Coordinated phosphorylation of insulin receptor substrate-1 by glycogen synthase kinase-3 and protein kinase CßII in the diabetic fat tissue

Ziva Liberman,1 Batya Plotkin,1 Tamar Tennenbaum,2 and Hagit Eldar-Finkelman1

1Department of Human Molecular Genetics and Biochemistry, Sackler School of Medicine, Tel Aviv University, Tel Aviv; and 2Faculty of Life Science, Bar Ilan University, Ramat-Gan, Israel

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Liberman Z, Plotkin B, Tennenbaum T, Eldar-Finkelman H. Coordinated phosphorylation of insulin receptor substrate-1 by glycogen synthase kinase-3 and protein kinase CßII in the diabetic fat tissue. Am J Physiol Endocrinol Metab 294: E1169–E1177, 2008.—Serine/threonine phosphorylation of insulin receptor substrate-1 (IRS-1) is an important negative modulator of insulin signaling. Previously, we showed that glycogen synthase kinase-3 (GSK-3) phosphorylates IRS-1 at Ser332. However, the fact that GSK-3 requires prephosphorylation of its substrates suggested that Ser336 on IRS-1 was the “priming” site phosphorylated by an as yet unknown protein kinase. Here, we sought to identify this “priming kinase” and to examine the phosphorylation of IRS-1 at Ser336 and Ser332 in physiologically relevant animal models. Of several stimulators, only the PKC activator phorbol ester PMA enhanced IRS-1 phosphorylation at Ser336. Treatment with selective PKC inhibitors prevented this PMA effect and suggested that a conventional PKC was the priming kinase. Overexpression of PKCα or PKCßII isoforms in cells enhanced IRS-1 phosphorylation at Ser336 and Ser332, and in vitro kinase assays verified that these two kinases directly phosphorylated IRS-1 at Ser336. The expression level and activation state of PKCßII, but not PKCα, were remarkably elevated in the fat tissues of diabetic ob/ob mice and in high-fat-diet-fed mice compared with that from lean animals. Elevated levels of PKCßII were also associated with enhanced phosphorylation of IRS-1 at Ser336 and elevated activity of GSK-3ß. Finally, adenoviral mediated expression of PKCßII in adipocytes enhanced phosphorylation of IRS-1 at Ser336. Taken together, our results suggest that IRS-1 is sequentilly phosphorylated by PKCßII and GSK-3ß at Ser336 and Ser332. Furthermore, these data provide evidence for the physiological relevance of these phosphorylation events in the pathogenesis of insulin resistance in fat tissue.

insulin signaling; insulin resistance; obesity

INSULIN RESISTANCE is the principal predictor for the development of type 2 diabetes and is associated with many complications, including obesity, polycystic ovary syndrome, hypertension, and cardiovascular disease (2, 34). Insulin resistance is a pathological state in which target tissues, particularly muscle fat and liver, fail to respond properly to normal concentrations of insulin (53, 61, 71). The immediate targets of the insulin receptor, the insulin receptor substrate proteins, in particular insulin receptor substrates 1 and 2 (IRS-1 and IRS-2), are key elements in mediating insulin signaling. The inappropriate regulation of these targets is believed to play a central role in insulin resistance. The IRS proteins become tyrosyl-phosphorylated by the insulin receptor tyrosine kinase, which in turn, initiates the recruitment of SH2 domain-containing molecules, such as the p85 regulatory subunit of PI 3-kinase, Grb-2, SHP2, Nck, and Crk (47, 59, 75). The tyrosine-phosphorylated IRSs then activate downstream effectors that mediate the metabolic and growth-stimulatory effects of insulin (47, 59, 75).

Recent studies indicate that phosphorylation of IRS-1 on serine/threonine residues produces inhibitory effects on insulin signaling (19, 25, 69). Some studies have correlated increased in vivo serine phosphorylation of IRS-1 with insulin resistance in muscle and liver (10, 11, 56, 58, 74). The protein kinases responsible for phosphorylation of IRS-1 include NH2-terminal c-Jun kinase (JNK) protein kinase C (PKC), mammalian target of rapamycin (mTOR), MAP kinase, inhibitor of κB kinase (IKKß), and 5′-AMP-activated protein kinase (AMPK) (1, 15, 23, 37, 42, 46, 57, 74). It is suggested that serine phosphorylation may sterically inhibit the interactions between IRS-1 and the NPEY motif of the insulin receptor, making IRS-1 a poorer substrate for the insulin receptor (16, 46); alternatively phosphorylation could initiate IRS-1 degradation (52).

Previously, we (19, 41) identified the serine/threonine kinase glycogen synthase kinase-3 (GSK-3) as an IRS-1 kinase and mapped its phosphorylation site on IRS-1 to the highly conserved serine residue Ser332. Phosphorylation at Ser332 produced inhibitory effects on IRS-1-mediated signaling (41). It is noteworthy that GSK-3 has recently been targeted in the drug discovery program for insulin resistance and type 2 diabetes. Indeed, treatment with selective GSK-3 inhibitors produced antidiabetic effects reflected by improved glucose homeostasis and glucose tolerance in rodent models (13, 24, 43, 49, 54). Enhanced GSK-3 phosphorylation of IRS-1 (at Ser332) may represent a molecular basis for the contribution of GSK-3 to insulin resistance and type 2 diabetes; nevertheless, this mechanism is not fully understood. The requirement of GSK-3 for prephosphorylation of its substrates at the +4 position (21, 55) implicated IRS-1-serine336 as the “priming” site. This requirement for ordered phosphorylation means that the GSK-3 inhibitory effect is dependent on the function of this unknown priming kinase. In addition, the physiological relevance of IRS-1 phosphorylation to the diabetic condition has not been determined. The present study addressed these two issues. We show that PKCßII primes IRS-1 for GSK-3 phosphorylation. We further provide evidence for a physiological role of serine-phosphorylated IRS-1, along with elevated levels of GSK-3 activity and PKCßII, in insulin-resistant fat tissue.
MATERIALS AND METHODS

Materials. Purified PKC proteins, PKC inhibitors GF-109203X and GO-6976, and MEK inhibitor U-0126 were purchased from Calbiochem (San Diego, CA). LY-333531 was synthesized by custom synthesis. Antibodies against the N-terminus of IRS-1, PKCo, PKCBII, PKCBI, anti-phospho-tyrosine antibody, PY-99, were from Santa Cruz Biotechnology (Santa Cruz, CA). GSK-3 (Ser#17) antibody was from Upstate Biotechnology (Lake Placid, NY). Polyclonal antibodies directed against phosphorylated IRS-1 (at either Ser332 or Ser336) were generated by Bethyl Laboratories (Montgomery, TX). Synthetic IRS-1 peptide, based on the IRS-1 sequence RREGGMS332RPAS336VDG, was synthesized by Genemed Synthesis (San Francisco, CA) and described previously (41). Radioactive materials were purchased from Amersham Pharmacia Biotech (Pittsburg, PA). All other materials were from Sigma (Rehovot, Israel).

Animals. The background strain of all animals used was C57Bl/6j mice. Lepob/ob (referred to here as ob/ob) were purchased from Harlan (Israel). High-fat diet-induced diabetic (HF) mice were generated in the laboratory as described previously (65, 68). In brief, 4-wk-old mice received either standard laboratory food or a high-fat diet containing 35% lard (Bioserve, Frenchtown, NJ), in which 55% of the calories came from fat. The animals were housed in individual cages with free access to water in a temperature-controlled facility with a 12:12-h light-dark cycle. Animals were used after 16 wk of high-fat diet feeding. Animals developed obesity, hyperglycemia, and insulin resistance (68). Animal care followed the protocol specified by the Institutional Animal Care and Use Committee.

Cell transfections. PTB-2 constructs that code residues 1–350 of IRS-1 were described previously (41). PKC constructs coding for rabbit PKCo and PKCBII, cloned in SRD plasmids were kindly provided by Nathan Daskal (Sackler School of Medicine, Tel Aviv University). HEK 293 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) and supplemented with 10% fetal calf serum. The cells were transfected transiently with IRS-1 or PTB-2 constructs (5 μg each), using the calcium phosphate method as described (18). Expression of IRS-1 was verified by Western blot analysis using IRS-1–specific antibody. Cells were treated with phorbol 12-myristate 13-acetate (PMA; 100 nM, 30 min), indicated PKC inhibitor (5 μM for 4 h), lithium (20 mM), or U-0126 (10 μM), as described in the text. CHO/IR/IRS-1 cells were transiently transfected with PKCBII plasmid using jetPEI transfection reagent (Polyplus-Transfection, J1/Kirch Cedex, France), following the manufacturer’s protocol. The cells were treated with insulin (20 nM) and lysates prepared as described below.

Cell extraction and Western blot analysis. Cells were lysed in ice-cold buffer G (20 mM Tris, pH 7.5, 10 mM β-glycerophosphate, 10% glycerol, 1 mM EDTA, 1 mM NaF, 5 mM sodium pyrophosphate, 0.5 mM orthovanadate, 1 mM benzamidine, 5 μg/ml leupeptin, 25 μg/ml aprotinin, 500 nM microcystine LR, and 1% Triton X-100). Cell extracts were centrifuged for 20 min at 15,000 g. Supernatants were collected and equal amounts of proteins (30 μg) were boiled with SDS sample buffer and subjected to gel electrophoresis (7.5–12% polyacrylamide gels). After transfer to nitrocellulose, specific antibodies were used to detect relevant proteins as described in text.

Phosphorylation of IRS-1 peptide by PKC and PKA. Purified PKC isoforms were incubated with 200 μM IRS-1 synthetic peptide in a reaction mixture of 20 mM Tris·HCl (pH 7.3), 10 mM MgCl2, 100 μM γ-[32P]ATP, DAG (3.7 ng/ml), and 40 μM phosphatidylserine (PS) at 30°C for 20 min. CaCl2 (1 mM) was added to PKCo and PKCB mixtures. The reactions were spotted on p81 paper (Whatman, Kent, UK), washed with phosphoric acid, and counted for radioactivity. PKC activity was confirmed using myelin basic protein (MBP) as a substrate.

PKC activity in CHO/IR/IRS-1 cells. Cells expressing PKCBII were treated with insulin (20 nM) for 15 min. cell lysates were prepared as described above. The resulting supernatants were passed through DE-52 (Whatman, Maidstone, UK) minicolumns that were equilibrated with column buffer (20 mM Tris, pH 7.5, 5 mM EGTA, 5 mM EDTA, 10% glycerol). PKCBII was eluted with column buffer including 0.25M NaCl. The partially purified PKC activity was determined by in vitro kinase assays as described above, except that MBP was used as a substrate, and some assays included the PKCβ inhibitor LY-333531 (100 nM).

Tissue extraction. Epididymal fat pads were homogenized with ice-cold buffer H (25 μg/ml each of 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophosphate, 50 mM NaF, 5% glycerol, 1% Triton X-100, leupeptin, aprotinin, and pepstatin A). Homogenates were centrifuged at 10,000 g, and supernatants were collected. Western blot analyses with indicated antibodies were performed. IRS-1 was immunoprecipitated from the supernatants of fat extracts with specific α-IRS-1 antibody. The immunoprecipitates were subjected to gel electrophoresis followed by immunoblot analysis with indicated antibodies. In another set of experiments, fat homogenates were centrifuged at 1,000 g, and supernatants were collected and subjected to another centrifugation at 100,000 g for 1 h at 4°C. The supernatants were collected as soluble fractions and referred to as the cytosol fraction. The pellets were dissolved in with ice-cold buffer H in a volume equal to that of soluble fractions; these are termed the membrane fraction. In additional experiments, the epididymal fat pads were incubated with PMA for indicated time points and with insulin (10 nM) for 15 min. Tissue homogenates were prepared as described, and IRS-1 was immunoprecipitated from tissue extracts as described.

Overexpression of PKCBII in fat tissue. Epididymal fat pads from C57Bl/6d mice were excised into small pieces and incubated with recombinant adenovirus coding for PKCBII or with a “control” virus encoding β-galactosidase (generated by Dr. Tamar Tennenbaum, Bar-Ilan University, Israel) together with lipofectamine (10 μg/ml; Invitrogen Life Technologies, Carlsbad, CA) in low glucose (5 mM) DMEM supplemented with 1% BSA, as previously described (67). Tissues were processed as described above.

Statistics and densitometry analyses. Gel band quantitation was performed by densitometry analysis, and statistics were calculated using Origin 6.0 Professional software (OriginLab, Northampton, MA) and Student’s t-test. For all analyses, P < 0.05 was considered statistically significant. Results are expressed as means ± SE.

RESULTS

IRS-1 Ser336 phosphorylation is primed by a classical PKC. The phosphorylation of IRS-1 at Ser332 by GSK-3 is dependent on its prior phosphorylation at Ser336 as is typically required in GSK-3 substrates (41). The experiment presented in Fig. 1A is in agreement with this. The IRS-1 mutant, S336A, in which phosphorylation at Ser336.

The amino acid sequence upstream of Ser336 (S332RPAS336) contains the motif RXXS, typical for recognition by the AGC family of protein kinases such as cAMP-dependent protein kinase (PKA), PKC, or S6 ribosomal protein kinases (44, 62). Thus, we sought to examine the effect of these kinases on the phosphorylation of IRS-1 at Ser336. We generated a polyclonal

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antibody, αpIRS-1Ser336, that recognizes only the Ser336-phosphorylated form of IRS-1. The specificity of this new antibody was validated (data not shown). Next, HEK 293 cells were transiently transfected with the PTB-2 construct that codes residues 1–350 of IRS-1 (41). We chose to use this short construct initially, as it allowed us to focus the study on the specific region phosphorylated by GSK-3. Cells were treated with PMA (a potent PKC activator), forskolin (a potent PKA activator), or a “general” stimulator serum (20% FCS). Phosphorylation of PTB-2 at Ser336 was evaluated by Western blot analysis using the αpIRS-1Ser336 antibody. Results indicated that, of these stimulators, only PMA enhanced Ser336 phosphorylation of the PTB-2 construct initially, as it allowed us to focus the study on the specific region phosphorylated by GSK-3. Cells were treated with PMA (a potent PKC activator), forskolin (a potent PKA activator), or a “general” stimulator serum (20% FCS). Phosphorylation of PTB-2 at Ser336 was evaluated by Western blot analysis using the αpIRS-1Ser336 antibody. Results indicated that, of these stimulators, only PMA enhanced Ser336 phosphorylation (Fig. 1B). Hence, it appeared that a PKC family member was likely the priming kinase(s).

PKC represents a large family of isoforms that are categorized into classical, novel, and atypical PKCs (45, 48). The amino acids surrounding Ser336 fulfill the substrate specificity requirement for PKC isoforms that include a basic residue (arginine) at position −3 and a hydrophobic residue (valine) at position +1 (36, 50). As atypical PKCs are insensitive to PMA, these isoforms are likely not the priming kinase. To further narrow the list of candidate PKC isoforms, the cells were treated with a broad-spectrum PKC inhibitor, GF-109203X, or with a selective inhibitor of the classical PKC isoforms (α, β, and γ), GO-6976, prior to stimulation by PMA. Treatment with either PKC inhibitor abolished PMA-induced phosphorylation at Ser336 (Fig. 1C). Inhibition of PKC by these inhibitors was verified by reduced phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS) protein (data not shown). Treatment with PMA enhanced GSK-3 phosphorylation of IRS-1 at Ser332 and the PKC inhibitors reduced this phosphorylation (Fig. 1C). This further suggested that PMA can prime IRS-1 for GSK-3 phosphorylation.

When cells were treated with a selective GSK-3 inhibitor, lithium (38), PMA-induced phosphorylation of Ser332 was abolished (Fig. 1D). This indicated that PMA-induced phosphorylation of Ser332 is mediated by GSK-3. We then examined whether other targets activated by PMA, such as the MAP kinase pathway, are involved. Cells were treated with the MEK inhibitor U-0126 prior to the stimulation with PMA. This treatment did not affect PMA-enhanced phosphorylation of Ser336 or Ser332 (Fig. 1D). Finally, we confirmed our results using “full length” IRS-1. Cells that expressed WT-IRS-1 were treated with PMA for 30 min and 24 h. A 30-min PMA treatment enhanced IRS-1 phosphorylation at Ser336 and Ser332 (Fig. 1E), whereas downregulation of PKC by the longer PMA treatment (24 h) reduced phosphorylation below basal levels (Fig. 1E). All together, the results suggest that a classical PKC isoform phosphorylate IRS-1 at Ser336 and facilitates GSK-3-phosphorylation at Ser332.

PKCα and PKCβII phosphorylate cellular IRS-1 at Ser336. Our results suggested that a classical PKC (i.e., PKCα and/or PKCβ) is likely involved in the phosphorylation of IRS-1 at Ser336. Thus, we decided to focus our studies on PKCα and...
PKCβ. First, we examined whether PKCα or PKCβ can directly phosphorylate IRS-1 at serine336. An IRS-1 peptide (RREGGMSRPAS336VDG) (41) was used as the substrate for in vitro kinase assays with purified PKC isoforms. Both PKCα and PKCβII phosphorylated the IRS-1 peptide, but PKCβII was a "more" effective kinase (Fig. 2A). Interestingly, other PKC isoforms, such as PKCδ, PKCe, and PKCγ, weakly phosphorylated the IRS-1 peptide (not shown). This further indicated that conventional PKCs can phosphorylate IRS-1 at Ser336.

Next, HEK 293 cells were transiently cotransfected with IRS-1 and PKCα or PKCβII constructs. The cells were treated with PMA or left unstimulated, and the phosphorylation of IRS-1 at Ser336 and Ser332 was determined. Overexpression of PKCα or PKCβII enhanced IRS-1-phosphorylation of Ser336 and Ser332 under both basal and PMA-stimulated conditions (Fig. 2B). This indicated that cellular PKCa/β mediate the phosphorylation of IRS-1. To further explore the potential role of PKCβ, the cells were treated with a selective PKCβ inhibitor, LY-333531 (33). Treatment with LY-333531 reduced PMA-induced phosphorylation of Ser336 to nearly basal levels (Fig. 2C). This suggested that PKCβ is the most important priming kinase of IRS-1 in cellular conditions.

Finally, we examined how a physiological activator of PKC, insulin, (6), affects IRS-1 phosphorylation. Insulin treatment of CHO cells overexpressing both the insulin receptor and IRS-1 indicated that insulin enhanced IRS-1 Ser336 phosphorylation after 2 min and persisted at longer time points (15 and 30 min; Fig. 2D). There was a slight increase in Ser332 phosphorylation after 2 min of insulin treatment, and phosphorylation substantially increased after 15 min. At longer time points (15–30 min), we could detect coordinated elevation in the phosphorylation of Ser336 and Ser332. To further examine whether PKC was activated by insulin, we examined phosphorylation levels of PKCα at Ser657, a phosphorylation associated with enhanced
PKCα activity (8) (Fig. 2D). We could not detect PKCβ in these cells. However, ectopically expressed PKCβII was activated by insulin (Fig. 2D). All together, insulin can activate both PKCα and PKCβ in these cells.

Elevated activity of PKCβII and GSK-3 in diabetic fat tissue. The results presented thus far indicate that PKCα and/or PKCβ phosphorylate IRS-1 at Ser336. However, the relevance of this phosphorylation to in vivo regulation in a diabetic system was unknown. Therefore, we focused on diabetic fat tissue in which GSK-3 is known to be hyperactive (20). Two model systems were used: obese, insulin resistant, ob/ob mice (76) and nutritionally-induced diabetic mice [HF mice (65, 68)]. Elevation in GSK-3β activity in the fat tissue from ob/ob and HF mice is demonstrated in Fig. 3A. Phosphorylation of GSK-3β at a key inhibitory site, Ser3 (66), is reduced, whereas phosphorylation at Tyr216, a site important for GSK-3 activation (26), is increased. There was no change in total GSK-3 protein levels between control and diabetic samples (Fig. 3A).

Remarkably, expression levels of PKCβII, but not PKCβI or PKCα, were elevated in the ob/ob and HF fat tissues (Fig. 3B). Phosphorylation of PKCα at Ser657 (8) was somewhat lower in the diabetic samples (Fig. 3B), indicating that PKCα activity was not elevated in this tissue. To further verify that PKCβII was active, we examined its localization in cell membranes (40). Indeed, levels of PKCβII associated with the fat cell membranes were specifically elevated in the ob/ob and HF samples (Fig. 3C). There was no elevation in levels of other PKC isoforms, including θ, ε, δ, and ζ, in the diabetic fat tissue (data not shown).

PKC phosphorylates IRS-1 at Ser336 in fat tissue. The results presented in Fig. 3 prompted us to focus our study on the potential role of PKCβII in the phosphorylation of IRS-1 in fat tissue. Epidydimal fat tissues isolated from C57Bl/6J mice were treated with PMA for various times, and the extent of IRS-1 phosphorylation was determined. Treatment with PMA led to rapid activation of Ser336 phosphorylation of IRS-1 within 5 min of exposure; this phosphorylation was consistently elevated during the 30-min treatment (Fig. 4A) and associated with enhanced phosphorylation at Ser332 (not shown). We next examined how this phosphorylation would affect insulin signaling. The tissue was treated with PMA (30 min) prior to stimulation with insulin (15 min). IRS-1 protein was immuno-precipitated from cell lysates, and tyrosine phosphorylation levels were determined (Fig. 4B). Indeed, pretreatment with PMA reduced insulin-induced tyrosine phosphorylation of IRS-1, supporting our notion that phosphorylation of Ser336/332 inhibits insulin signaling. We next sought to examine the role of PKCβII in the phosphorylation of IRS-1 in this tissue. The isolated fat tissues were exposed to recombinant adenovirus encoding PKCβII or to a control adenoviral vector encoding β-galactosidase. Levels of expression of PKCβII and phos-
phorylation of IRS-1 were determined in the infected tissues. As shown in Fig. 4C, IRS-1 phosphorylation at Ser\(^{336}\) was significantly enhanced in PKC\(\beta\)-II-infected adipocytes. This suggested that IRS-1 phosphorylation at Ser\(^{336}\) is modulated by PKC\(\beta\)-II in fat tissue.

IRS-1 phosphorylation at Ser\(^{32}\) and Ser\(^{336}\) is elevated in diabetic fat tissue. Our studies have indicated so far that PKC\(\beta\)II is elevated in diabetic fat tissue and is capable of phosphorylating IRS-1 specifically at Ser\(^{336}\). We next evaluated IRS-1 serine phosphorylation in the diabetic fat tissue. IRS-1 was immunoprecipitated from fat tissue extracts prepared from control, ob/ob, and HF animals. The immunoprecipitates were subjected to immunoblot analysis using the anti-phospho-IRS-1 antibody. A specific increase in IRS-1 phosphorylation at Ser\(^{336}\) was clearly detected in ob/ob and HF samples (Fig. 5A). Total amounts of IRS-1 were lower in the diabetic fat tissues than in the control tissue (Fig. 5A), indicating that, in fact, a larger pool of IRS-1 molecules were phosphorylated at Ser\(^{332/336}\) in these tissues. Results indicated 2- and 1.6-fold increases in phosphorylations at Ser\(^{332}\) and Ser\(^{336}\), respectively, in the diabetic fat tissues relative to control (Fig. 5B). Interestingly, the extent of IRS-1 phosphorylation was closely correlated with the degree of obesity developed in the models (Fig. 5C).

Fig. 4. Modulation of IRS-1 serine phosphorylation by PKC in fat tissue. A: epididymal fat tissues were incubated with PMA (100 nM) for indicated times as described in MATERIALS AND METHODS. Tissue extracts were prepared and subjected to gel electrophoresis followed by immunoblot analysis with anti-phospho-IRS-1Ser\(^{336}\). Densitometry analysis of IRS-1-phosphorylated bands is shown in bottom. Results are means of data from 4 independent experiments ±SE. *P < 0.05. B: same as A, except that tissue was treated with PMA for 30 min prior to stimulation with insulin (10 nM) for an additional 15 min. IRS-1 was immunoprecipitated from tissue extracts, and samples were subjected to immunoblot analysis using anti-phospho-tyrosine and anti-IRS-1 antibody. C: epididymal fat tissues were infected with adenovirus coding for PKC\(\beta\)II or \(\beta\)-galactosidase (\(\beta\)-gal), as described in MATERIALS AND METHODS. Tissue extracts were prepared and subjected to gel electrophoresis followed by immunoblot analysis with anti-phospho-IRS-1Ser\(^{336}\), anti-PKC\(\beta\)II, and anti\(\beta\)-actin antibodies. Densitometry analysis of phosphorylated IRS-1 is shown at right, and results are means ±SE of 4 independent experiments, *P < 0.05. Representative gels from 2 of 4 experiments are shown.

Fig. 5. IRS-1 Ser\(^{332/336}\) phosphorylation is elevated in fat tissue of diabetic animals. A: IRS-1 proteins were immunoprecipitated from fat tissue extracts from ob/ob, HF, and control animals. Immunoprecipitates were subjected to Western blot analysis using anti-phospho-IRS-1Ser\(^{332}\), anti-phospho-IRS-1Ser\(^{336}\), and anti-IRS-1 antibodies. Total IRS-1 is shown at bottom. Gels representative of samples 2 animals from groups of 9–12 animals are shown. B: ratio of phosphorylated to total IRS-1 as calculated from densitometry analysis. Ratio obtained from control was designated 1, and results are presented as fold of control. Results are means of samples from 9–12 animals ±SE, ***P < 0.01. C: blood glucose levels, plasma insulin, body weight, and fat pad mass of ob/ob, HF, and control animals are listed. Values are means ±SE for samples from 9–12 animals, *P < 0.05.
**DISCUSSION**

Serine/threonine phosphorylation of IRS-1 is a negative feedback regulator in insulin signaling. Evidence that the kinase GSK-3 phosphorylated of IRS-1 at Ser\(^{332}\) increased our understanding the factors that contribute to insulin resistance (41). However, GSK-3 phosphorylation at Ser\(^{332}\) requires prior phosphorylation of IRS-1 at Ser\(^{336}\), the nature of the priming kinase was unknown. We realized that the priming kinase likely belonged to the AGC-serine/threonine protein kinase family, and by using a novel anti-phospho-IRS-1 (Ser\(^{336}\)) antibody, we identified PMA-sensitive PKC isoforms as potential candidates for the priming kinase. The use of inhibitors with specificity for certain PKC isoforms and PKC-overexpressing cell systems implicated classical PKCs \(\alpha\) or \(\beta\). The expression level and kinase activity of PKC\(\beta II\), but not PKC\(\alpha\), was significantly upregulated in the fat tissue of two diabetic animal models, ob/ob and HF mice. This was also associated with elevated levels of IRS-1 phosphorylation at Ser\(^{336}\) and Ser\(^{332}\) in samples from the diabetic animals compared with normal tissue. Using adenoviral-mediated gene delivery, we confirmed that PKC\(\beta II\) can phosphorylate IRS-1 at Ser\(^{336}\) in the fat tissue. Our results suggested that PKC\(\beta II\) is likely the in vivo priming kinase responsible for IRS-1 phosphorylation at Ser\(^{336}\) at least under diabetic conditions in the fat tissue.

We further noticed that levels of PKC\(\beta II\), GSK-3 activity, and IRS-1 phosphorylation, were consistently higher in the fat tissue from ob/ob animals compared with HF animals, implying a link between these parameters and the severity of obesity and/or insulin resistance (Fig. 4C). Phosphorylation of IRS-1 at Ser\(^{336}\) and Ser\(^{332}\) was previously shown to constrain insulin action (41); consistently, prior treatment with PMA reduced insulin-induced tyrosine phosphorylation of IRS-1 (Fig. 4B). We thus suggest that PKC\(\beta II\)/GSK-3-mediated phosphorylation of IRS-1 represents a mechanism that contributes to insulin resistance in fat tissue. This sequential phosphorylation can represent another important level of regulation of insulin signaling. Namely, as long as Ser\(^{336}\) is not phosphorylated by PKC, GSK-3 cannot promote the phosphorylation of Ser\(^{332}\). On the other hand, phosphorylation of both sites is expected to produce a stronger and effective impact on insulin signaling. Hence, the activity of both kinases is an important and essential factor in contributing to insulin resistance.

The PKC family, and in particular DAG-PKC, were previously shown to have regulatory roles in insulin signaling, and their expression levels and/or activities were associated with insulin resistance in type 2 diabetes in patients and animal models (14, 27, 29, 64). PKC isoforms may be differentially regulated by high concentrations of glucose or free fatty acids in various cell types such as muscle and adipocytes (4, 35, 64). It is also apparent that the contribution of PKC isoforms to insulin signaling and/or insulin resistance is tissue specific. For example, PKC\(\beta\) and PKC\(\delta\) are implicated in skeletal muscle insulin resistance (3, 22, 31, 60), PKC\(\alpha\) and -\(\epsilon\) are elevated in diabetic liver (14), and PKC\(\zeta\) has an essential role in insulin-induced glucose transport in adipocytes (5, 6). Our studies highlight the specific role of PKC\(\beta II\) in diabetic fat tissue, although we do not exclude the possibility that additional PKC isoforms may be involved.

The roles for GSK-3 and PKC\(\beta II\) as negative modulators in insulin signaling were previously described, and both protein kinases are promising drug targets for treatment of insulin resistance and type 2 diabetes (17, 39). PKC\(\beta\) has been strongly implicated in the pathogenesis of diabetic vascular complications and has been shown to be a key regulator in hyperglycemia-induced retina, renal, and cardiovascular dysfunction (28, 39, 72). Elevated levels of PKC\(\beta II\) were detected in vascular smooth muscle and skeletal muscle of obese diabetic patients (32, 39). Use of the selective PKC\(\beta\) inhibitor LY-333531 restores insulin-stimulated glucose transport in rodent models (30, 51, 70). However, the roles of PKC\(\beta II\) and GSK-3 in insulin-resistant fat tissue are less clear. Evidence from knockout studies indicated that PKC\(\beta\) plays important role in metabolic regulation of fat tissue and energy expenditure (5, 7, 9, 63). Loss of PKC\(\beta\) improved insulin sensitivity, reduced fat mass, and protected against weight gain (7, 63). Elevation of GSK-3 activity was detected in diabetic fat tissues of rodents and humans and is correlated with adipogenesis and weight gain (12, 20). Here, we suggest new roles for PKC\(\beta II\) and GSK-3 in a concerted regulation of insulin action in fat tissue. Specifically, our view is that, under hyperglycemic/hyperlipidemic conditions, PKC\(\beta II\) and GSK-3 activities are elevated in fat tissue and ensure continuous phosphorylation of IRS-1 at Ser\(^{336}\) and Ser\(^{332}\). This, in turn, impairs insulin action in the fat tissue.

In summary, our work highlights the cooperative activities of PKC\(\beta II\) and GSK-3 in phosphorylation of IRS-1 and the role of this phosphorylation in mediating insulin resistance. We suggest that combined inhibition of PKC\(\beta II\) and GSK-3 may represent a new strategy for treatment of insulin resistance and type 2 diabetes.

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**REFERENCES**


