Insulin Signaling in Vascular Endothelial Cells: A Key Role for Heterotrimeric G Proteins Revealed by siRNA-Mediated Gβ1 Knockdown†

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ABSTRACT: Activation of insulin receptors stimulates the phosphoinositide 3-kinase (PI3-K)/Akt signaling pathway in vascular endothelial cells. Heterotrimeric G proteins appear to modulate some of the cellular responses that are initiated by receptor tyrosine kinases, but the roles of specific G protein subunits in signaling are less clearly defined. We found that insulin treatment of cultured bovine aortic endothelial cells (BAEC) activates the α isoform of PI3-K (PI3-Kα) and discovered that purified G protein Gβ1γ2 inhibits PI3-Kα enzyme activity. Transfection of BAEC with a duplex siRNA targeting bovine Gβ1 leads to a 90% knockdown in Gβ1 protein levels, with no effect on expression of other G protein subunits. siRNA-mediated Gβ1 knockdown markedly and specifically potentiates insulin-dependent activation of kinase Akt, likely reflecting the removal of the inhibitory effect of Gβγ on PI3-Kα activity. Insulin-induced tyrosine phosphorylation of insulin receptors is unaffected by Gβ1 siRNA. By contrast, Gβ1 knockdown leads to a significant decrease in the level of serine phosphorylation of the insulin receptor substrate IRS-1. We explored the effects of siRNA on several serine/threonine protein kinases that have been implicated in insulin signaling. Gβ1 siRNA significantly attenuates phosphorylation of the 70 kDa ribosomal protein S6 kinase (p70S6K) in the basal state and following insulin treatment. We also found that IGF-1-initiated activation of Akt is significantly enhanced after siRNA-mediated Gβ1 knockdown, while IGF-1-induced p70S6K activation is markedly suppressed following transfection of Gβ1 siRNA. We propose that Gβ1 participates in the activation of p70S6K, which in turn promotes the serine phosphorylation and inhibition of IRS-1. Taken together, these studies suggest that Gβ1 plays an important role in insulin and IGF-1 signaling in endothelial cells, both by inhibiting the activity of PI3-Kα and by stimulating pathways that lead to activation of protein kinase p70S6K and to the serine phosphorylation of IRS-1.

Cellular responses to insulin have been extensively characterized in diverse tissues, including liver, muscle, and fat, but the role of insulin in vascular endothelial cell signaling is less completely understood. Activation of the insulin receptor tyrosine kinase stimulates autophosphorylation of the receptor on specific tyrosine residues, which promotes the binding and tyrosine phosphorylation of a family of insulin receptor substrates (IRS)[1] and leads to the activation of diverse downstream protein kinases and other modulators (*1*). A key downstream modulator of insulin responses is the phosphoinositide 3-kinase (PI3-K) (*2*, *3*). The PI3-Ks belong to a family of receptor-activated lipid kinases that phosphorylate the D-3 position on the inositol ring in phosphoinositides, which in turn activate specific phospholipid-dependent kinases (PDKs) and lead to the phosphorylation and activation of protein kinase Akt. Activated kinase Akt phosphorylates several substrate proteins, such as the endothelial isoform of nitric oxide synthase (eNOS), glycogen synthase 3β (GSK3β), and mammalian target of rapamycin (mTOR), and appears to be a key determinant of insulin-mediated responses, including cell growth, cell survival, and glucose uptake (*1*−*3*).

Cellular responses to insulin receptor (IR) activation are dependent upon the receptor-mediated tyrosine phosphorylation of IRS proteins (*1*−*3*). However, in contrast to the stimulation of IRS by tyrosine phosphorylation, the phosphorylation of IRS proteins on serine and threonine residues attenuates insulin signaling by decreasing the level of insulin-stimulated tyrosine phosphorylation of IRS (*4*). Activation of intracellular serine/threonine kinases that phosphorylate IRS may provide a mechanism for receptor-mediated inhibition of insulin signaling and may be altered in the clinical

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[1] Abbreviations: siRNA, small interfering RNA; Gβ1 siRNA, small interfering RNA targeted to the bovine Gβ1 mRNA; BAEC, bovine aortic endothelial cells; PI3-K, phosphatidylinositol 3-kinase; IR, insulin receptor; IRS, insulin receptor substrate(s); PIP3, phosphatidylinositol 3-monophosphate; PDK, phospholipid-dependent kinase; mTOR, mammalian target of rapamycin; eNOS, endothelial isoform of nitric oxide synthase; GSK3β, glycogen synthase kinase 3β; p70S6K, 70 kDa ribosomal protein S6 kinase; rpS6, ribosomal protein S6; S1P, sphingosine 1-phosphate; VEGF, vascular endothelial growth factor; IGF-1, insulin-like growth factor-1; SE, standard error.

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syndrome of insulin resistance (*1*−*4*). For example, PI3-K/Akt-dependent activation of the protein kinase mTOR leads to the phosphorylation of the 70 kDa ribosomal protein S6 kinase (p70S6K). In addition to phosphorylating ribosomal protein S6 (rpS6), p70S6K promotes the serine/threonine phosphorylation of IRS proteins and thereby inhibits insulin signaling (*3*−*8*). Thus, the targets of kinase Akt may both stimulate and attenuate insulin responsiveness, and the receptor-dependent modulation of cellular kinase pathways may play a pathophysiological role in the mechanisms of insulin resistance.

Vascular endothelial cells transduce signals following the activation of a broad array of cell surface receptors, including receptor tyrosine kinases as well as G protein-coupled receptors (*9*). In addition to insulin, the PI3-K/Akt pathway in endothelial cells can be activated by other receptor tyrosine kinases, including receptors for insulin-like growth factor-1 (IGF-1), which share similar signaling pathways with the responses elicited by insulin receptor activation in endothelial cells (*10*, *11*). Other key endothelial responses are modulated by receptors for vascular endothelial growth factor (VEGF), as well as by G protein-coupled receptors such as the $S1P_1$ receptor, which binds the lipid ligand sphingosine 1-phosphate (S1P) (*9*). The existence of common intracellular targets for these distinct receptor-modulated signaling pathways may provide an opportunity for "cross-talk" involving the regulation of a common intermediate, which may allow for the integration of cellular responses to receptor activation (*12*). The metabolic consequences of insulin receptor activation share features with the responses elicited by some G protein-coupled receptors in the same cells (*12, 13*).

The molecular mechanisms for cross-talk between insulin or IGF-1 receptors and G protein-coupled receptors in vascular endothelial cells remain incompletely characterized (*12*). Following receptor-stimulated binding of GTP to the α subunits of heterotrimeric G proteins, the α and $\beta\gamma$ subunits dissociate from one another and are then able to modulate distinct downstream signaling pathways until GTP hydrolysis on the α subunit leads to reassociation of the $\alpha\beta\gamma$ heterotrimer (*14*). In vascular endothelial cells, the G protein-coupled $S1P_1$ receptor promotes the activation of the β isoform of PI3-K (PI3-Kβ); this signaling pathway is dependent upon the activation of PI3-Kβ by G$\beta\gamma$ subunits (*15*). By contrast, the growth factor VEGF activates both the α and β isoforms of PI3-K, apparently without the direct involvement of G protein $\beta\gamma$ subunits; the VEGF signaling pathway, but not the S1P response, is dependent on tyrosine kinase activation. The role of G protein $\beta\gamma$ subunits in insulin signaling to PI3-K in vascular endothelial cells is less clearly defined. In these studies, we have used siRNA methods and an in vitro kinase assay to elucidate the role of a specific G protein β subunit that plays a key role in insulin-mediated responses.

EXPERIMENTAL PROCEDURES

Materials. Fetal bovine serum was purchased from HyClone Laboratories (Logan, UT); all other cell culture reagents and media were from Life Technologies, Inc. LipofectAMINE 2000 transfection reagent was from Invitrogen. Polyclonal antibodies against PI3-Kα, IRS-1, Gβ1, Gβ2, Gβ4, and Gαq were from Santa Cruz Biotechnology (Santa Cruz, CA). Rapamycin and polyclonal antibodies directed against phospho-IRS-1 (mouse Ser302), phospho-Akt (Ser473), Akt, phospho-GSK3β (Ser9), phospho-mTOR (Ser2448), mTOR, phospho-p70S6K (Thr389), p70S6K, phospho-rpS6 (Ser235), and rpS6 were from Cell Signaling Technologies (Beverly, MA). Monoclonal antibodies against phosphotyrosine (4G10), IGF-1 receptor β subunit, or Gαi2 and polyclonal antibodies against the insulin receptor β subunit were from Upstate Biotechnology (Lake Placid, NY). Anti-eNOS and anti-GSK3β monoclonal antibodies were from Transduction Laboratories (Lexington, KY). The Super Signal chemiluminescence detection reagents and secondary antibodies conjugated with horseradish peroxidase were from Pierce (Rockford, IL). Protein concentrations were determined with the Bio-Rad protein assay kit. Tris-buffered saline and phosphate-buffered saline were from Boston Bioproducts (Ashland, MA). VEGF and wortmannin were from Calbiochem (La Jolla, CA). S1P was purchased from Biomol (Plymouth Meeting, PA). [^{32}P]ATP was from Perkin-Elmer (Boston, MA). Insulin and all other reagents were from Sigma.

Cell Culture. Bovine aortic endothelial cells (BAEC) were obtained from Cell Systems (Kirkland, WA) and maintained in culture in Dulbecco's modified Eagle's medium (DMEM) supplemented with FBS (10%, v/v) as described previously (*15*). Cells were plated onto 0.2% gelatin-coated culture dishes and studied prior to cell confluence between passages 5 and 9.

Sf9 cells were cultured in suspension in Grace's insect medium supplemented with FBS (10%, v/v) and 50 μg/mL gentamicin at 27 °C with constant shaking (125 rpm).

Purification of Recombinant Gβ1γ2 Subunits from Sf9 Cells. The purification of recombinant Gβ1γ2 subunits from Sf9 cells was performed essentially as described previously (*16*, *17*). Sf9 cells were infected with recombinant baculoviruses encoding G protein subunits β1, γ2, and His6-αq, generously provided by U. Mende (Brigham and Women's Hospital). Ni−NTA column chromatography was performed as described for purification of Gαq. After adsorption of the oligomer to a Ni−NTA column, the Gβ1γ2 subunit was eluted using AlF_4^- to promote subunit dissociation. Mono Q column chromatography was used to purify the Gβ1γ2 subunits to homogeneity. The purified Gβ1γ2 protein was characterized by trypsin digestion, as previously described (*18*).

Lipid Kinase Assay. The enzyme activity of immunopurified PI3-K in BAEC was determined in vitro as described previously (*15*). Purified Gβ1γ2 (1.35 mg/mL) was diluted into a buffer containing Hepes (20 mM, pH 8.0), NaCl (100 mM), $MgCl_2$ (1 mM), dithiothreitol (1 mM), EDTA (1 mM), and CHAPS (0.7%, w/v). An aliquot (2 μL) of purified Gβ1γ2 (or its vehicle) was added to an enzyme reaction solution (40 μL), including phosphatidylinositol as a substrate. The enzyme reaction was initiated by the addition of 5 μL of 2 mM ATP supplemented with 10 μCi of [^{32}P]ATP (3000 Ci/mmol). The R_f value of lipid spots corresponding to phosphatidylinositol 3-monophosphate [PI(3)P, the principal product of the PI3-K enzyme reaction] was ∼0.8 in this TLC system (*15*−*19*). Quantitation of incorporation of ^{32}P into PI(3)P was carried out using a Cyclone phosphorimaging system (Packard).

siRNA Preparation and Transfection. A Gβ1 siRNA duplex was designed on the basis of established characteristics of siRNA targeting constructs (*20*, *21*). The sequence of Gβ1 siRNA corresponds to bases 225−243 from the open reading frame of the bovine Gβ1 mRNA (*22*) (5′-GGA UGG UAA ACU UAU UAU C-dTdT-3′). The small interfering RNA duplex oligonucleotides were purchased from Ambion, Inc. (Austin, TX). A nonspecific control siRNA from Dharmacon (Lafayette, CO) was used as a negative control (5′-AUU GUA UGC GAU CGC AGA C-dTdT-3′). BAEC were transfected with siRNA as described previously (*23*) and analyzed 48 h after transfection.

Drug Treatment and Cell Lysates. Insulin was dissolved in 0.01 N HCl and stored at −20 °C; the same volume of 0.01 N HCl was used as a vehicle control. VEGF and S1P were prepared as previously reported (*15*, *23*). Culture medium was changed to serum-free medium, and incubations proceeded overnight prior to all experiments. Drug treatments and cell lysate preparation were performed as described previously (*15*, *23*, *24*).

Immunoprecipitation and Western Blot Analyses. For immunoprecipitation, BAEC lysates were incubated with the precipitating antibodies and protein A or G/agarose slurries according to the manufacturer's protocol. The immunoprecipitates or the cell lysates were resolved by SDS−PAGE and transferred to nitrocellulose membranes; immunoblots were probed with antibodies using protocols provided by the suppliers. Densitometric analyses of the Western blots were performed using a ChemiImager 400 device (AlphaInnotech); for all experiments showing densitometry of Western blots, the ordinate is in arbitrary units.

Statistical Analysis. Statistical analyses were performed using one-way ANOVA, followed by the Tukey's test for multiple comparisons. A p value of <0.05 was considered significant. All experiments were repeated at least three times.

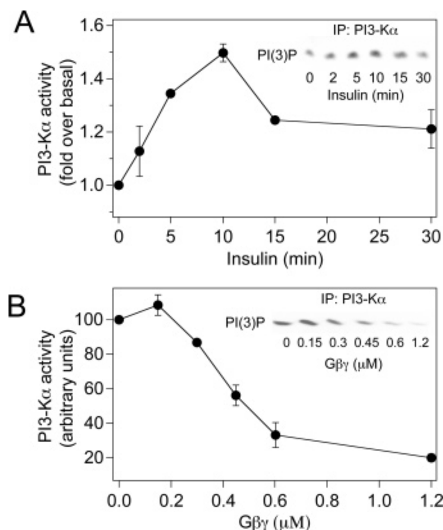

FIGURE 1: Activation and inhibition of PI3-Kα in BAEC. Shown are the results of in vitro PI3-K activity assays in cell lysates derived from BAEC. Cell lysates were immunoprecipitated (IP) with an antibody specific to the α isoform of PI3-K (PI3-Kα); immunoprecipitates were subjected to a lipid kinase assay as described in detail in Experimental Procedures. After enzyme reaction, the radiolabeled lipid products were separated by TLC and subjected to autoradiography. The lipid spots corresponding to PI(3)P, the PI3-K product, are shown. Panel A presents time course experiments of PI3-Kα activity in insulin-treated BAEC. Cells were treated with insulin (1 μM) for up to 30 min as indicated. The inset shows the representative data of a PI3-Kα activity assay, which was performed three times with equivalent results. The signal intensity of PI(3)P in each data point was analyzed and normalized by the signal present at time zero. Each data point in the graph represents the mean ± the SE derived from three independent preparations. In panel B, the in vitro PI3-Kα activity assay was performed in the presence of varying concentrations of G protein $\beta\gamma$ subunit as indicated. Equal amounts of BAEC lysates were immunoprecipitated (IP) with the antibody specific for PI3-Kα. The inset shows a representative experiment analyzing formation of PI(3)P, the PI3-K product. Shown in the graph are pooled data, which were analyzed and normalized by the signal present in the highest concentration of G$\beta\gamma$ (1.2 μM). Each data point in the graph represents the mean ± SE pooled from three independent preparations.

RESULTS

Lipid Kinase Activity Assay of PI3-Kα Immunoprecipitated from BAEC. We first studied the insulin-induced activation of PI3-K isoforms in BAEC, using specific antibodies for different PI3-K isoforms to immunoprecipitate the enzymes, which were then analyzed using lipid kinase activity assays (*15*). Our preliminary experiments revealed that insulin promoted activation principally of the PI3-Kα isoform in these cells, without any substantive effects on the PI3-Kβ isoform. We therefore performed more detailed analyses of insulin-dependent PI3-Kα. As shown in Figure 1A, insulin treatment of BAEC rapidly increases PI3-Kα activity, reaching maximum stimulation 10 min after drug treatment. The role of G$\beta\gamma$ subunits in the modulation of PI3-Kα has not been clearly defined (*15*), and we therefore purified G$\beta\gamma$ subunits for use in lipid kinase assays with PI3-Kα. The combination of Gβ1 with γ2 is quite common, and the Gβ1γ2 subunit has been broadly implicated in signal pathways in endothelial cells and in other cell types (*25*−*29*). We expressed and purified Gβ1γ2 subunits from Sf9 cells transfected with recombinant baculoviruses expressing these proteins, as described previously (*16*, *17*). We added these purified G$\beta\gamma$ subunits to the immunoprecipitated PI3-Kα derived from BAEC and performed lipid kinase assays. To prevent any effect of detergent in this buffer containing 0.7% CHAPS, in all experiments, different quantities of G$\beta\gamma$ protein are always supplemented with the detergent-contained buffer to yield a constant detergent concentration at varying concentrations of G$\beta\gamma$. As shown in Figure 1B, the addition of G$\beta\gamma$ subunits dramatically decreases the formation of PI(3)P by the BAEC-derived PI3-Kα. The EC_{50} for G$\beta\gamma$ inhibition of PI3-Kα activity is ∼450 nM. Taken together, these results suggest that G protein $\beta\gamma$ subunits may play an inhibitory role in insulin-mediated PI3-Kα activation in BAEC. To explore the roles of G protein $\beta\gamma$ subunits in PI3-Kα regulation, we decided to use siRNA methods to "knock down" expression of specific G proteins.

Downregulation of Gβ1 Expression in BAEC Using siRNA. We designed a siRNA duplex targeted to the bovine Gβ1 mRNA (Gβ1 siRNA), with a 5′-GGA UGG UAA ACU UAU UAU C-dTdT-3′ sequence, corresponding to bases 225−243 from the open reading frame of the bovine Gβ1 mRNA (*22*). We analyzed the ability and specificity of this Gβ1 siRNA to knock down Gβ1 expression by transfecting BAEC with increasing concentrations of this siRNA duplex. Figure 2 shows immunoblots from BAEC transfected with Gβ1 or control siRNA, in which cell lysates are probed for

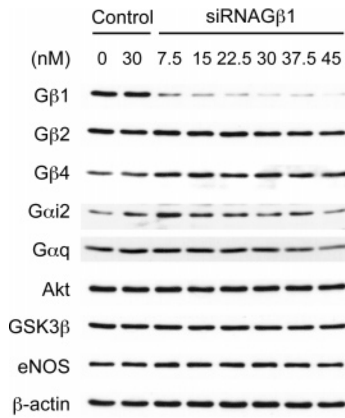

FIGURE 2: siRNA-mediated specific downregulation of Gβ1 expression in BAEC. BAEC were transfected with the indicated concentrations of duplex siRNA targeted against Gβ1 or a random sequence (control siRNA). Forty-eight hours after transfection, cells were harvested and lysed and then subjected to SDS−PAGE. The expression of Gβ1 and other proteins was analyzed in immunoblots probed with isoform-specific antibodies as indicated. The protein levels of β-actin are shown as a loading control. Shown are the results of a representative experiment, which was repeated three times with equivalent results.

expression of Gβ1 as well as a broad range of other G proteins. Gβ1 expression was efficiently knocked down in a dose-dependent manner by transfection with Gβ1 siRNA; transfection with a control siRNA did not affect the expression of Gβ1 (Figure 2). There was no significant change in the expression of other Gβ subunits, and levels of Gα proteins were similarly unaffected by Gβ1 siRNA. Treatment of BAEC with Gβ1 siRNA also has no significant effect on the expression of a wide range of signaling molecules in BAEC such as Akt, GSK3β, and eNOS; expression of β-actin protein was similarly unaffected. Subsequent experiments used Gβ1 siRNA or control siRNA at 30 nM, a concentration that typically resulted in a 90% decrease in Gβ1 protein levels with nominal effects on expression of other signaling proteins in these cells.

Downregulation of Gβ1 Expression Enhances Insulin Signaling in BAEC. We next used the Gβ1 siRNA to explore whether Gβ1 knockdown affects insulin signaling in BAEC. We used phosphorylation state-specific Akt antibodies to determine the time course of insulin-stimulated Akt activation in BAEC. As shown in Figure 3, Gβ1 siRNA markedly potentiates insulin-stimulated phosphorylation of kinase Akt, while the kinase Akt total protein level shows no change following Gβ1 knockdown. The level of insulin-stimulated phosphorylation of kinase Akt is significantly increased following Gβ1 siRNA treatment ($p < 0.001$, $n = 5$). We also analyzed the dose response to insulin in BAEC treated with Gβ1 siRNA (Figure 4). As in the time course experiment, the insulin dose response showed that Gβ1 siRNA has no effect on total Akt expression; however, insulin-stimulated Akt phosphorylation is significantly enhanced following Gβ1 knockdown ($p < 0.05$, $n = 5$). Thus, both the time course and dose−response experiments reveal enhanced insulin signaling after Gβ1 knockdown in BAEC. These findings are consistent with the lipid kinase assay (Figure 1), which showed that Gβγ subunits inhibit PI3-Kα activity in vitro.

To explore the specificity of the effects of Gβ1 siRNA on signaling to Akt in BAEC, we analyzed responses to other agonists that promote Akt phosphorylation. VEGF and S1P are potent agonists for receptor-mediated Akt phosphorylation in these cells. As shown in Figure 5, Gβ1 siRNA does

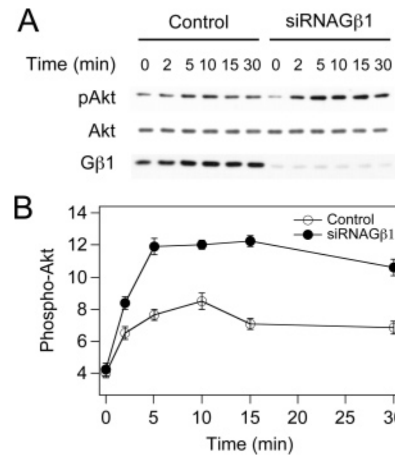

FIGURE 3: Enhanced insulin-mediated Akt phosphorylation after Gβ1 knockdown in BAEC. Panel A shows immunoblots of BAEC lysates prepared from cells transfected with siRNA targeted against Gβ1 or control siRNA and treated with insulin (1 μM) for the indicated times. The cell lysates were resolved by SDS−PAGE and analyzed in immunoblots probed with a phospho-specific antibody to show the level of Akt phosphorylation. Knockdown by Gβ1 siRNA was confirmed by blotting with Gβ1 antibody, as shown. Equal loading was confirmed by immunoblotting with anti-Akt antibodies. Shown are the results of a representative experiment, which was repeated five times with equivalent results. Panel B presents the results of densitometric analyses from pooled data, showing the level of Akt phosphorylation in Gβ1 siRNA or control siRNA transfected cells after treatment with insulin for different periods of time. Each data point in the graph represents the mean ± SE derived from five independent experiments. Compared with that of control siRNA transfected cells, the level of insulin-induced phosphorylation of Akt was increased significantly in Gβ1 siRNA transfected cells ($p < 0.001$).

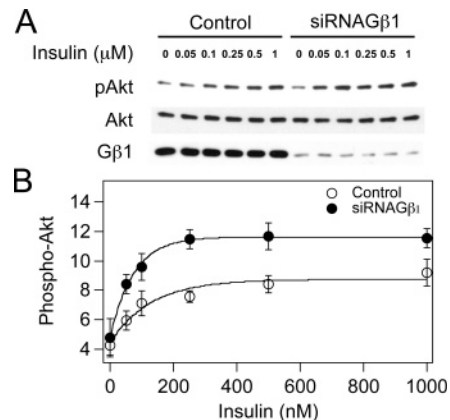

FIGURE 4: Increased insulin sensitivity after Gβ1 siRNA transfection in BAEC. Panel A presents immunoblots of BAEC lysates prepared from cells transfected with Gβ1 siRNA or control siRNA and treated for 10 min with increasing concentrations of insulin as indicated. The level of Akt phosphorylation and the expression of Gβ1 were detected with specific antibodies. Equal loading was confirmed by immunoblotting with anti-Akt antibodies. Shown are the results of a representative experiment, which was repeated five times with equivalent results. Panel B shows pooled data following densitometric analyses, quantitating the level of Akt phosphorylation in Gβ1 siRNA or control siRNA transfected cells after treatment with insulin at different concentrations. Each data point in the graph represents the mean ± SE derived from five independent experiments. Compared with that of control siRNA transfected cells, the level of insulin-induced phosphorylation of Akt increased significantly in Gβ1 siRNA transfected cells ($p < 0.05$).

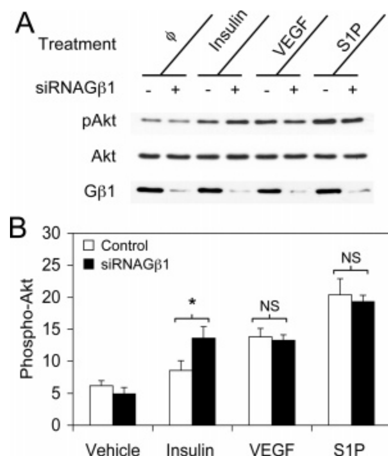

FIGURE 5: Differential effects on downstream receptor pathways after Gβ1 knockdown in BAEC. Panel A shows immunoblots of BAEC lysates prepared from cells transfected with Gβ1 siRNA or control siRNA, treated separately with insulin (1 μM) for 10 min, with VEGF (10 ng/mL) for 5 min, and with S1P (100 nM) for 5 min, and probed with specific antibodies, as shown. This figure presents the results of a representative experiment, which was repeated five times with equivalent results. Panel B presents the densitometric analyses from pooled data, showing the level of Akt phosphorylation in Gβ1 siRNA or control siRNA transfected cells after treatment with insulin, VEGF, and S1P. Each data point in the graph represents the mean $\pm$ SE derived from five independent experiments. The asterisk indicates $p < 0.05$ for Gβ1 siRNA vs control siRNA transfected cells (determined by ANOVA). NS means not significant. ϕ means vehicle.

not significantly affect Akt phosphorylation in response to either VEGF or S1P, while the response to insulin is potentiated ($p < 0.05$, $n = 5$).

Downregulation of Gβ1 Expression Decreases the Level of Serine Phosphorylation of Insulin Receptor Substrate 1. To further explore the mechanisms whereby Gβ1 siRNA specifically potentiates insulin-induced Akt activation, we analyzed the effects of Gβ1 siRNA on phosphorylation of the insulin receptor (*1*−*3*). We used a phosphotyrosine antibody to probe immunoblots prepared from the immunoprecipitated insulin receptor isolated from insulin-treated BAEC. As shown in panels A and B of Figure 6, the level of tyrosine phosphorylation of the insulin receptor was significantly increased after insulin treatment ($p < 0.05$, $n = 3$); Gβ1 siRNA has no substantive effect on either basal or insulin-stimulated tyrosine phosphorylation of the insulin receptor. The principal IRS isoform in these cells is IRS-1 (*30*), but we were unable to convincingly demonstrate insulin-mediated tyrosine phosphorylation of IRS-1, despite using a variety of different antibodies and methods in immunoblots and immunoprecipitations in BAEC lysates. In contrast to these negative results for tyrosine phosphorylation of IRS-1, we found that Gβ1 siRNA significantly reduces the level of serine phosphorylation of IRS-1, while IRS-1 total protein levels show no change (Figure 6C,D). We used phosphorylation state-specific antibodies to probe lysates from insulin-treated BAEC transfected with either Gβ1 or control siRNA. We found that Gβ1 siRNA specifically attenuated both basal and insulin-promoted serine phosphorylation of IRS-1; significant effects were seen in phosphorylation at serine 307 (equivalent to serine 302 in mouse IRS-1) at a basal level ($p < 0.01$, $n = 5$) and after insulin stimulation ($p < 0.001$, $n = 5$). Phosphorylation at this site has been shown to inhibit insulin signaling (*3*−*8*); it seems plausible that the Gβ1 siRNA-mediated reduction in the level of IRS-1 serine phosphorylation may represent another mechanism whereby Gβ1 siRNA potentiates insulin responses in endothelial cells.

Downregulation of Gβ1 Expression Inhibits Activation of p70S6K in BAEC. A wide range of serine/threonine protein kinases are activated following insulin receptor stimulation, and a subset of these kinases have been implicated in phosphorylation of IRS-1 (*5*). We used phosphorylation state-specific antibodies to explore the effects of Gβ1 siRNA on a broad array of insulin-activated protein kinases. We also employed two well-characterized protein kinase inhibitors to explore the intervening kinase pathways: wortmannin was used as an inhibitor of the PI3-K pathway, and rapamycin was used to inhibit the mTOR pathway (*31*). As shown in Figure 7A, insulin-mediated phosphorylation of the serine/threonine kinase (and kinase Akt substrate) GSK3-β is not affected by Gβ1 siRNA; phosphorylation of GSK3-β is completely blocked by the PI3-K inhibitor wortmannin, whereas the mTOR inhibitor rapamycin has no substantive effect. However, the levels of phosphorylation of p70S6K and rpS6 (*32*) are decreased significantly following Gβ1 siRNA transfection, both in the basal state and following insulin treatment. It is plausible that a decrease in p70S6K activity following Gβ1 siRNA may explain the reduction in the level of serine phosphorylation of IRS-1at Ser307 (mouse Ser302) (Figure 7A), as this kinase has been implicated in IRS-1 phosphorylation at this specific site (*3*−*8*). As shown in Figure 7A, phosphorylations of both p70S6K and rpS6 are blocked by rapamycin, suggesting that mTOR is involved as an upstream modulator of these proteins (*32*). However, Gβ1 siRNA does not appear to significantly affect phosphorylation of mTOR itself (Figure 7A). We analyzed in detail the effects of Gβ1 siRNA by performing densitometry on immunoblots from each experiment and show in panels B and C of Figure 7 the analyses of pooled data for those responses that were reproducibly perturbed by Gβ1 siRNA. As noted above, we found that transfection of Gβ1 siRNA led to enhanced insulin-initiated phosphorylation of kinase Akt ($p < 0.05$, $n = 5$, Figure 7B; see also Figures 3 and 4); importantly, Gβ1 siRNA transfection led to a significant ($p < 0.001$, $n = 5$) decrease in the level of phosphorylation of the kinase p70S6K, both in the basal state and following insulin treatment (Figure 7C). Moreover, both the basal and insulin-induced phosphorylation of p70S6K was completely abrogated by the mTOR inhibitor, rapamycin, which did not affect phosphorylation of Akt, whereas the PI3-K inhibitor wortmannin had an only partial effect on phosphorylation of p70S6K under conditions that completely suppressed kinase Akt phosphorylation.

Downregulation of Gβ1 Expression Enhances IGF-1 Signaling in BAEC. Ligand-induced autophosphorylation of IGF-1 receptors promotes the recruitment of IRS-1 proteins and activates downstream PI3-K/Akt signaling (*10*, *11*). As shown in Figure 8, IGF-1 treatment of BAEC significantly increases the level of Akt phosphorylation in a dose-dependent manner; the magnitude of IGF-1-dependent Akt phosphorylation is smaller than that elicited by insulin treatment, but phosphorylation occurs at a lower ligand concentration. We used Gβ1 siRNA to explore whether Gβ1 knockdown enhances IGF-1 signaling in BAEC. As shown

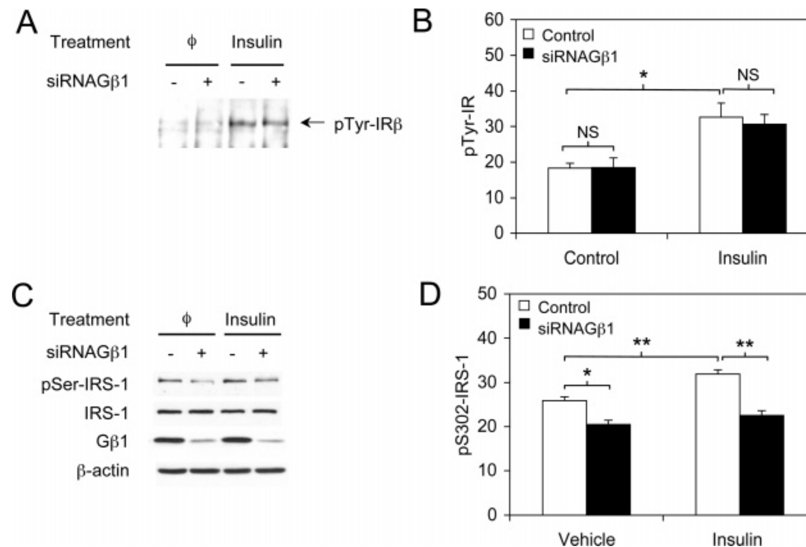

FIGURE 6: Phosphorylation of insulin receptor and IRS-1 after insulin treatment following Gβ1 downregulation in BAEC. BAEC lysates were prepared from cells transfected with Gβ1 siRNA or control siRNA and treated with insulin (1 μM) for 10 min. Panel A shows an immunoblot of the insulin receptor (IR) β subunit immunoprecipitated (IP) from BAEC lysates using the polyclonal antibody for the IR β subunit as described in Experimental Procedures. The immunoprecipitates were resolved by SDS−PAGE and analyzed in immunoblots probed with antibody (4G10) against phosphotyrosine. Shown are the results of a representative experiment, which was repeated three times with equivalent results. Panel B presents the densitometric analyses from pooled data, showing the level of tyrosine phosphorylation of insulin receptor in Gβ1 siRNA or control siRNA transfected cells after treatment with insulin. Each data point in the graph represents the mean ± SE derived from three independent experiments. Panel C shows BAEC lysates resolved by SDS−PAGE and analyzed in immunoblots probed with antibodies against phospho-IRS-1 at Ser307 (mouse Ser302). The expression of IRS-1 and Gβ1 was assessed with specific antibodies. Equal loading was confirmed by immunoblotting with β-actin antibodies. Shown are the results representative of experiments repeated five times with equivalent results. Panel D presents the densitometric analyses from the pooled data, showing the level of IRS-1 phosphorylation at Ser307 (mouse Ser302) as analyzed in either Gβ1 siRNA or control siRNA transfected cells after treatment with insulin. Each data point in the graph represents the mean ± SE derived from five independent experiments. The asterisk indicates $p < 0.01$ for Gβ1 siRNA vs control siRNA transfected cells (determined by ANOVA), and two asterisks indicate $p < 0.001$ for Gβ1 siRNA vs control siRNA transfected cells (determined by ANOVA). NS means not significant. ϕ means vehicle.

in Figure 9, transfection of BAEC with Gβ1 siRNA leads to a significant increase in the level of IGF-1-stimulated Akt phosphorylation ($p < 0.001$, $n = 5$), with no substantive change in the expression of total Akt protein. Analyses of IGF-1 dose responses revealed an EC_{50} of 2 nM, with no substantive change in EC_{50} following Gβ1 siRNA treatment (Figure 10). However, the degree of activation of p70S6K after IGF-1 treatment is reduced significantly following Gβ1 knockdown in a time-dependent and dose-dependent manner (Figures 9 and 10). As shown in Figure 11, Gβ1 siRNA has no substantive effect on either basal or IGF-1-stimulated tyrosine phosphorylation of the IGF-1 receptor. However, Gβ1 siRNA markedly attenuates the phosphorylation of IRS-1 at Ser307 (mouse Ser302) at basal ($p < 0.01$, $n = 5$) as well as after IGF-1 stimulation ($p < 0.001$, $n = 5$).

DISCUSSION

These studies have applied siRNA methods in establishing a role for G protein Gβ1 in the modulation of insulin responses in vascular endothelial cells (*9*). We found that insulin potently activates the α isoform of PI3-K in endothelial cells, and we showed that the activity of this lipid kinase is markedly inhibited by purified Gβ1γ2 (Figure 1). These studies revealed that Gβγ-mediated inhibition of PI3-Kα activity is seen at a concentration of Gβγ significantly higher than that required for Gβγ-mediated activation of PI3-Kβ activity (*15*, *33−35*). It seems plausible that different PI3-K isoforms may be differentially regulated by Gβγ, and moreover, it has been proposed (*23*) that local concentrations of Gβγ may be increased in circumscribed regions of the cell such as plasmalemmal caveolae. The differential effects of Gβγ on PI3-Kα versus PI3-Kβ activity suggest that the isoform-specific catalytic subunits that distinguish these members of the PI3-K protein family might play a key role in the interactions of Gβγ and PI3-K proteins. The molecular determinants of these interactions remain incompletely understood. We next explored the effects of specific G protein βγ subunits on insulin signaling in intact endothelial cells. We developed a duplex siRNA directed against Gβ1, which is a robustly expressed Gβ isoform in endothelial cells, and transfected this siRNA to specifically and potently knock down Gβ1 expression (Figure 2). Transfection of Gβ1 siRNA had no substantive effects on expression of other Gα or Gβ subunits, and there were no alterations in the abundance of other signaling molecules such as Akt, eNOS, and GSK3β (Figure 2). However, Gβ1 siRNA markedly potentiated the insulin-induced activation of kinase Akt in BAEC (Figures 3 and 4); this finding is consistent with our observation that purified Gβ1γ2 inhibits PI3-Kα activity. We propose that there is a tonic inhibition of PI3-Kα by Gβ1γ2, which can be revealed following knockdown of Gβ1 protein using siRNA, which leads to enhanced phosphorylation of kinase Akt, a kinase downstream from PI3-K (*1−3*). We were intrigued to note that an increased level of Akt phosphorylation does not result in enhanced eNOS phosphorylation at serine 1177 (data now shown), a site that is modified by kinase Akt as well as numerous other protein kinases, including PK-A, PK-G, AMPK, and CAMK-II (*36*). We speculate that insulin might modify these other kinases (and/or insulin-modulated phos-

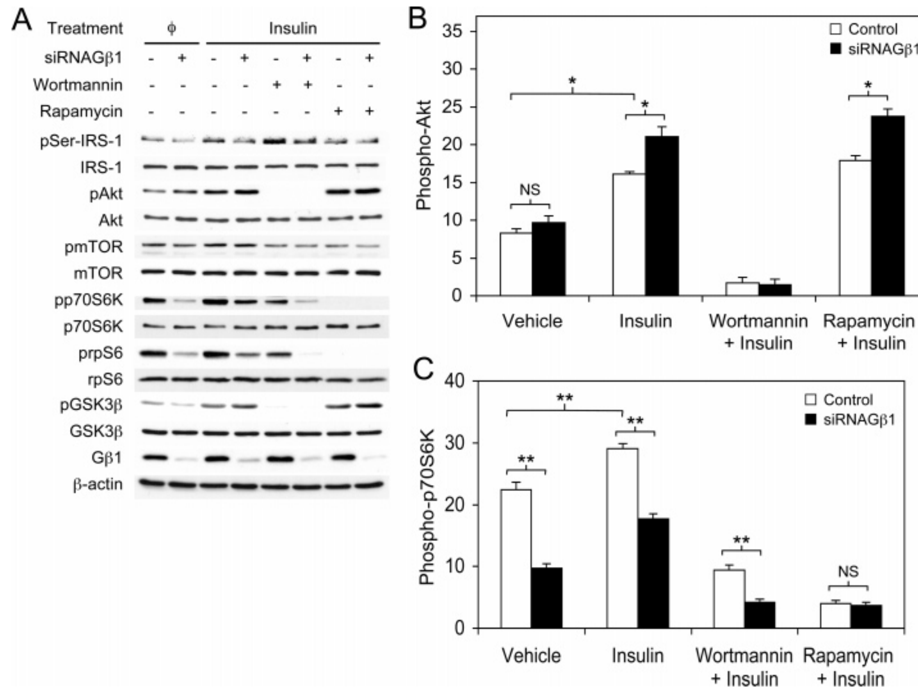

FIGURE 7: Phosphorylation of insulin-modulated protein kinases after Gβ1 knockdown in BAEC. BAEC lysates were prepared from cells transfected with Gβ1 siRNA or control siRNA and treated with insulin (1 μM) for 10 min in the absence or presence of inhibitors wortmannin (200 nM) or rapamycin (20 nM). Pretreatment of the cells with the inhibitors is described in Experimental Procedures. Panel A shows immunoblots of BAEC lysates probed with specific antibodies to determine levels of expression and phosphorylation of proteins as indicated. Shown are the results of a representative experiment, which was repeated five times with equivalent results. Panel B presents pooled data from densitometric analyses, showing phosphorylation of Akt under each condition. Panel C shows densitometric analyses from the pooled data, showing the level of p70S6K phosphorylation at threonine 389. Each data point represents the mean $\pm$ SE derived from five independent experiments. The asterisk indicates $p < 0.05$ for Gβ1 siRNA vs control siRNA transfected cells (determined by ANOVA), and two asterisks indicate $p < 0.001$ for Gβ1 siRNA vs control siRNA transfected cells (determined by ANOVA). NS means not significant. ϕ means vehicle.

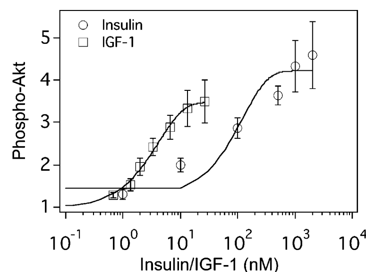

FIGURE 8: Phosphorylation of kinase Akt following insulin or IGF-1 treatment of endothelial cells. BAEC were treated with varying concentrations of insulin or IGF-1 as shown, and Akt phosphorylation was quantitated by densitometric analyses of cell lysates in immunoblots probed with phospho-specific Akt antibodies. Shown are the pooled data from densitometric analyses, quantitating the level of Akt phosphorylation in BAEC treated for 10 min with increasing concentrations of insulin or IGF-1 as indicated, and normalized to the signal observed in the absence of ligand. Each data point in the graph represents the mean $\pm$ SE derived from five independent experiments.

phoprotein phosphatases), leading to a blunting of the contribution of kinase Akt to eNOS phosphorylation at serine 1177. The effect of Gβ1 siRNA in enhancing PI3-K/Akt signaling appears to be specific to insulin-modulated responses. Neither VEGF- nor S1P-mediated activation of Akt was affected by Gβ1 siRNA (Figure 5). Since S1P signaling is entirely G protein-mediated (*15*), we hypothesize that Gβ subunits other than Gβ1 may take part in S1P signaling.

The inhibitory effect of Gβ1 on insulin-mediated activation of PI3-K/Akt may be sufficient to explain the results seen following Gβ1 siRNA transfection in these cells. However, these observations do not fully explain the specificity of the effects of Gβ1 siRNA on insulin-modulated pathways. We found that Gβ1 siRNA has no substantive effect on tyrosine phosphorylation of the insulin receptor (Figure 6A). However, we found that the level of serine phosphorylation of the key insulin receptor substrate IRS-1 at Ser307 (mouse Ser302) was significantly reduced following transfection of endothelial cells with Gβ1 siRNA (Figure 6B). It has been shown previously that IRS-1 serine phosphorylation markedly attenuates its ability to transduce insulin signals (*1*, *3*−*8*). The family of insulin receptor substrate (IRS) proteins can serve as docking proteins that activate multiple signaling pathways, including the PI3-K/Akt signaling pathway. Therefore, the Gβ1 siRNA-promoted decrease in the level of IRS-1 phosphorylation might be expected to enhance insulin-mediated signaling, as we found in these studies (Figures 3 and 4). The observation that Gβ1 siRNA attenuates IRS-1 serine phosphorylation may provide important clues about the identity of additional potential targets for Gβ1 in the modulation of endothelial cell insulin signaling. Several protein kinases have been implicated in serine phosphorylation of IRS-1 (*5*). For example, the protein kinase GSK3-β, which itself is phosphorylated by kinase Akt, has been previously shown to phosphorylate IRS-1 at specific serine residues (*32*). However, we found that GSK3-β phosphorylation is entirely unaffected by Gβ1 siRNA-mediated knockdown. The kinase p70S6K is another insulin-modulated kinase that appears to be centrally involved in modulation of IRS-1 serine phosphorylation (*3*−*8*). Specifically, phosphorylation at the Ser302 site in mouse IRS-1

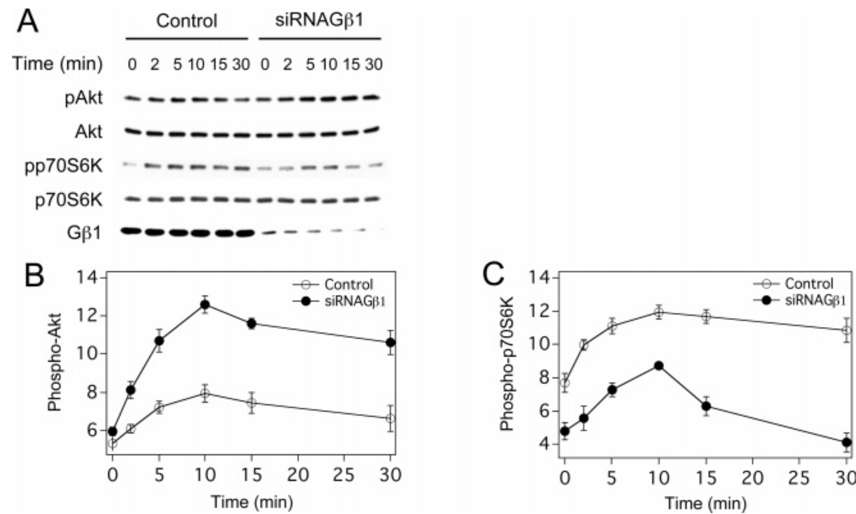

FIGURE 9: Effects of Gβ1 siRNA on IGF-1-induced Akt phosphorylation in endothelial cells. Panel A shows the results of immunoblot analyses of cell lysates prepared from BAEC transfected with siRNA targeted against Gβ1 or control siRNA and treated with IGF-1 (6.7 μM) for the indicated times. The cell lysates were resolved by SDS−PAGE and analyzed in immunoblots probed with the phospho-specific antibodies to show the level of Akt or p70S6K phosphorylation. Knockdown by Gβ1 siRNA was confirmed by blotting with Gβ1 antibody, as shown. Equal loading was confirmed by immunoblotting with anti-Akt antibodies. Shown are the results of a representative experiment, which was repeated five times with equivalent results. Panels B and C present the results of densitometric analyses from pooled data, showing the level of Akt and p70S6K phosphorylation in Gβ1 siRNA or control siRNA transfected cells after treatment with IGF-1 for different periods of time. Each data point in the graph represents the mean ± SE derived from five independent experiments. Compared with that of control siRNA transfected cells, the level of IGF-1-induced phosphorylation of Akt was increased significantly in Gβ1 siRNA transfected cells ($p < 0.001$), whereas IGF-1-induced phosphorylation of p70S6K was markedly suppressed in Gβ1 siRNA transfected cells ($p < 0.001$).

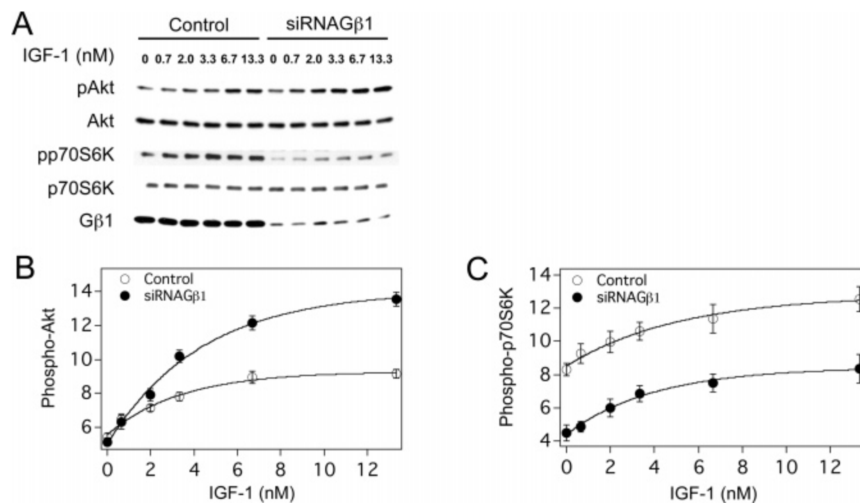

FIGURE 10: Differential effects of Gβ1 siRNA on IGF-1 signaling to kinase Akt and p70S6K. Panel A presents immunoblots of BAEC lysates prepared from cells transfected with Gβ1 siRNA or control siRNA and treated for 10 min with increasing concentrations of IGF-1 as indicated. The level of Akt or p70S6K phosphorylation and the expression of Gβ1 were detected with specific antibodies. Equal loading was confirmed by immunoblotting with anti-Akt antibodies. Shown are the results of a representative experiment, which was repeated five times with equivalent results. Panels B and C show pooled data following densitometric analyses, quantitating the level of Akt and p70S6K phosphorylation in Gβ1 siRNA or control siRNA transfected cells after treatment with IGF-1 at different concentrations. Each data point in the graph represents the mean ± SE derived from five independent experiments. Compared with that of control siRNA transfected cells, the level of IGF-1-induced phosphorylation of Akt increased significantly in Gβ1 siRNA transfected cells ($p < 0.05$), whereas IGF-1-induced phosphorylation of p70S6K was markedly suppressed in Gβ1 siRNA transfected cells ($p < 0.001$).

has been reported to show negative effects in insulin signaling (*7*, *8*). Our studies have established that Gβ1 siRNA markedly attenuates the phosphorylation of kinase p70S6K and rpS6 and show that the mTOR inhibitor rapamycin abrogates phosphorylation of p70S6K and rpS6 (Figure 7). The PI3-K inhibitor wortmannin completely blocks phosphorylation of kinase Akt and GSK3β but does not eliminate the serine phosphorylation of either IRS-1 or p70S6K. By contrast, the mTOR inhibitor rapamycin does not affect phosphorylation of Akt or GSK3β but completely blocks the insulin-modulated increase in the level of IRS-1 serine phosphorylation and completely abrogates phosphorylation of p70S6K and rpS6. We feel that these findings, taken together, suggest that mTOR is involved in the activation of the p70S6K pathway in these cells and that Gβ1 activates the p70S6K pathway: Gβ1 siRNA blocks the phosphorylation of p70S6K as well as the phosphorylation of two of its key substrates, ribosomal protein S6 and IRS-1 (Figure 7). A recent report showed that GβL, a G protein β subunit-like protein, is a positive regulator of the mTOR/p70S6K

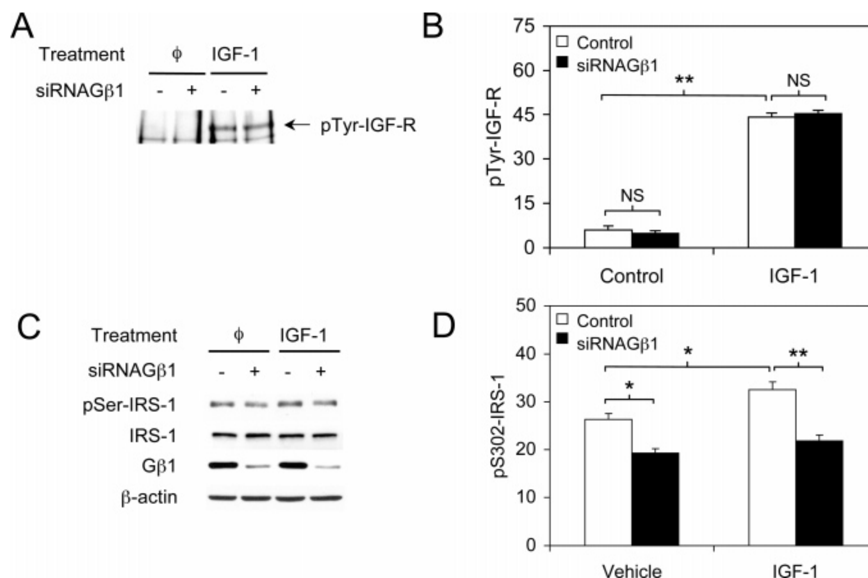

FIGURE 11: Ligand-induced phosphorylation of the IGF-1 receptor and IRS-1 in endothelial cells transfected with Gβ1 siRNA. BAEC lysates were prepared from cells transfected with Gβ1 siRNA or control siRNA and treated with IGF-1 (6.7 nM) for 10 min. Panel A shows an immunoblot of the IGF-1 receptor β subunit immunoprecipitated (IP) from BAEC lysates using a specific monoclonal antibody as described in Experimental Procedures. The immunoprecipitates were resolved by SDS−PAGE and analyzed in immunoblots probed with antibody (4G10) directed against phosphotyrosine. Shown are the results of a representative experiment, which was repeated five times with equivalent results. Panel B presents the densitometric analyses from pooled data, showing the level of tyrosine phosphorylation of the IGF-1 receptor in BAEC transfected with Gβ1 siRNA or control siRNA and treated with IGF-1. Each data point in the graph represents the mean ± SE derived from five independent experiments. Panel C shows results of immunoblot analyses of BAEC lysates from cells treated with IGF-1 (6.7 nM, 10 min); immunoblots were probed with antibodies against phospho-IRS-1 at Ser307 (mouse Ser302). The expression of total IRS-1 and Gβ1 was assessed with their specific antibodies. Equal loading was confirmed by immunoblotting with β-actin antibodies. Shown are the results representative of experiments repeated five times with equivalent results. Panel D presents the densitometric analyses from the pooled data, showing the level of IRS-1 phosphorylation at Ser307 (mouse Ser302) as analyzed in either Gβ1 siRNA or control siRNA transfected cells after treatment with IGF-1. Each data point in the graph represents the mean ± SE derived from five independent experiments. The asterisk indicates $p < 0.01$ for Gβ1 siRNA vs control siRNA transfected cells (determined by ANOVA), and two asterisks indicate $p < 0.001$ for Gβ1 siRNA vs control siRNA transfected cells (determined by ANOVA). NS means not significant. ϕ means vehicle.

pathway (*37*). The nucleotide sequence alignment between the Gβ1 siRNA and the corresponding sequence for GβL reveals no significant similarity, and it is highly implausible that the Gβ1 siRNA leads to any GβL knockdown. Insulin and IGF-1 receptors are distinct but structurally similar receptor tyrosine kinases that share many common steps in their downstream signaling pathways (*10*, *11*). Although insulin and IGF-1 have the highest affinity for their cognate receptors, these ligands can bind to either receptor with a lower affinity. As for insulin, IGF-1 signaling involves receptor autophosphorylation and subsequent tyrosine phosphorylation of IRS-1 proteins and other receptor substrate proteins. Although insulin-like growth factor 1 receptors may be more abundant than insulin receptors in some endothelial cells (*38*), we found that IGF-1 elicited a less robust response for kinase Akt activation in BAEC (Figure 8). As we found for insulin (Figures 3 and 4), siRNA-mediated G protein β1 downregulation significantly enhanced IGF-1 activation of kinase Akt (Figures 9 and 10). In addition, following Gβ1 downregulation activation of IGF-1 receptors was not affected; however, Gβ1 siRNA suppressed both basal and IGF-1-stimulated phosphorylation of IRS-1 at serine 302 (Figure 11). These results support our hypothesis that a decreased level of serine phosphorylation of IRS proteins is responsible for the enhanced insulin signaling after G protein $\beta\gamma$ knockdown in BAEC. Taken together, our findings suggest that responses to both insulin and IGF-1 are negatively modulated by G protein β1 in endothelial cells.

These studies indicate further that the targets of G protein β1 in insulin (or IGF-1)-mediated signaling in endothelial cells may importantly involve the suppression of p70S6 kinase activity. Activation of p70S6K involves a complex series of phosphorylations that occur on multiple serine or threonine residues on this protein (*32*). Numerous protein kinases have been implicated in phosphorylation of these sites, and the detailed mechanisms remain incompletely understood. Full activation of p70S6K appears to require phosphorylation of a specific threonine residue by PDK1, a Ser/Thr protein kinase that is stimulated following PI3-K activation. mTOR also phosphorylates and activates p70S6K, leading to modulation of protein translation. Thus, there are numerous potential targets for Gβ1 in this complex regulatory pathway, including p70S6K itself, protein kinases that phosphorylate p70S6K, as well as the myriad and enigmatic phosphoprotein phosphatases that have been implicated in insulin signaling (*5*, *32*).

It has been demonstrated that heterotrimeric G proteins can directly associate with insulin/IGF-1 receptors, and previous reports have revealed that insulin receptor phosphorylation can be activated by Gαi2 and G$\beta\gamma$ subunits (*12*, *39*). The findings presented here suggest an additional level of complexity in the role of G protein subunits in insulin receptor signaling. G protein-coupled receptors, such as the $S1P_1$ receptor, also lead to G$\beta\gamma$ modulation of PI3-K, but for the $S1P_1$ receptor, this pathway leads to G$\beta\gamma$-dependent activation of PI3-Kβ. By contrast, the findings presented here

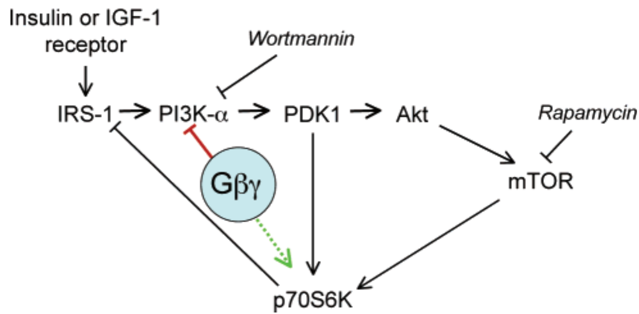

FIGURE 12: Scheme for G protein Gβγ-mediated inhibition of insulin or IGF-1 signaling in endothelial cells. In this model, G protein Gβγ directly inhibits PI3-Kα, thereby suppressing the ligand-mediated activation of lipid-activated kinase PDK1 and decreasing the level of phosphorylation and activation of its key substrates kinase Akt and mTOR. PDK1 and mTOR both serve to phosphorylate and activate p70S6K, which in turn leads to the enhanced serine phosphorylation of IRS-1. Gβγ plays a role also in the activation of p70S6K such that siRNA-mediated downregulation of Gβ1 suppresses p70S6K phosphorylation even though PI3-K activation is enhanced by Gβ1 siRNA. Inhibition of PI3-K by wortmannin suppresses insulin-mediated p70S6K phosphorylation, but Gβ1 siRNA leads to further inhibition of this response. Treatment of endothelial cells with the mTOR inhibitor rapamycin completely inhibits phosphorylation of p70S6K, suggesting a key role for the mTOR pathway in p70S6K activation and indicating that Gβ1 siRNA elicits effects on p70S6K that are at least in part independent of its inhibition of PI3-Kα activation. Please see the text for details.

indicate that Gβγ can also inhibit the distinct PI3-Kα isoform. These complex receptor−G protein−lipid kinase interactions may facilitate cross-talk between different signaling pathways initiated by discrete stimuli. Our experiments provide several lines of evidence implicating G protein Gβ1 in the tonic inhibition of insulin and IGF-1 signaling. Figure 12 shows a model suggested by the findings reported in these studies. In this scheme, G protein β1 directly attenuates PI3-Kα activity (Figure 1) and also suppresses insulin/IGF-1 signaling by activating p70S6K and thereby enhancing serine phosphorylation of IRS-1, and thereby inhibiting insulin or IGF-1 receptor responses. The involvement of Gβ1 in insulin signaling provides a mechanism for cross-talk between receptor tyrosine kinases and G protein-coupled receptors, and Gβ1 represents a potential target for pharmacological intervention in syndromes of insulin resistance.

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