreement with the fact that ET$_A$ receptors are the predominant ET-1 receptors in vascular SMCs (52). However, Gαi inhibitor PTX is unable to block ET-1's effect (Fig. 7). It has been reported that ET-1 can also stimulate tyrosine and/or serine phosphorylation of intracellular proteins independent of PTX-sensitive G-proteins in various types of cells (28,30,34). Furthermore, we have shown that the inhibitory effect of ET-1 on insulin-stimulated PI 3-kinase activity in vascular SMCs is partly modulated by PKC activation, since the PKC inhibitor GF109203X neutralizes some of ET-1's effect. The role of PKC in regulating insulin-stimulated PI 3-kinase is supported by the findings that PMA alone, without ET-1, also inhibits insulin-induced PI 3-kinase activity to an extent similar to ET-1. In contrast, PKC inhibitor GF109203X enhances PI 3-kinase activity. Thus, activation of the ET$_A$-PKC pathway by ET-1 may be involved, at least in part, in its inhibitory effect on insulin-induced PI 3-kinase pathway.

It has been proposed that increased serine phosphorylation of IR proteins may lead to the inhibition of insulin signaling (6,47). Although ET-1 increases serine phosphorylation of IR in vascular SMCs, ET-1 does not alter IR tyrosine phosphorylation induced by insulin, suggesting IR kinase activity is not affected. It has been previously reported that phorbol esters can increase serine phosphorylation of IR without affecting its tyrosine phosphorylation (60) or its tyrosine kinase activ-
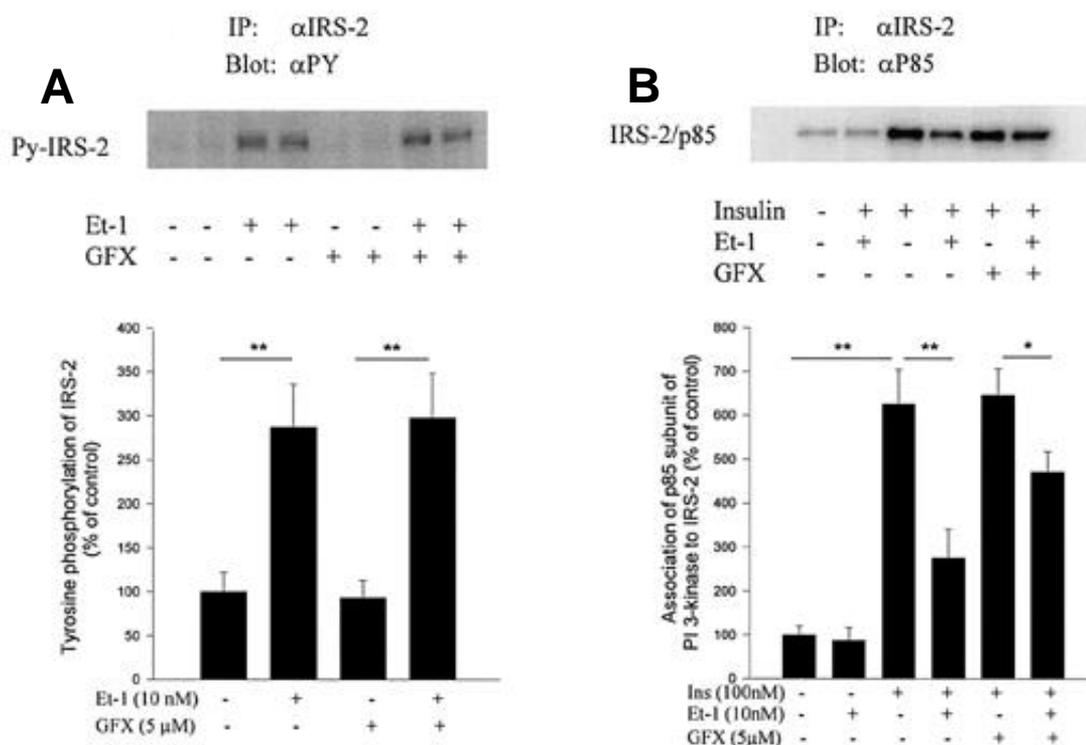
FIG. 9. Effects of PKC inhibitor GF10923X on ET-1–induced tyrosine phosphorylation of IRS-2, and the dissociation of IRS-2 and PI 3-kinase. *A*: Effect of ET-1 on basal and ET-1–induced tyrosine phosphorylation of IRS-2. Vascular SMCs were treated with or without GF10923X (5 μmol/l) for 20 min before addition of ET-1 (10 nmol/l) for 2 min and then 100 nmol/l of insulin for 3 min. Cell lysates (2 mg protein) were immunoprecipitated with αIRS-2 antibody. Tyrosine phosphorylation of αIRS-2 was detected with αPY antibody. *B*: Effect of GF10923X on the formation of IRS-2/PI 3-kinase complex. Cells were preincubated with or without GF10923X (5 μmol/l) for 20 min before the addition of ET-1 (10 nmol/l) for 2 min and then insulin (100 nmol/l) for another 3 min. Cell lysates (2 mg protein) were immunoprecipitated with αIRS-2 antibody. IRS-2–associated p85 subunit of PI 3-kinase was detected with αp85 antibody. Upper panels show representative phosphoimages. Lower panels show the data (means ± SD) of three independent duplicate experiments. *$P < 0.05$, **$P < 0.01$.

ity (61). Similarly, activation of PKC by PMA does not alter IR tyrosine phosphorylation, but it decreases insulin-induced tyrosine phosphorylation of IRS-2 and its association with the p85 subunit of PI 3-kinase in vascular SMCs (Fig. 8). It is still possible that serine/threonine phosphorylation of IR induced by ET-1 does not affect IR autophosphorylation but may induce a conformational change that may interfere with its binding to IRS proteins and thus contribute to the inhibitory effect of ET-1 on activation of PI 3-kinase by insulin.

Increases in serine phosphorylation of IRS proteins under various conditions can impede insulin signaling on the PI 3-kinase pathway (8,9,19,48,49). In this study, ET-1 also increases IRS-2 serine phosphorylation, inhibits the docking of PI 3-kinase to IRS-2, and decreases IRS-2–associated PI 3-kinase activity in vascular SMCs. It is not known which serine kinases may be directly involved in IRS-2 phosphorylation induced by ET-1. Interestingly, the decreased association of PI 3-kinase to IRS-2 is partially reversed when cells are pretreated with PKC inhibitor GF109203X. It is possible that activation of PKC by ET-1 may directly or secondarily mediate serine phosphorylation of IRS-2, thus modulating the protein-protein interactions between IRS-2/PI 3-kinase or IR/IRS-2, and lead to the inhibition of insulin-stimulated PI 3-kinase activity in SMCs. Our finding is consistent with a recent report that suggests that activation of endogenous PKC by PMA leads to phosphorylation of serine 612 in IRS-1, and this serine phosphorylation is responsible for the inhibition of IRS-1–associated PI 3-kinase activity (8).

In addition to the increase in serine phosphorylation of IR and IRS-2, ET-1 also increases tyrosine phosphorylation of IRS-2 in vascular SMCs. The finding that ET-1 increases tyrosine phosphorylation yet decreases its association to the p85 subunit of PI 3-kinase (Fig. 4) is surprising, since increases in IRS-2 tyrosine phosphorylation generally lead to increases in IRS/p85 subunit association and activation of PI 3-kinase (4). It is not known whether ET-1–induced tyrosine phosphorylation in IRS-2 may negatively affect its affinity to the p85 subunit of PI 3-kinase and thus contribute to the inhibition of insulin-stimulated PI 3-kinase activation. It is possible that ET-1's direct effect on tyrosine phosphorylation of IRS-2 may compensate for the decrease in insulin-induced tyrosine phosphorylation of IRS-2 as a result of an ET-1–induced increase in serine phosphorylation of IRS-2 or IR. It remains to be determined which tyrosine kinase is involved in tyrosine phosphorylation of IRS proteins in ET-1–stimulated vascular SMCs. Previously, ET-1 has been shown to induce the tyrosine phosphorylation and activation of protein kinases such as pp60 c-Src (28), pp125 focal adhesion kinase (30), and p42/p44 mitogen-activated protein kinases (31). ET-1 also mediates tyrosine phosphorylation and transactivation of the epidermal growth factor receptor (51) as well as tyrosine phosphorylation of other cytosolic docking proteins such as Shc (62). Interestingly, other G-protein–coupled receptor agonists, such as thrombin and angiotensin II, have also been reported to induce tyrosine phosphorylation of IRS proteins (56–58). Recently, it has been suggested that both Gαi- and

Gαq protein–coupled receptors could activate Src family tyrosine kinases c-Src and Fyn (63–65). It is possible that ET-1 may activate G-protein–dependent cytosolic tyrosine kinases and thus phosphorylate tyrosine residues in IRS proteins. However, inhibition of PKC with GF109203X does not alter ET-1–induced tyrosine phosphorylation of IRS-2, suggesting activation of common PKC isoforms by ET-1 is not involved in stimulation of the responsible tyrosine kinase.

Thus, the present study has provided new evidence to show that both IRS-1 and IRS-2 can mediate insulin's effect on PI 3-kinase activation in the vascular SMCs, although IRS-2 appears to be favored. This is different from adipose tissues (M.F. White, unpublished observations) and skeletal muscle (personal communication of Dr. Laurie J. Goodyear), which have much greater expression of IRS-1 than IRS-2. In addition, the new evidence suggests that ET-1 can modulate insulin signaling and actions by increasing serine phosphorylation of the IR and serine/tyrosine phosphorylation of IRS-2. We have also demonstrated that the PKC activator PMA is able to mimic ET-1's effect of inhibiting insulin-induced PI 3-kinase, whereas the PKC inhibitor GF109203X diminishes ET-1's effect. Our data also suggest that ET-1's inhibitory effect on PI 3-kinase activation by insulin is due, at least in part, to PKC activation causing phosphorylation of IR and IRS proteins. Although the PKC inhibitor GF109203X partially prevents ET-1's inhibitory effect on insulin-induced docking of PI 3-kinase to IRS-2, it does not reverse tyrosine phosphorylation of IRS-2 induced by ET-1, suggesting that ET-1 can alter IR/IRS-2 phosphorylation by at least two pathways that are PKC dependent and independent. The PKC-dependent pathway may mediate IR/IRS-2 serine phosphorylation. The PKC-independent pathway may involve tyrosine kinase, which causes an increase in tyrosine phosphorylation of IRS proteins.

It is interesting to note that, unlike other tissues, PKC activities are clearly demonstrated to be increased in the vasculature of diabetic animals. Increased ET-1 levels in plasma have been reported in diabetic patients (35). The results from the present study suggest that the activation of PKC by hyperglycemia and ET-1 in the vascular tissues inhibits insulin's activation of PI 3-kinase, which can have biological significance, such as the inhibition of glycogen synthesis (data not shown). Nitric oxide production may also be affected in the vasculature, since Zeng and Quon (15) have suggested that insulin's activation of nitric oxide synthase is mediated by the PI 3-kinase pathway. These results have provided a potential molecular explanation for the observation that insulin's vasodilatory effects are blunted in diabetic patients or animals (17,18). In addition, the identification of ET-1 as a vasotropic factor that can induce insulin resistance in the vascular wall has suggested a new target for therapeutic intervention to prevent cardiovascular diseases in diabetic patients.

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