INHIBITION OF INSULIN SIGNALING IN ENDOTHELIAL CELLS BY PROTEIN KINASE C INDUCED PHOSPHORYLATION OF P85 SUBUNIT OF PI3-KINASE

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*Running title: Inhibition of insulin signaling by protein kinase C

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Background: Insulin actions are decreased in endothelial cells causing vascular dysfunction in diabetic and insulin resistant states.

Results: IRS2 and p85 subunit of PI3K are targets of PKC and angiotensin activation inhibiting insulin signaling.

Conclusion: PKC and angiotensin activation inhibit selective insulin activation of Akt/eNOS in endothelial cells.

Significance: We provide a biochemical mechanism by which PKC activation inhibits insulin signaling and protective actions in endothelial cells.

SUMMARY

Insulin’s regulation of endothelial function is consistently abnormal in insulin resistant states and diabetes. Protein Kinase C (PKC) activation has been reported to inhibit insulin signaling selectively in endothelial cells via the IRS/PI3K/Akt pathway to reduce the activation of endothelial nitric oxide (eNOS). In this study, we observed that PKC activation differentially inhibited insulin receptor substrate 1/2 (IRS1/2) signaling of insulin’s activation of PI3K/eNOS by decreasing only tyrosine phosphorylation of IRS2. In addition, PKC activation, by general activator and specifically by angiotensin II, increased the phosphorylation of p85/PI3K which decreases its association with IRS1 and activation. Thr86 of p85/PI3K was identified to be phosphorylated by PKC activation and confirmed to affect IRS1 mediated activation of Akt/eNOS by insulin and VEGF using deletion mutant of Thr86 region of p85/PI3K. Thus, PKC and angiotensin induced phosphorylation of Thr86 of p85/PI3K may partially inhibit the activation of PI3K/eNOS by multiple cytokines and contribute to endothelial dysfunction in metabolic disorders.

Insulin resistance is one of the major risk factors for developing atherosclerosis and is characterized by the loss of insulin action in multiple tissues, including adipocytes, skeletal muscle and hepatocytes (1). Besides these traditional metabolic tissues, insulin action on vascular endothelium has been shown to be physiologically significant in regulating hemodynamic functions, by the activation of endothelial nitric oxide (eNOS) (2-4). Pathophysiologically, the loss of insulin action on the vascular endothelium can cause endothelial dysfunction, which correlates to increased risk of coronary artery disease and even insulin resistance (5). The importance of insulin’s actions on endothelial function were clearly established.
recently by the findings that mice with double knockout of apolipoprotein E (apoE−/−) and insulin receptor (IR−/−) on the endothelial cells developed atherosclerosis at 2-3 times greater severity than apoE−/− mice (6).

The mechanism of insulin’s physiological actions on the vascular endothelium is predominately mediated via the IRS/PI3K/Akt pathway, leading to the activation of eNOS and the expression of cytokines such as vascular endothelial growth factor (VEGF) and heme oxygenase-1 (HO-1) (4;7-9). In insulin resistant states most commonly associated with obesity and diabetes, selective inhibition of the IRS/PI3K/Akt pathway has been demonstrated, probably due to abnormal elevation of free fatty acid and glucose levels (10). One mechanism causing the selective inhibition of insulin’s actions is likely due to PKC activation, which has been shown to be a consistent feature in the endothelium in response to diabetes or insulin resistance (11). Inhibition of PKCβ isoforms has been reported to decrease severity of atherosclerosis in apoE−/− mice (12).

We suggest that one mechanism for PKC activation to increase atherosclerosis is the inhibition of insulin’s action selectivity in the endothelial cells (13). PKC activation has been reported to selectively inhibit insulin activation of Akt and eNOS in endothelial cells by decreasing the phosphorylation of Akt at Ser473 and eNOS at Ser1177 (1179 for bovine) (13). However, the specific step(s) of insulin signaling between insulin receptors (IR) to Akt, which are being inhibited by PKC activation, have not been identified. Previously, studies in non-endothelial cells have shown that PKC activation can decrease insulin-induced tyrosine phosphorylation of IR and IRS1 by increasing serine (Ser) or threonine (Thr) phosphorylation at several sites on these proteins (14-20). However, we have reported that PKC activation can also inhibit vascular endothelial growth factor (VEGF)-induced phosphorylation of Akt and eNOS in endothelial cells, suggesting that phosphoinositide 3-kinase (PI3K) subunits can also be targets of the inhibiting actions of PKC activation (13).

In this study, we have characterized the effects of PKC activation on multiple steps of insulin signaling cascade, from the receptor to Akt phosphorylation, in endothelial cells. The results showed that the inhibitory effects of PKC activation in endothelial cells differ from other types of cells in the lack of changes on IR and IRS1 tyrosine phosphorylation. However, we have identified a novel phosphorylation site on p85 subunit of PI3K, which is responsive to PKC activation and blunts insulin activation of Akt/eNOS in endothelial cells.

**EXPERIMENTAL PROCEDURES**

All chemicals otherwise noted were purchased from Sigma-Aldrich Co. (St. Louis, MO). Antibodies were purchased from Cell Signaling Technology Inc. (Danvers, MA), Upstate Millipore, (Billerica, MA), or Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Antibodies against p85, IRS1, IRS2, phosphotyrosine (pY) (4G10®) were from Upstate. Phospho-/total-eNOS, phospho-/total-Akt and ERK, and HA-tag are from Cell Signaling. All the phospho-IRS1 antibodies were purchased either from Cell Signaling or Upstate. Polyclonal antibody against insulin receptor (IR) beta was from Santa Cruz and monoclonal antibody for immunoblotting was from Cell Signaling. Anti-GST-HRP conjugated and Protein A/G PLUS agarose were purchased from Santa Cruz. Amersham ECL™ and ECL Plus Western Blotting Detection Reagents were purchased from GE Healthcare (Piscataway, NJ). GF109203X (Bisindolylmaleimide I, GFX) and PD98059 were purchased from Calbiochem (EMD) (Gibbstown, NJ). Phosphatidylinositol (#109853) was purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). VEGF was purchased from R&D Systems Inc. (Minneapolis, MN), Dulbecco's Modified Eagle Medium with 100mg/dl glucose (DMEM-L) was provided by Joslin Media Core with penicillin G and streptomycin included. Angiotensin was purchased from Sigma-Aldrich Co. (St. Louis, MO).

**Cell culture**

Bovine aortic endothelial cells (BAEC) were isolated from bovine aorta purchased from a local vendor and propagated in DMEM–L and 10% horse serum. BAEC were used at passage 2 to 5. Mouse endothelial cells were isolated from lungs of IRS2KO mice (Fasshauer et al, 2000) or wild-type C57/B6 mice. Lungs were digested with collagenase 0.2% (w/v) in DMEM/0.1% BSA. The dissociated cells were cultured in DMEM with...
100 mg/dl glucose, 10% horse serum, endothelial cell growth supplement (ECGS) 50 µg/ml and heparin 100 µg/ml for 3 days, and were then resuspended and incubated with ICAM-2 rat anti-mouse antibody (BD Pharmingen, San Jose, CA) with Dynabeads sheep anti-rat IgG (Dynal/Invitrogen, Carlsbad, CA) in PBS/0.1% BSA. The endothelial cells were magnetically isolated when passing through Dynal MPC-15 Magnetic Particle Concentrator (Dynal/Invitrogen, Carlsbad, CA). Cells were standardized with 0.1% BSA (Sigma Aldrich, St. Louis, MO) for 24 hr, then pretreated with 100nM of PMA for 20 min, and stimulated with 100nM of insulin for 5 min before washing and lysing the cells.

Adenoviral infection
To overexpress IRS1 and IRS2, adenoviral vector that encodes full length cDNA of rat IRS1 and IRS2 were used as previously described (21). Adenovirus encoding Lac Z was used as a control. The MOI used to infect the cells was 25.

Immunoprecipitation and immunoblot studies.
Cells were lysed in modified RIPA buffer (50mM Tris-HCl (pH 7.4), 150mM NaCl, 1% NP40, EDTA 1mM, NaF 1mM) containing protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 2 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM Na3VO4; Sigma). The protein amount was measured with BCA kit (Bio-Rad). For immunoprecipitation, the lysates were immunoprecipitated with the indicated antibody and immobilized on protein A/G PLUS-agarose beads (Santa Cruz). The lysates (50 µg protein) or the precipitant were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride or nitrocellulose membrane, and blocked with 5% skim milk. Antigens were detected using the indicated antibodies and then with horseradish peroxidase-conjugated secondary antibodies and detected with the ECL system (GE Healthcare).

Transfection assays
Transfection of siRNA or DNA and siRNA-DNA was performed using Lipofectamine 2000 (Invitrogen). The sequence of SiRNA for IRS2 is sense: 5’ GGGCUGAGGAAGCGGACUUt 3’; The sequence of SiRNA for p85 is sense: 5’ GGGGAGGAGGAUUGACUuaa 3’. One day before transfection, cells were plated in Opti-MEM Reduced Serum Medium (Invitrogen) in 6-well dishes. On the day of transfection, siRNA or DNA or siRNA-DNA molecule-Lipofectamine™ 2000 complex was added into cells 48 hrs before harvesting.

Far-western analysis
BAEC with overexpression of IRS1 were exposed to insulin and/or PMA (100nM) as described above. Total cell lysates were immunoprecipitated with IRS1 (100nM) antibody and separated on SDS-PAGE. Membranes used for transfer were incubated with 1 µg/ml of synthetic p85α-GST in PBST+5% BSA at 4°C overnight, followed by anti-GST-HRP (sc459) in PBST+5% skim milk. The signals were visualized with chemiluminescence and film.

PI3K activity assay
Method for PI3K assay was performed as previously described (21).

Isolation of p85α from BAEC for TOF-MS/MS
BAEC (20 plates of p150 culture dishes for each experiment) were infected with 1–2x10^8 ifu/dish of adenovirus encoded with human HA-tagged p85α. On the day after infection, cells were starved in 0.1% BSA DMEM-L for 24 hrs and then incubated with 100nM of PMA for 20 min before washing with ice cold PBS and harvesting with IP lysis buffer. Samples were centrifuged at 10,000 rpm for 25 min at 4°C. Supernatant yielded approximately 100mg of protein which was incubated with 1:600 of anti-HA antibody at 4°C for 24 hrs and then centrifuged at 10,000 rpm for 25 min at 4°C. Then supernatant was incubated with protein A/G agarose (Santa Cruz) for 2 hrs at 4°C. The beads were collected, washed and boiled with sample buffer. Denatured protein was separated on SDS-PAGE (7.5%). A p85α band was identified by Coomassie staining and parallel immunoblotting.

LC-MS/MS analysis-

p85 was immunoprecipitated from bovine aortic endothelial cell lysates and immunoprecipitate was separated by SDS-PAGE. P85 protein was visualized by staining with Coomassie Brilliant Blue G-250 stain (Bio-Rad) and the gel slice containing p85 was digested with sequencing grade modified trypsin (Promega). The resultant tryptic digest was separated by capillary HPLC (C18, 75µM i.d.
Picofrit column, New Objective) and analyzed using a LTQ ion trap mass spectrometer (ThermoElectron). Synthetic phosphopeptide, corresponding to tryptic sequences derived from p85 was obtained from Boston Biomolecules, Inc. and analyzed using the LC-MS/MS protocol described above. Spectra containing a neutral ion loss of -98 and 49 were analyzed using Sequest (BioWorks 3.3, Thermo Electron) with fragment ion tolerance <0.5 and amino acid modification variables including phosphorylation (80 Dal) of Ser, Thr, and Tyr.

Statistical analysis
Data are presented as the mean +/- SD. Comparisons between two groups were performed with unpaired students’ t test. Multiple comparisons were performed with one-way ANOVA and Student-Newman-Keuls Method was used for post-hoc tests. P values less than 5% were considered statistically significant.

RESULTS
Characterization of PKC’s inhibitory effects on insulin’s signaling cascade.

The effect of PKC activation on insulin’s induction of Akt and ERK phosphorylation was studied using PMA, which mimics diacylglycerol (DAG) and can activate conventional and novel PKC isoforms (22). As reported, insulin (100nM) increased phosphorylation of S473-Akt (p-Akt S473) by 6.7 ± 1.8 fold which was inhibited completely by the addition of PMA (Fig. 1.1). However, the addition of GFX, a general PKC inhibitor, prevented the actions of PMA on insulin’s induction of p-Akt S473. Insulin alone increased phosphorylation of ERK1/2 (p-ERK1/2), by 1.7 ± 0.3 fold which were increased further by 9.5 ± 1.0 fold in the presence of PMA. Addition of GFX inhibited the enhancing actions of PMA on insulin-induced p-ERK1/2 (Fig. 1.1).

To identify the step(s) of the insulin signaling pathway, which is targeted by PKC activation in BAEC, we characterized in sequence from insulin receptor (IR) to p-Akt S473. Analyzing IR in BAEC showed that insulin significantly increased tyrosine phosphorylation (p-Tyr) of IRβ subunit, which was not reduced by the addition of PMA, suggesting that PKC’s target is post IR in BAEC (Fig. 1.2).

To clearly evaluate whether PKC activation can alter IRS1, we overexpressed IRS1 by 10 fold which enhanced insulin’s effect on p-Akt S473 by 2.3 fold (p<0.01) (Figs. 1.3, 1.4). PMA blocked insulin-induced p-Akt S473 in wild type (WT) cells and IRS1-overexpressed BAEC by 62% (p<0.01). Changes in phosphorylation of Thr308-Akt were parallel to that of Ser473-Akt in BAEC. Phosphorylation of eNOS Ser1179, (p-eNOS S1179) downstream of Akt was also enhanced with the overexpression of IRS1, which was increased with the addition of insulin by 6.6 fold and inhibited by PMA by 59% (p<0.01), in parallel with changes in p-Akt S473 (Fig. 1.5).

Using the BAEC overexpressing with IRS1 or IRS2, insulin significantly increased p-Tyr of IRS1 (p-Tyr IRS1) by 7.0 fold and IRS2 (p-Tyr IRS2) by 8.7 fold. The addition of PMA did not inhibit insulin-induced p-Tyr IRS1, but it significantly inhibited p-Tyr IRS2 by 39% (Fig. 2.1).

The lack of inhibitory effect of PKC activation on IRS1 tyrosine phosphorylation, although it still decreased insulin’s action on p-Akt, was surprising since PKC has been reported to increase Ser/Thr phosphorylation of IRS1 in non-endothelial cells and inhibit its signaling function (10). To confirm that IRS1 mediated insulin’s signaling can be inhibited by PKC activation in BAEC, we reduced IRS2 levels with siRNA by 59% in BAEC (Supplemental Figure 1), and observed that insulin still strongly induced p-Akt Ser473 by 8.5 fold which was also inhibited in the presence of PMA by 76% (Fig. 2.2). To confirm that IRS1 can clearly mediate insulin activation of Akt/eNOS, lung vascular endothelial cells were isolated from IRS2 knockout (IRS2KO) mice and studied. As shown in Fig. 2.3, insulin activation of p-Akt in IRS2KO endothelial cells was reduced compared to WT mice but PMA still inhibited insulin activation in endothelial cells from WT (by 94%) and IRS2KO (by 77%) mice significantly. These results suggest that PKC activation can inhibit IRS1 and IRS2 mediated signaling for the activation of p-Akt/p-eNOS by different mechanisms in endothelial cells.

To clarify the discrepancy between the lack of effects of PKC activation on insulin induced total p-Tyr of IRS1 and inhibition of p-Akt, the inhibitory effects of PKC activation on several steps of insulin’s signaling cascade from IRS1 to
p-Akt were characterized. First, we assessed the extent of PMA’s inhibitory effects at the pre- or post-p110/PI3K step by overexpressing constitutively active p110/PI3K in BAEC (BAEC-myr-p110A) (Fig. 3.1). The addition of PMA to BAEC-myr-p110A inhibited p-Akt by only 44%, indicating that approximately 50% of PKC’s inhibitory effects are targeted to steps before the activation of p110/PI3K. Since IRS1 appears to mediate a majority of insulin’s activation of p-Akt in BAEC and did not exhibit a decrease in its total tyrosine phosphorylation with PKC activation, we characterized IRS1 and its association with p85/PI3K by assessing the various known phosphorylation sites on IRS1 which have been shown to affect IRS1 functions (Table 1). Surprisingly, Ser307, the best characterized residue to be phosphorylated by PKC activation and decrease IRS1 function, did not increase in phosphorylation level with PMA (23). Residue Ser612 of IRS1, which is known to be phosphorylated by MAPK or PKC, increased by 1.9±0.3-fold (p<0.05) with the addition of PMA in BAEC (19). However, the phosphorylation of Tyr-608 which is adjacent to Ser612 and needed for binding to p85/PI3K, did not show a concomitant inhibition by PKC activation when stimulated by insulin (24). Moreover, pre-treatment of BAEC with PD98059, a MEK inhibitor, completely blocked the phosphorylation of Ser612/IRS1 induced by PMA, but it did not change the inhibitory effect of PMA on insulin induced IRS1-associated PI3 kinase activity and p-Ser 473 of Akt (Table 1).

Assessing PI3 kinase activities and interactions with IRS1

Since PMA did not alter insulin induced tyrosine phosphorylation of IRβ subunit and IRS1, we assessed PI3K activities induced by insulin with and without the addition of PMA. As shown in Fig. 3.2, insulin increased IRS1-associated PI3K activity by 6.3 fold which was significantly inhibited by 35 ±12.8% with the addition of PMA (p<0.001). In addition, we characterized the association between IRS1 and p85α/β/PI3K isoforms and found that insulin increased the association between IRS1 and p85α/β subunit of PI3K by 4.2 fold which was deceased by 35% (p<0.005) in the presence of PMA when the complex was immunoprecipitated with antibodies to IRS1 (Fig. 3.3). Similarly, when immunoprecipitation was performed with antibodies to p85α/β, insulin increased IRS1 association with p85α/β/PI3K by 9.9 fold which was reduced by 43% in the presence of PMA (p<0.001) (Fig. 3.4).

To confirm that the change in p85/PI3K is partially responsible for PKC induced inhibition of interactions between IRS1 and p85, we performed far-western analysis using recombinant p85α-GST fusion protein. As shown in Fig. 4, insulin increased p-Tyr of IRS1 in BAEC overexpressing IRS1 which was not reduced by PMA, but the association between IRS1 and endogenous p85/PI3K was decreased by 35% (Fig. 3.3). In contrast, the association between recombinant p85α-GST protein and endogenous IRS1 was not inhibited by PMA (Fig. 4). No association was observed between IRS1 and carboxyl-terminal peptide of p85α as a control. These data suggest that PKC affects endogenous p85α/β subunit of PI3K in BAEC and decreases its binding to IRS1.

Analysis of p85α/β/PI3K phosphorylation sites with PKC activation.

To determine potential phosphorylation sites on p85/PI3K which are induced by PMA, p85α was overexpressed in BAEC by adeno viral vector infection containing p85α and exposed to PMA for 30 min. and p85α was immunoprecipitated by anti-p85α antibodies and separated by gel electrophoresis. We identified MS2 spectra corresponding to the phosphopeptide ISPPT*PK using LC-MS/MS analysis of the tryptic digest of p85 isolated from PMA-stimulated BAEC were identified (Fig. 5). MS2 spectra from the p85-derived tryptic peptide and the corresponding synthetic peptide (ISPPT*PK) displayed similar distributions of y and b fragment ions for 1+ and 2+ precursor ions. In addition, MS2 spectra for 1+ precursors from the tryptic and synthetic peptides contained a prominent fragment, a 721 m/z, which is consistent with neutral ion loss. To document further that PKC activation by PMA increased the phosphorylation of Thr86 of p85α in BAEC, antibodies were raised against phosphorylated Thr86/p85α peptides. The specificity of the antibody was validated against p-Thr86 peptide in vitro. As shown in Fig. 6, PMA increased the level of p-Thr86/p85α in BAEC by...
The increase of p-Thr86/p85α was inhibited completely by the addition of PKC inhibitor GFX.

The functional role of p-Thr86/p85α was evaluated by overexpressing a plasmid containing human p85α deletion mutant, lacking 13 amino acids of p85α (84-96) (Fig. 7.1) In BAEC overexpressing deletion mutant p85α (84-96) pro-1, insulin increased its association with IRS1 by 1.5-fold similar to control BAEC. The addition of PMA decreased insulin induced association between IRS1/p85α in the control cells by 72%. In the BAEC overexpressing deletion mutant p85α (84-96) pro-1, the inhibiting effects of PMA on the association between IRS1 and p85α was significantly reduced (Fig. 7.2). The effect of overexpressing pro-1 p85α mutant was characterized further on insulin’s activation of Akt (p-Akt). Since overexpression of p85α subunit inhibits p110/PI3K activities, we reduced the amount of endogenous p85α subunit using siRNA of p85α in BAEC with simultaneous transfection of pro-1 mutant of p85α. In these conditions, the levels of endogenous p85α in BAEC was reduced by 67% and the amount of pro-1 mutant (p85α) was significantly increased (Fig. 7.3). P-Akt levels were increased by 7.5 fold and 7.0 fold with insulin in control BAEC and in cells transfected with siRNA of p85α, respectively. The addition of PMA inhibited insulin induced p-Akt levels completely in wild type BAEC. In BAEC transfected with siRNA of p85α and pro-1 (p85α mutant), insulin again increased p-Akt levels by 8.0 fold (p<0.05). However, the inhibitory effect of insulin induced PMA p-Akt levels was significantly reduced (Fig. 7.3).

**PKC’s activation by PMA and Angiotensin on p-Akt**

VEGF can also increase the levels of p-Akt after binding to vascular endothelial growth factor receptor 2 (VEGFR-2/KDR) and activating PI3K (25). However, unlike insulin, KDR can bind and activate p85/PI3K without IRS1/2. Thus, PKC activation by PMA with increased phosphorylation of p85/PI3K should also inhibit the actions of VEGF. As shown in Fig. 8, VEGF increased p-Akt by 10.6 fold (p<0.001), which was inhibited completely by the addition of PMA. Further, the adding of PKC inhibitor GFX alone or with VEGF did not alter p-Akt levels. However, GFX prevented the inhibitory actions of PMA on VEGF’s activation of p-Akt. To support Thr86/p85 as the site for PMA’s inhibiting effects on VEGF’s activation of p-Akt, the effect of overexpressing p85α deletion mutant (pro-1) in BAEC was studied. In control BAEC, VEGF increased p-ser473Akt by 10 fold which was significantly inhibited by PMA. In BAEC overexpressing p85α siRNA, the endogenous levels of p85αβ were significantly decreased. However, VEGF was still able to increase p-Akt levels by 10 fold which again was inhibited by PMA by 90% even though the levels of p85αβ were significantly decreased. In BAEC, transfected with p85 siRNA and p85α deletion mutant (pro-1), the amount of p85αβ were elevated by 11.7 fold above their endogenous levels. In contrast to wild type BAEC, VEGF was able to increase p-Akt levels equally with and without PMA in BAEC transfected with p85α deletion mutant (pro-1) (Fig. 8A).

We also studied the effect of angiotensin II (AngII, 100nM) to inhibit insulin’s actions and induce phosphorylation of phospho-Ser/Thr-p85α/β. As shown in Fig. 8B, AngII increased phospho-Ser/Thr-p85α/β by 2.1-fold similar to PMA which was prevented completely by GFX, a general PKC inhibitor. Insulin did not have any effect on phospho-Ser/Thr-p85α/β in BAEC overexpressing IRS1. In BAEC overexpressing IRS1, AngII also inhibited insulin’s activation of pAkt similarly to PMA. AngII’s inhibition of insulin-induced p-Akt was also prevented by GFX (Fig. 8C). The effect of AngII to activate PKC isoforms was characterized and showed that PKCa isoform was the predominant isoform to be translocated from cytosol to the membrane by at least 2-fold similarly to PMA. (Figure 8D) AngII also caused translocation of PKCb and δ (data not shown). PKCα also can activate PKC βI, βII, δ and ε isoforms at 20 mins. without causing downregulation. (Supplemental Figure 4) To confirm, further, the effect of PKCa isoform in mediating the phosphorylation of phospho-Ser/Thr85α/β, we overexpressed adenoviral vector...
containing PKCα, βI, δ and ε isoforms. The results, as shown in Fig. 8E, suggested that the overexpression of PKCα isoform induced the phosphor-Ser/Thr p85α to a greater extent than PKC βI, δ and ε isoforms.

DISCUSSION

We have reported that metabolic abnormalities due to insulin resistance and diabetes can cause selective inhibition of insulin signaling via the IRS/PI3K/Akt pathway and a decrease of insulin’s activation or expression of eNOS (10). In contrast, these metabolic abnormalities enhanced insulin’s signaling via the MAPK pathway which are known to increase the expression of PAI-1, endothelin 1 and extracellular matrix in the arterial wall (26-29). This hypothesis of selective diminution of insulin’s actions via the IRS/Akt pathway may lead to accelerated atherosclerosis as observed in insulin resistance and diabetes has been supported recently by the report that the deletion of insulin receptors specific to the endothelial cells increased the severity of atherosclerotic lesions in apoE null mice by 2-3 fold (6).

PKC activation, especially, the β isoform, has been associated with endothelial dysfunction and atherosclerosis (12;13). Mice with double knockout of PKC-β isoform and apoE exhibited significantly less atherosclerosis than apoE null alone (12). Treatment with PKC-β selective inhibitor improved forearm blood flow in people with hyperglycemia and also ameliorated endothelial dysfunction in insulin resistant rodents (30). Multiple reports have shown that PKC activation can increase serine/threonine phosphorylation of IR and IRS1/2 and decrease their levels of tyrosine phosphorylation and functions induced by insulin (15-20;31). Conventional and novel PKC α/β/δ/ε and τ isoforms, activated by diacylglycerol (DAG), due to hyperlipidemia or hyperglycemia (19), have been reported to cause insulin resistance (15-20;31) mainly in liver, muscle or adipose tissue where increase in threonine/serine phosphorylation of IR or IRS.

In the endothelial cells, these results indicate PKC activation can inhibit several steps of insulin’s signaling pathways such as IRS2, p85/PI3K and unknown post-PI3K steps. Our results also support the idea that IRS1 and IRS2 participates significantly in mediating insulin’s activation of Akt and eNOS. For this study, we have focused on IRS1’s role since elucidating all PKC’s targets are beyond the scope of a single report.

Our data clearly established that IRS1 is functionally important in endothelial cells since endothelial cells from IRS2KO mouse still responded to insulin. In addition, global or endothelial cell targeted IRS1KO mouse exhibited vascular endothelial dysfunction (32). Interestingly, PKC activation inhibited insulin signaling cascade differently between IRS1 and IRS2 in endothelial cells. Further studies are in progress to provide explanation for the differential effects of PKC activation on p-Tyr of IRS1 and IRS2. Although we didn’t observe any decrease of insulin-induced tyrosine phosphorylation of IRS1 by PKC, we did find decreased binding of IRS1 to p85/PI3K and PI3 kinase activity. Activation of PKC and MAPK have been reported to phosphorylate serine 612 on IRS1 (15) which in mouse liver, can lead to the attenuation of downstream insulin signaling by decreasing the adjacent 608/IRS1 tyrosine phosphorylation which is essential for docking with p85/PI3K (24). Our study in endothelial cells also showed that PKC activation increased phosphorylation of serine 612/IRS1, but this did not inhibit insulin-induced phosphorylation of tyrosine 608/IRS1. Moreover MEK inhibitor which completely blocked the phosphorylation of serine 612 induced by PMA did not inhibit PMA’s effect on insulin’s activation of PI3K activity and p-Akt levels. These results indicate that phosphorylation of serine 612/IRS1 induced by PMA is not responsible for inhibitory effect of PKC on PI3K/Akt pathway in vascular endothelial cells. Another well documented effect of PKC activation is the phosphorylation at serine 307 of IRS1. PKC-τ has been reported to phosphorylate serine 307/IRS1, followed by decrease in its tyrosine phosphorylation in skeletal muscle and muscular insulin resistance (23). Further, Ser24, 318, 357, and 1101 of IRS1 have all been reported to be phosphorylated by PKC (15-20;31). However, PMA did not increase the phosphorylation of these sites on IRS1 in endothelial cells.
PI3K is a key node for insulin signaling and is a critical member of various growth factors and cytokine signaling such as VEGF/Akt signaling. PI3K consists of heterodimer of regulatory subunit and catalytic subunit and it is fully activated when regulatory subunit binds to phosphotyrosine residue of adapter proteins like IRS1 at the submembrane area of a cell (33). A p85 subunit of PI3K is not only an abundant regulatory subunit but it has a unique regulatory function for this enzyme (34). Excessive amounts of p85 monomer can inhibit the activity of PI3K by competing with PI3K heterodimer for binding to phosphotyrosines (34). The presence of excessive amounts of p85 monomer and its inhibitory function could explain the findings that its reduction by siRNA did not reduce the actions of insulin and VEGF. (34) Although a great deal is known about the regulation of PI3K only a few of the Ser/Thr phosphorylation sites on PI3K have been reported especially those due to PKC activation. One is Ser 608 on p85α, which is autophosphorylated by catalytic subunit of p110/PI3K and serves as a negative regulator of the enzyme. The other residue is Ser83 on p85α which is phosphorylated by protein kinase A and kinase activity is enhanced (35). In this study, we have identified a novel phosphorylation site; Thr86 on p85α which was phosphorylated with PKC activation induced by PMA and angiotensin. Our data suggests that it is phosphorylated by PKC and serves as a negative regulator because deletion of this region causes the loss of PKC’s effect on insulin induced activation on PI3K. Our results suggest that the phosphorylation of Thr86 reduces the binding of p85α to IRS1 and possibly IRS2 (Supplemental Figure 2). However, Thr86/p85α is not within or adjacent to the SH2 domain which are known as binding sites to phosphotyrosines. However, it is within one of the two proline rich domains, which is reported to bind with SH3 domain (36). It is not well understood what proteins are associated with p85α through proline rich domains. Since various molecules including p85α itself have SH3 domains, it is possible that changes in interaction with a protein with SH3 domain will reduce the affinity with IRS1. Thus, it is possible that a conformational change, induced by the phosphorylation of Thr86/p85α, can lead to a change in subcellular sites and decrease of the kinase activity with IRS1. Since proline rich domain is positively charged and phosphorylation may change this site to negative, it is possible for Thr86 to modify its interactions with IRS1 and result in decreases in PI3K activation.

Since p85/PI3K activation is critical for the intracellular signaling of many cytokines and hormones, it was not surprising that the inhibitory action of PKC activation also reduced VEGF’s signaling via PI3K/Akt. Previously, we have identified PKCB isoforms as most likely to inhibit insulin’s actions in endothelial cells via inhibition of insulin’s actions on Akt and eNOS. This is supported by recent reports that PKCB isoforms selective inhibitor or PKCBKO/apoE−/− mouse have decreased severity of atherosclerosis. It is also likely that activation of other PKC isoforms may even enhance VEGF action and angiogenesis (37). AngII appears to activate multiple PKC isoforms with PKCα isoform being the most effective in phosphorylating Thr86 of p85α/PI3K. Since AngII has been reported to inhibit insulin activation of p-AKT in myocardium, it is likely that one of the mechanisms by which AngII is inhibiting insulin’s action selectively to cause endothelial dysfunction is by activating PKC, predominantly α isoform and phosphorylating Thr86/p85α/PI3K.

In summary, this report has characterized several potential sites by which PKC activation can inhibit insulin’s actions via p-IRS/PI3K/Akt pathway in endothelial cells. One such novel specific site was identified to be Thr86 on p85/PI3K which interfered with its association with IRS1 to decrease insulin and VEGF’s action on Akt and eNOS. Since elevation of fatty acid levels and enhancement of AngII actions can activate PKC, we suggest that increased phosphorylation on p85/PI3K by PKC activation could be partially responsible for endothelial dysfunction observed in these pathological conditions.
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REFERENCES


**FIGURE LEGENDS**

**FIGURE 1.1.** Effect of PKC activation on insulin signaling cascade.
Effect of PMA on insulin induced p-Akt and p-ERK
BAEC were pretreated with PMA and stimulated with insulin with and without PKC inhibitor GFX (5µm). The density of the bands were detected by enhanced chemiluminescence and quantitated densitometrically. (n=4, *: p<0.01)

**FIGURE 1.2.** Effect on insulin-stimulated p-Tyr of IRβ
BAEC were treated with PMA and/or insulin. The samples were immunoprecipitated (IP) with anti-insulin receptor beta antibody and immunoblotted by anti-phosphotyrosine antibodies (IB). Bar graph shows the ratios of phospho-tyrosine to IRβ signal. (n=3) (N.S.= not significant)

**FIGURE 1.3.** Immunoblot effect of insulin induced p-Akt on Ser473 and Thr308 in BAEC overexpressing IRS1

**FIGURE 1.4.** Densitometrical quantitation of Akt phosphorylation on Ser473 in BAEC overexpressing IRS1
Each bar shows the ratio of phospho-serine 473 to total Akt (n=4, *: p<0.01, #: p<0.05).

**FIGURE 1.5.** Effect of insulin and PMA on p-eNOS at Ser1179 in BAEC overexpressing IRS1
(n=4, *: p<0.01)
IB = immunoblot analysis

**FIGURE 2.** Comparative analysis of PKC activation on IRS1 and IRS2 activation

**FIGURE 2.1** Effect on insulin induced p-Tyr of IRS1 and IRS2
BAEC were treated with PMA and/or insulin. Samples were IP with anti-IRS1 or IRS2 antibody and IB with antibodies to IRS1, IRS2 or p-Tyr. Bar graph shows the ratios of phospho-tyrosine to IRS1 (n=4) or IRS2 signal (n=3) (N.S. = not significant).

**FIGURE 2.2.** Effect of IRS2 knockdown on insulin activation of p-Akt
BAEC transfected with IRS2, siRNA or eGFP were pretreated with and without PMA or insulin. Results show IB analysis by antibodies to Akt and p-Akt as indicated. (n=3, *: p<0.01, #: p<0.05)

**FIGURE 2.3.** Effect of insulin on endothelial cells from IRS2KO mouse
Lung endothelial cells from wild type mouse (WT) and IRS2KO mouse were pretreated with PMA, and stimulated with insulin (*p<0.01, #p<0.05). Immunoblotted (IB) with antibodies to IRS2, p-Ser473-Akt or total-Akt (t-Akt) (N=4, *p<0.01, #p<0.05).

**FIGURE 3.** Regulation of insulin-induced PI3 kinase activity and association with IRS1 by PKC activation

**FIGURE 3.1.** Effect of constitutively active p110/PI3K (myr-P110A) on p-Akt. BAEC, transfected with GFP or constitutively active p110/PI3K plasmid, were treated with insulin or PMA. P110α, p-Akt and t-Akt were assessed by immunoblot analysis in three separate experiments. (n=3)

**FIGURE 3.2.** Effect of insulin and PMA on PI3K activity associated with IRS1
Left: Representative thin layer chromatography with arrows point to 32P incorporation into phosphatidyl inositol. (PIP)
Right: Bar graph shows densitometrical quantitation of 32P phosphorylated phosphatidyl inositol. (n=3, *: p<0.001)

**FIGURE 3.3.** Effect of PKC activation on IRS1- associated p85α/β in IRS1-overexpressed cells
Top: Immunoprecipitation (IP) with anti-IRS1 antibodies followed by immunoblotting (IB) with indicated antibodies.
Bottom: Densitometry quantitation of p85α/β to IRS1 levels. (n=3, *: p<0.001, #: p<0.005)

**FIGURE 3.4.** Effect of PKC activation on the amount of p85- associated IRS1 in IRS1-overexpressed cells
Top: IP with anti-p85α/β antibodies, followed by IB with indicated antibodies are shown.
Bottom: Densitometry quantitation of IRS1 to p85α/β signal ratio are shown (n=3, *: p=0.001, #: p<0.001)

**FIGURE 4.** Far-Western (FW) analysis of IRS1 association with recombinant p85α-GST, endogenous p85α/β
Upper panels - Representative blots of immunoprecipitation (IP) with indicated probe or antibodies.
Lower panels - Bar graph of densitometry quantitations of p85α-GST signals.
C = control, I = Insulin, PMA = phorbol myristate acetate, PI = Insulin(+) PMA(+). N.S. = not significant, p-Tyr = antibody to phosphotyrosine

**FIGURE 5.** MS2 spectra corresponding to p85α phosphorylation at Threonine 86.
Panel A - Shows the MS2 spectra of a tryptic peptide (identified as ISPPT*PK) from gel-purified p85 isolated from BAEC. Spectra shown is from a 1+ precursor with 819.2 m/z. This precursor displayed a prominent neutral ion loss at 721.2m/z. (* indicates the phosphorylated residue, Thr86). The phosphopeptide sequence was assigned using Sequest.
Panel B - Shows the MS2 spectra of 2+ ion at 410.2 m/z, which co-elutes with 819.2. The MS2 of this 2+ precursor provided 1+ and 2+ ions associated with ISPPT*PK fragmentation. These spectra and sequence assignments were confirmed in 3 separate experiments.

Panels C and D shows MS2 spectra of synthetic peptide ISPPT*PK as a 1+ and 2+ precursor, respectively.

FIGURE 6. Effect of PKC activation on phosphorylation Thr86/p85α levels in BAEC

Cells were treated with PMA or GFX (PKC inhibitor). Total cell lysates were separated on SDS-PAGE and immunoblotted with antibodies as directed at p-thr86/p85α. Densitometry of immunoblot analysis from three different experiments. (n=3)

FIGURE 7. Effect of p85α deletion mutant on insulin’s and PMA’s actions

FIGURE 7.1. Schema of wild type p85α and p85 deletion mutant p85 pro-1 deletion mutant: human p85α with deletion of 13 amino acids (84-96)

FIGURE 7.2. Effect of p85 deletion mutant (p85 pro-1) on association to IRS1 with and without insulin and PMA.

Top: Immunoprecipitation (IP) with anti-IRS1 antibodies followed by immunoblotting (IB) with indicated antibodies are shown.

Bottom: Densitometry analysis of p85α/β to IRS1 ratio in bar graph (n=3).

N.S. = not significant

FIGURE 7.3. BAEC transfected with p85siRNA and/or p85 pro-1 deletion mutant and pretreated with PMA, and stimulated with insulin. Total cell lysates were separated on SDS-PAGE and IB with antibodies as indicated (n=3). N.S. = not significant

FIGURE 8. Inhibitory effect of Angiotensin II on insulin signaling via increased p-Thr86/p85α in BAECs.

Panel A - BAECs were treated with or without PMA and followed by stimulation of VEGF after transfected with p85siRNA and/or p85 pro-1 deletion mutant. (B-C) BAEC cells were treated with Angiotensin II at 100nM for 2hr or PMA for 20min and followed by 100nM of Insulin stimulation.

Panels B and C - The same amount of total proteins (60 µg) was separately applied for Immunoblot analysis using the antibodies specific for p-Thr85/p85α (B) and pAkt (C). The p-Thr85/p85α level was quantified by densitometry and normalized by p85α level, and expressed as % of untreated (mean ± SD, n = 3). **P < 0.01.

Panel D - Under same conditions as (B) and (C). The cells were then harvested and fractionated. The membrane and cytosolic protein were applied for Immunoblot analysis using the specific antibody against PKCα. The membranes were stripped and re-blotted with antibodies for Pancadherin and β-actin.

Panel E - Different PKC isoforms were overexpressed in BAECs by using adenovirus or GFP adenovirus as a control. The cells were treated with 100 nM of PMA and lysed. Total cell lysates were blotted with indicated antibodies. Densitometry of immunoblot analysis from four different experiments (n=4, * p<0.05).
Table 1  Effect of insulin and PMA on various phosphorylation sites on IRS1

<table>
<thead>
<tr>
<th>Residue</th>
<th>treatment with insulin</th>
<th>treatment with PMA</th>
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</thead>
<tbody>
<tr>
<td>Ser 307</td>
<td>No change</td>
<td>No change</td>
</tr>
<tr>
<td>Ser 612</td>
<td>No change</td>
<td>1.9 ± 0.3 fold increase *</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(p&lt;0.05)</td>
</tr>
<tr>
<td>Tyr 608</td>
<td>2.6 ± 0.3 fold increase (p&lt;0.05)</td>
<td>No effect on insulin-stimulated increase</td>
</tr>
<tr>
<td>Tyr 891</td>
<td>No change</td>
<td>No change</td>
</tr>
</tbody>
</table>

* Reversal of phosphorylation with PD98059 didn’t affect PMA’s inhibitory effect assessed by PI3Kinase activity and Akt phosphorylation
Figure 1.1

phosphoS473-Akt

total-Akt

phospho-ERK

total-ERK

pAkt / tAkt (A.U.)

pERK / tERK (A.U.)

Insulin
PMA
GFX

- - + + - + - + +

* * N.S.

* *
Figure 1.2

IP; IRβ  IB; pY
IB; total IRβ

pY/IRβ (A.U.)

Insulin  -  +  -  +
PMA      -  -  +  +

N.S.
Figure 1.3

<table>
<thead>
<tr>
<th>Protein</th>
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<th>(+)</th>
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<tr>
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<tr>
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<tr>
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<td>pT308-Akt</td>
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<tr>
<td>total-Akt</td>
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</table>

<table>
<thead>
<tr>
<th>Treatment</th>
<th>(-)</th>
<th>(+)</th>
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<tbody>
<tr>
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<td>+</td>
</tr>
<tr>
<td>PMA</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
Figure 1.4

[Graph showing the effect of insulin and PMA on IRS1 overexpression. The x-axis represents Insulin (-) and (+) and PMA (-) and (+), while the y-axis represents pS473 Akt / total Akt (A.U.).]

IRS1 overexpression (-)
IRS1 overexpression (+)

* indicates a significant difference from the negative control group.
# indicates a significant difference from the positive control group.
Figure 1.5

IRS1 overexpression

pS1179-eNOS

total-eNOS

Insulin
- + - + - + - +
PMA
- - + + - - + +
Figure 2.1

**IRS1 overexpressed cells**

- IP: IRS1  IB: pY
- IP: IRS1  IB: IRS1

**IRS2 overexpressed cells**

- IP: IRS2  IB: pY
- IP: IRS2  IB: IRS1

Insulin

- -  +  -  +

PMA

- -  -  +  +

**pY/IRS1 (A.U.)**

<table>
<thead>
<tr>
<th>Insulin</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
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</table>

**p<0.01**

**pY/IRS2 (A.U.)**

<table>
<thead>
<tr>
<th>Insulin</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
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<tbody>
<tr>
<td>PMA</td>
<td>-</td>
<td>-</td>
<td>+</td>
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**N.S.**
Figure 2.2

<table>
<thead>
<tr>
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<th>eGFP control</th>
<th>IRS2 siRNA</th>
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<tbody>
<tr>
<td>phosphoS473-Akt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>t-Akt</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The figure shows the expression levels of phosphoS473-Akt and t-Akt under different conditions: Insulin and PMA.

- **Insulin**: - - + + - - + +
- **PMA**: - - + + - - + +

The bars represent the activity (A.U.) with * indicating a statistically significant difference compared to the control and # indicating an alternative statistical comparison.

The graph includes error bars to indicate the variability in the data.
Figure 2.3

<table>
<thead>
<tr>
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<th>IRS2 KO</th>
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<tbody>
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<td><img src="image2.png" alt="Image" /></td>
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<tr>
<td>t-Akt</td>
<td></td>
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**Graph:**

<table>
<thead>
<tr>
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<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
<th>-</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Legend:**
- **WT** (Wild Type)
- **IRS2 KO** (IRS2 Knockout)

**Graph Details:**
- **p-Akt/t-Akt (A.U.):**
  - Insulin:
    - WT: 
      - Control: 0 (white bar)
      - + Insulin: 8 (white bar with error bars)
    - IRS2 KO: 
      - Control: 0 (black bar)
      - + Insulin: 8 (black bar with error bars)
  - PMA:
    - Control: 0 (white bar)
    - + PMA: 4 (white bar with error bars)

**Significance:**
- *: Significant difference
- #: Significant difference compared to control
Figure 3.1

Insulin and PMA treatments were used to study the phosphorylation of P110α, Akt, and P110α. The figure shows the fold change in expression levels compared to the control. The graph indicates a significant increase in expression levels with Insulin and PMA treatment, with a p-value of <0.05.

- GFP
- myr-P110 A

P110α
p-Akt
t-Akt

Insulin: - + + - + + - - - - - -
PMA: - - + - - + - - + + + +
Figure 3.2

[Image of gel electrophoresis with bands for PIP, Insulin, and PMA treatments.]

Arbitrary ratio of densitometry

Insulin  -  +  -  +  +
PMA       -  -  +  +  +

![Graph showing bar chart with asterisks indicating statistical significance.]

* * *
Figure 3.3

[Image of Western blot analysis with bands labeled IP; IRS1 IB; p85α/β and IP; IRS1 IB; IRS1. Below the images, a bar graph shows the levels of p85α/β/IRS1 in A.U. for different treatments: - Insulin, + PMA, with statistical symbols (*) and (#) indicating significance.]
Figure 3.4

IP; p85α/β  IB; IRS1

IP; p85α/β  IB; p85α/β

Insulin
-  +  -  +
PMA
-  -  +  +

IRS1 / p85α/β (A.U.)

#  #  *
Figure 4

IRS1 overexpressed

IP; IRS1, FW; \textsuperscript{recombinant} p85\textsubscript{α}-GST

IP; IRS1, IB; p85\textsubscript{α}/β

IP; IRS1, IB; pY

IP; IRS1, IB; IRS1

Insulin

- + - + - + - +

PMA

- - + + - - + +

Recombinant p85\textsubscript{α}-GST signal (A.U.)

Control Insulin PMA PMA+Insulin
Figure 6

Phospho-P85(T86) and P85 expression levels in the presence of PMA and GFX. The activity is quantified by fold of control (A.U.) and is statistically significant with P<0.05 in both conditions. The bar graph shows the fold of control with error bars indicating standard deviation.
Figure 7.1

human p85α

Pro1

deleted aa 84-96: PPTPKPRPPRPLP
Figure 7.2

**GFP**

IP: IRS1, IB: P85a

IP: IRS1, IB: IRS1

Cell lysis, IB: P85a

---

**P85 pro1**

P85a/IRS1 (A.U.)

- N.S.
- p<0.05

Insulin
- - + + - + +

PMA
- - + - - - +
Figure 7.3

<table>
<thead>
<tr>
<th></th>
<th>control</th>
<th>P85 siRNA</th>
<th>P85 siRNA + P85 mutant</th>
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<td>phosphoS473-Akt</td>
<td><img src="phosphoS473-Akt.png" alt="Image" /></td>
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p<0.05 N.S. 

p<0.01 

p<0.01 

p<0.01 

p<0.05 

N.S.

Insulin: - + - + - + - + - + - + - +

PMA: - - + + - - + + - - + + +
**Figure 8**

### A

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<tr>
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<td>phoso Akt (S473)</td>
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<td>P85α/β</td>
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### B

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<tr>
<td>PMA (100nM)</td>
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### C

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<tr>
<td>Insulin (100nM)</td>
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<tr>
<td>Insulin (100nM)</td>
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<tr>
<td>Pan-Cadherin</td>
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<td>PKCa</td>
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### E

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<tr>
<td>p85</td>
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</table>

*; P<0.05 and **; P<0.01 (mean ± SD, n = 3)
Inhibition of insulin signaling in endothelial cells by protein kinase C induced phosphorylation of P85 subunit of PI3-kinase
Yasuhiro Maeno, Qian Li, Kyoungmin Park, Christian Rask-Madsen, Benbo Gao, Motonobu Matsumoto, Yingjie Liu, I-Hsien Wu, Morris F. White, Edward P. Feener and George L. King

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