Abstract

Intracellular insulin signaling involves a series of alternative and complementary pathways created by the multiple substrates of the insulin receptor (IRS) and the various isoforms of SH2 domain signaling molecules that can interact with these substrates. In this study, we have evaluated the roles of IRS-1 and IRS-2 in signaling to the phosphatidylinositol (PI) 3-kinase pathway in the ob/ob mouse, a model of the insulin resistance of obesity and non–insulin-dependent diabetes mellitus. We find that the levels of expression of both IRS-1 and IRS-2 are decreased ∼50% in muscle, whereas in liver the decrease is significantly greater for IRS-2 (72%) than for IRS-1 (29%). This results in differential decreases in IRS-1 and IRS-2 phosphorylation, docking of the p85α regulatory subunit of PI 3-kinase, and activation of this enzyme in these two insulin target tissues. In ob/ob liver there is also a change in expression of the alternatively spliced isoforms of the regulatory subunits for PI 3-kinase that was detected at the protein and mRNA level. This resulted in a 45% decrease in the p85α form of PI 3-kinase, a ninefold increase in the AS53/p55α, and a twofold increase in p50α isoforms. Thus, there are multiple alterations in the early steps of insulin signaling in the ob/ob mouse, with differential regulation of IRS-1 and IRS-2, various PI 3-kinase regulatory isoforms, and a lack of compensation for the decrease in insulin signaling by any of the known alternative pathways at these levels. (J. Clin. Invest. 1997. 100:3164–3172.) Key words: diabetes • insulin receptor kinase • insulin resistance • phosphatidylinositol 3-kinase • insulin receptor substrate

Introduction

Insulin resistance and obesity are classical features of type II, non–insulin-dependent diabetes mellitus (NIDDM). These characteristics are present in most humans with the disease, as well as in the most commonly used animal models, such as the ob/ob mouse, the db/db mouse, and the Zucker fatty rat (1–4). Previous studies examining the early steps in the insulin action cascade have shown that insulin binding is decreased in both the liver and muscle of ob/ob mice due to a decrease in the insulin receptor number (1, 5). This results in a decrease in autophosphorylation of the receptor β subunit, as well as a decrease in insulin receptor substrate-1 (IRS-1) phosphorylation and IRS-1–associated phosphatidylinositol (PI) 3-kinase activity (5, 6).

Over the past few years, there has been an increase in our understanding of the molecular mechanisms of insulin action with increasing evidence of a complex signaling network using alternative or complementary pathways and multiple molecular isoforms of key signaling molecules (7–9). For example, the recent cloning of IRS-2 (4PS) has demonstrated a second subunit of the receptor that shares many features present in IRS-1 (10). In mice made IRS-1–deficient by homologous recombinant gene targeting, IRS-2 serves as an alternate substrate and allows for significant residual insulin and IGF-1 signaling (11–13).

IRS-1 and IRS-2 link the insulin receptor to its final biological actions via a series of intermediate effectors which bind to the tyrosine-phosphorylated motifs in IRS proteins via SH2 domains. One of the best studied SH2 domain proteins is the enzyme PI 3-kinase. This enzyme is stimulated by a number of growth factors, and, in the case of insulin, has been shown to play a critical role in many of the metabolic effects of the hormone, including stimulation of glucose transport, activation of glycogen synthase, and inhibition of the key enzyme of gluconeogenesis, phosphoenol pyruvate carboxykinase (PEPCK) (7, 14). PI 3-kinase is composed of a regulatory subunit that binds to IRS-1/IRS-2 via SH2 domains and a 110-kD catalytic subunit that phosphorylates PI and its 4′ and 4′,5′ phosphorylated derivatives in the D-3 position of the inositol ring (15–23). Initially, only two subunits of 85 kD were characterized as regulatory subunits of PI 3-kinase. These were referred to as p85α and p85β and are the products of different genes (20–22). However, recent studies from our laboratory (24) and others (25–27) have revealed several additional isoforms of p85α. These include 53- and 50-kD isoforms termed AS53 (also termed p55α) and p50α, respectively, which are the result of alternative splicing of the p85α gene. Furthermore, a novel 55-kD protein (p550β), the product of a different gene, has been characterized as a regulatory subunit of PI 3-kinase (28). These isoforms have a distinct tissue expression and alterations in their primary sequence that have been suggested to play a role in specificity of signaling or, in some cases, differential localization within the cell (24–28).

In this study, we have characterized the role of these alternative signaling pathways in the insulin resistance of the obese hyperglycemic (ob/ob) mouse. We find that not only are there quantitative differences in the regulation of the IRS proteins,
but also dramatic changes in the isoforms of PI 3-kinase expressed in the liver of the ob/ob mouse, which contribute to multiple levels of alteration in insulin signaling in this insulin-resistant state.

Methods

Materials. Reagents for SDS-PAGE and immunoblotting were from Bio-Rad (Richmond, CA). Heps, PMSF, aprotinin, leupeptin, benzamidine hydrochloride, DTT, ATP, Triton X-100, Tween 20, glycerol, and BSA (fraction V) were from Sigma Chemical Co. (St. Louis, MO). NP-40 was from Calbiochem (La Jolla, CA). plates from Merck (Gibbstown, NJ), protein A–Sepharose 6MB from Pharmacia (Uppsala, Sweden), [32]P-protein A from ICN Biomedicals (Costa Mesa, CA), and [γ-32P]ATP and [α-32P]dCTP from NEN-DuPont (Wilmington, DE). Di-nitroctyllose paper (BA 85, 0.2 μm) and NYT-RAN nylon membrane were from Schleicher & Schuell (Keene, NH). Sodium pentobarbital was from Abbott Laboratories (South Pasadena, CA) and human recombinant insulin (Humulin R) was from Eli Lilly & Co. (Indianapolis, IN). Monoclonal antiphosphotyrosine (pY) antibody (4G10), anti-PI 3-kinase (anti-p85), and antibodies to the COOH-terminal sequence (TYASINFQKQPEDRQ) corresponding to residues 1221–1235 of the rat liver IRS-1 protein were obtained as previously described (29). Antibodies to recombinant IRS-2 and murine p85 cDNA were a generous gift of Morris White (Joslin Diabetes Center, Boston, MA). Ultraspec RNA isolation system was purchased from Biotecx (Houston, TX). Poly(A)+ mRNA isolation kit was from Ambion (Austin, TX). An unidirectional mouse brain dT-primed cDNA library from the Uni-ZAP XR vector, Pfu-polymerase, and the PCR-script cloning vector were from Stratagene (La Jolla, CA). dNTPs were from Pharmacia. The PCR II cloning system was from Invitrogen (Carlsbad, CA). A random labeling kit was from Amershon (Arlington Heights, IL).

Animals. Male obese hyperglycemic mice (C57Bl/6J ob/ob) and their lean matched controls (ob+/+) were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice (8–10-wk old) were fed standard rodent chow and water ad libitum. Food was withdrawn 10–12 h before experiments. Mice were anesthetized with 100 mg/kg of sodium pentobarbital injected intraperitoneally. Following loss of pedal and corneal reflexes, 5 U (0.2 mg) of regular human insulin or its diluent was injected into the portal vein. The liver, gastrocnemius, and quadriceps muscles were excised 1 and 3 min after insulin (or its diluent) injection, respectively, and frozen in liquid nitrogen.

Immunoprecipitation and Western blotting. The frozen tissue was homogenized in ice-cold lysis buffer ([1% Triton X-100, 10% glycerc, 1% NP-40, 50 mM Hepes, pH 7.4, 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 5 mM sodium vanadate, aprotinin (10 μg/ml) leupeptin (5 μg/ml), benzamidine (1.5 mg/ml) and PMSF (34 μg/ml)] using a Polytron PTA 20 S generator operated at maximum speed for 30 s. The insoluble material was removed by centrifugation at 55,000 rpm in a 70 Ti rotor (Beckman Instruments, Inc., Fullerton, CA) for 60 min, and equal protein amounts of the supernatant were subjected to immunoprecipitation for 2 h using the indicated antibodies. Subsequently, protein A–Sepharose was added for another hour. The samples were processed for SDS-PAGE electrophoresis and Western blotting as previously described (6). Under the conditions used, the α-IRS-1 antibody precipitated 65% of the IRS-1 in muscle and 59% of the IRS-1 in liver. Based on other studies, this is associated with no detectable IRS-2. The α-IRS-2 is less effective in precipitation (30–37%) depending on cell type, and does coprecipitate a small amount (< 7%) of IRS-1.

In vivo phosphorylation of PI. Immune complexes were incubated in a 65-μl reaction containing 440 μM ATP (30 μCi [32P]ATP and 5 μg of PI). After 10 min at room temperature with constant shaking, the reaction was stopped by the addition of 20 μl 8 N HCl and 160 μl CHCl3/methanol (1:1). The samples were centrifuged, and the lower organic phase was removed and applied to a silica gel TLC plate (Merck, St. Louis, MO) coated with 1% potassium oxalate. TLC plates were developed in CHCl3/CH3OH/H2O/NH4OH (60:47:13:3), dried, and visualized by autoradiography. The γ32P-labeled 3-phosphatidylinositolides were quantitated using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Cloning of cDNA probes. A murine p85α-specific subclone spanning 423 nucleotides (nt) downstream of the start codon was generated by PCR using 100 ng of mouse p85α cDNA as a template, a 5'-primer corresponding to nt 556–577 (5'-agtg caa gag gcc tac cag-3') and a 3'-primer corresponding to nt 958–979 (5'-ccct aat gtc ccc gac-3) of the p85α cDNA sequence (20). In addition, a 572-nt subclone corresponding to the COOH-terminal SH2 domain of p85α was produced by PCR. The 5'-primer (5'-aga aag ctc gtt gaa gca-3') was identical to nt 1979–2015 and the 3'-primer (5'-gac gca aag ctg gct ttc-3') corrected to nt 2552–2569 of p85α (20). Both PCR products were cloned into the PCR II vector. The complete coding sequence of murine AS53 was cloned by PCR using 1 μg of DNA from a λ mouse brain cDNA library as a template with 5'-primer identical to nt 1–20 of the rat AS53/p55α sequence (5'-agtc acc aag gct tgg ac-3) and a 3'-primer (5'-tct ctc cct ctc tgc tgt atc-3) corresponding to nt 1348–1365 of the rat AS53/p55α (25). The resulting 1365-nt PCR product was cloned in PCR II. Sequencing of the mouse AS53-cDNA revealed > 91% identity to the nucleotide sequence of rat AS53/p55α (25). An AS53-specific subclone was generated by PCR using the same template and 5'-primer, and 3'-primer corresponding to nt 89–106 to rat AS53/p55α (5'-ctg ggt gat cca ttc tta-3'). The 106-nt PCR product was cloned blunt-ended into PCRscript. A subclone specific for mouse p50α was produced by PCR using 1 μg of murine genomic DNA as a template and 5'-primer identical to nt −182 to −164 of the untranslated murine p50α sequence (5'-ccg gat gag ctc cgc ggc-3) and a 3'-primer corresponding to nt 1–22 of the p50α-coding sequence (5'-cag tat ctc gat tgg t-3) (27). The resulting 204-nt PCR product was cloned into PCRscript. A p55IRK-specific subclone (28) was generated from the λ mouse brain cDNA library by PCR using 5'-gac agc gag ggg gat ccc cag att cct ccc ggg c-3 (nt 1361–1383) and 5'-cct ccc cag cta tgg gta ctt c-3 (nt 1170–1370) as 5'- and 3'-primers, respectively. The 396-nt PCR product was cloned into PCR II. Likewise, part of murine p85β (28) was cloned by PCR using primers 5'-gtt gtt gtt gtt gtt cct gct gtt gtt gtt ccc ggg cgc ccc g-3 (corresponding to nt 866–887 and 1640–1661, respectively). The 795-nt fragment was cloned into PCRscript. A subclone for murine pit100a was generated by PCR using the same template and primers 5'-tgc gtc cta cta gta gtt gtt gtt gtt gtt ccc g-3 (corresponding to nt 2701–2720) and 5'-tac cct ctc gct ggt gtt gtt gtt gtt ggg g-3 (nt 3191–3207) (30). The 506-nt PCR product was cloned into PCRscript. The identity of PCR products was confirmed by sequencing.

RNA extraction and Northern blotting. The Ultraspec RNA isolation system was used to extract total RNA from frozen tissue samples and mRNA was purified using the poly(A)+ mRNA isolation kit. 40 μg of total RNA or 2 μg of poly(A)+ RNA was separated in a denaturing 1% agarose gel and subjected to Northern blotting using γ32P-labeled cDNA probes generated by random priming as previously described (29). Blots were exposed overnight with intensifier screens to Kodak XAR film.

Results

Protein expression and phosphorylation of IRS-1 and IRS-2. IRS-1 and IRS-2 are the major substrates of the insulin receptor kinase in muscle and liver and serve as the primary docking proteins for PI 3-kinase in the insulin signaling network. To determine the level of these relatively nonabundant proteins, homogenates of liver and muscle of the ob/ob mice and their lean littermates were subjected to immunoprecipitation using anti-IRS-1 and anti-IRS-2 antibodies followed by Western immunoblotting with the same antibodies. In both tissues the
of both proteins was decreased in ob/ob mice. In the liver of ob/ob mice, IRS-1 protein expression was decreased by only 29%, whereas IRS-2 was decreased by 72% (P < 0.0001) (Fig. 1 A). In ob/ob muscle, expression of IRS-1 and IRS-2 was decreased almost identically by 51% (P < 0.001 for both) (Fig. 1 B).

To assess tyrosine phosphorylation of these IRSs, liver and muscle homogenates were obtained from control and ob/ob animals before and after insulin stimulation. Lysates were subjected to immunoprecipitation with anti–IRS-1, anti–IRS-2 antibodies, and anti-pY antibodies by Western immunoblotting with anti-pY antibodies. Insulin stimulation resulted in a threefold increase in both IRS-1 and IRS-2 phosphorylation in liver of the control mice (Fig. 2, A and B). In the liver of ob/ob mice, insulin-stimulated IRS-1 phosphorylation was decreased by 53% (P = 0.001), whereas IRS-2 phosphorylation was decreased by 74% (P < 0.0001) relative to control animals (Fig. 2, A and B). Total IRS phosphorylation pattern as assessed by immunoprecipitation and immunoblotting by anti-pY antibodies and quantification of the bands in the 180–185-kD range showed a threefold increase in phosphorylation upon insulin stimulation in the control group and was decreased by 70% in livers of the ob/ob animals (Fig. 2 C). In muscle, insulin-stimulated phosphorylation of IRS-1 by sevenfold and IRS-2 by fourfold in the control lean (ob/+) mice (Fig. 2, D and E). Insulin-stimulated IRS-1 phosphorylation in muscle was decreased by 68% (P = 0.001) in ob/ob mice as compared with their lean controls (Fig. 2 D), while IRS-2 phosphorylation was decreased by 72% in muscle of these obese diabetic mice (P < 0.002) (Fig. 2 E). Phosphorylation of all high molecular weight IRSs (pp185) increased 14-fold upon insulin stimulation in ob/+ mice. Total substrate phosphorylation pattern upon insulin stimulation was decreased by 72% (P < 0.0001) in muscle of ob/ob animals compared with their lean littermates (Fig. 2 F).

**p85α association and PI 3-kinase activity.** To assess p85α association with IRS-1 and IRS-2, liver and muscle homogenates were subjected to immunoprecipitation with anti–IRS-1, anti–IRS-2, or anti-pY antibodies followed by Western immunoblotting with anti-p85α antibodies as described in Methods. As previously described (17, 18), after tyrosine phosphorylation, there is an increase in p85α docking that parallels the increase in phosphorylation. In liver of lean ob/+ mice, this amounted to a threefold stimulation of association of p85α with IRS-1 and a twofold increase in association for IRS-2 (Fig. 3, A and B). After insulin stimulation, p85α association to IRS-1 in liver of ob/ob mice was reduced by 58% (P = 0.0008) compared with control animals (Fig. 3 A). Similarly, association of p85α to IRS-2 was reduced by 64% (P < 0.0001) (Fig. 3 B). Western immunoblotting of anti-pY immunoprecipitates with anti-p85α antibodies revealed a reduction of 47% (P < 0.03) of p85α docking to all pY substrates in liver of ob/ob mice relative to ob/+ mice (Fig. 3 C). In the muscle of control mice, insulin stimulation resulted in a 5-fold increase of p85α association with IRS-1, a 2.5-fold increase in association with IRS-2, and a 15-fold increase in association in total anti-pY precipitates (Fig. 3, D–F). In ob/ob mice, a different pattern was observed. Insulin-stimulated association of p85α with IRS-1 was reduced by 53% (P < 0.0001) and association with total pY proteins was decreased by 56.5% (P = 0.005), whereas p85α association with IRS-2 was reduced by only 18.3% (P = 0.08).

PI 3-kinase activity showed changes concordant with p85α association with IRS-1 and IRS-2 in liver and muscle. Thus, in liver of ob/ob mice there was a 61% reduction in IRS-1–associated PI 3-kinase activity after insulin stimulation as compared with lean control animals (P < 0.002) (Fig. 4 A) and a 44% reduction in IRS-2–associated PI 3-kinase activity (P < 0.004) (Fig. 4 B). PI 3-kinase activity associated with anti-pY immunoprecipitates in ob/ob mice was also reduced by ~44% (P < 0.02) relative to lean control littermates (Fig. 4 C). In skeletal muscle, IRS-1–associated PI 3-kinase activity was markedly reduced by 63% (P < 0.0001) in the obese animals as compared with their control littermates (Fig. 4 D), whereas IRS-2–associated PI 3-kinase activity was decreased only slightly by 35% (Fig. 4 E), which was similar to the decrease in p85α docking, but was not statistically significant. However, total PI 3-kinase activity in anti-pY precipitates in muscle was reduced by 64% (P < 0.002), consistent with a greater role for IRS-1 than IRS-2 in PI 3-kinase in muscle (Fig. 4 F).

Expression of p85α and p85α isoforms. As noted in the Introduction, recent studies have indicated that, in addition to p85α, many tissues express other types of regulatory subunits of PI 3-kinase in the molecular mass range between 50 and 55 kD (24–28). These PI 3-kinase subunits are derived by alternative splicing of the p85α gene or are the products of separate genes and are expressed in muscle, liver, and other tissues at variable

![Figure 1](image-url)IRS-1 and IRS-2 protein expression in liver and muscle of ob/ob and ob/+ control mice. Tissue extraction was performed as described in Methods. Equal amounts of protein were subjected to immunoprecipitation with COOH-terminal IRS-1 and IRS-2 antibodies, separated by SDS-PAGE and Western immunoblotting using the same antibodies. The bar graphs show data quantification by ImageQuant (Molecular Dynamics) for results in liver (A) and muscle (B). Data are the mean ± SEM of 3 independent experiments using 12 animals for each group. They are expressed as relative to control values which were set at 100%.
levels. To determine whether the reduction in PI 3-kinase activity and p85α association with the IRS phosphoproteins in ob/ob mice was associated with a change in p85α or p85α isoform expression, homogenates of liver and muscle were immunoblotted with p85α antibodies either directly or after immunoprecipitation with a polyclonal antibody recognizing the common SH2 domain region of p85α, AS53/p55α, and p50α. In muscle homogenates, all three species were detected, with a slight predominance of the lower molecular mass species over p85α. However, there was no change in expression of p85α or its isoforms, when ob/ob mice were compared with their lean littermates (data not shown). By contrast, in liver of lean mice, only p85α and p50α could be detected, and p85α was the more prominent of these two by immunoblotting with a common antibody (Fig. 5). In liver of ob/ob mice, this pattern was changed. Thus, p85α expression was reduced by 45%, and there was a twofold increase in the level of p50α (P < 0.0001). Even more striking was the appearance of an additional band migrating between 53 and 55 kD. This band almost certainly corresponds to AS53 rather than p55PIK, since it was strongly positive with an antibody to the common SH2 domains of p85α, AS53, and p50α (Fig. 5). The level of AS53 in liver of ob/ob mice by immunoblotting was increased ninefold (P < 0.0001).

**Northern blotting.** Since specific antibodies to the alternatively spliced forms of p85α are not yet available, we turned to Northern blot analysis as a method to confirm which isoforms of PI 3-kinase regulatory subunit were overexpressed and whether the changes in protein expression were secondary to changes at the mRNA level. Using a probe spanning the COOH-terminal SH2-domain that is common to p85α, AS53, and p50α, transcripts of 7.4, 6.4, 4.4, 2.8, and 1.7 kb were labeled on Northern blots of liver (Fig. 6 A). Using a probe to the specific region of p85α containing the SH3 domain and bcr-region, the band at 7.4 kb was clearly labeled (Fig. 6 B), while an AS53-specific probe labeled the transcript at 2.8 kb (Fig. 6 C), and the p50α-specific probe labeled transcripts at 6.4, 2.8, and 1.7 kb (Fig. 6 D). When compared with lean controls, there was a 50% decrease in the level of the 7.4-kb transcript in liver of ob/ob mice corresponding to the decrease in p85α protein (Fig. 6 A). While an AS53-specific probe labeled the transcript at 2.8 kb (Fig. 6 C), and the p50α-specific probe labeled transcripts at 6.4, 2.8, and 1.7 kb (Fig. 6 D). When compared with lean controls, there was a 50% decrease in the level of the 7.4-kb transcript in liver of ob/ob mice corresponding to the decrease in p85α protein (Fig. 6 A). In contrast, the transcripts running at 6.4, 2.8, and 1.7 kb, corresponding to AS53 and p50α, were clearly increased in ob/ob liver RNA (Fig. 6 A, C, and D). Using the specific probes, the AS53 signal was upregulated fourfold in liver of ob/ob mice and the p50α mRNA species increased by threefold (Fig. 6 C and D). No significant changes were observed in the levels of mRNA for the p85β regulatory subunit and the p110α catalytic subunit (Fig. 6 F and G). The levels of P55PIK were very low in liver RNA and also did not change significantly (Fig. 6 E), but were clearly detectable in brain RNA at 5.8 kb (not shown).
Insulin action at the cellular level is a complex network characterized by both alternative pathways and isoforms of the key proteins involved. At the first intracellular level are the multiple substrates of the insulin receptor. IRS-1 through IRS-4, Shc, and Gab-1 are all phosphorylated by the insulin receptor kinase and, with the exception of Shc, each of these possess multiple phosphorylation sites allowing for the interaction with several SH2 domain–containing proteins simultaneously (7, 9, 10, 16, 31–33). These include the tyrosine phosphatase SHP2 (SHPTP2), the tyrosine kinase Fyn, the adaptor molecule GRB2, and the regulatory subunits of PI 3-kinase (7, 16, 34–37). At least seven potential forms of PI 3-kinase regulatory subunit are now known to exist. The first subunits described were p85α and p85β and are products of separate genes (20–22). Both of these contain two SH2 domain–containing proteins simultaneously (7, 9, 10, 16, 31–33). These include the tyrosine phosphatase SHP2 (SHPTP2), the tyrosine kinase Fyn, the adaptor molecule GRB2, and the regulatory subunits of PI 3-kinase (7, 16, 34–37). At least seven potential forms of PI 3-kinase regulatory subunit are now known to exist. The first subunits described were p85α and p85β and are products of separate genes (20–22). Both of these contain two SH2 domain–containing proteins separated by the p110 catalytic subunit binding region in the COOH-terminal half of the molecule, and a SH3 domain and bcr homology region in the NH2-terminal half. Two alternative splicing events of p85α result in removal of the NH2-terminal half and replacement with either 6 or 34 unique amino acids resulting in proteins termed p50α and AS53 (also termed p55α) (24–27). p85α, AS53, and possibly p50α can each also occur in two forms as a result of an alternative splice in the inter-SH2 domain region which replaces one amino acid with a nine-

amino acid insert in a domain near the autophosphorylation site of the regulatory subunit and near the site of p110 binding (24). Finally, there is another PI 3-kinase regulatory subunit, derived from a third and separate gene, which encodes a protein of 55 kD, termed p55PK. p55PK is ~70% homologous to the p85 family members, being most similar to AS53 (28). Although PI 3-kinase activation has been implicated as a key intermediate in many metabolic actions of insulin, including regulation of glucose transport and gene expression, the functional differences among these various regulatory subunits is unclear, but may include involvement in specific signaling pathways, as well as subcellular localization (7).

Alterations in the early steps of insulin signaling have been recognized as an important component of many insulin-resistant states. Decreased insulin binding, decreased receptor kinase activity, decreased IRS-1 protein, and decreased IRS-1–associated PI 3-kinase activity have all been demonstrated in ob/ob mice and other models of NIDDM (1, 5, 6, 38, 39). However, in view of the alternative substrates and pathways of insulin signaling, it is possible that there could be compensatory changes in either IRSs or various isoforms of PI 3-kinase which might minimize the impact of the alterations in IRS-1–linked signaling. The present data indicate that both IRS-1 and IRS-2 are downregulated in liver and muscle of ob/ob mice, although to a different extent. In liver, the decreases in IRS-2 protein levels and phosphorylation are greater than that for
IRS-1. This is accompanied by parallel decreases in association with IRS-1 and IRS-2 with p85α and corresponding PI 3-kinase activities. In muscle, the decreases in IRS-1 and IRS-2 protein and phosphorylation levels are similar, while the reduction in IRS-1–associated PI 3-kinase activity is greater than that of IRS-2. The reduction of total pY-associated PI 3-kinase activity in liver was similar to that of IRS-1– and IRS-2–associated PI 3-kinase activity, whereas in muscle, the decrease in total pY-associated PI 3-kinase activity more closely paralleled the reduction of IRS-1–associated PI 3-kinase activity, consistent with previous observations (13, 40) that in liver, both IRS-1 and IRS-2 act as major IRSs and provide linkage to PI 3-kinase. Furthermore, in liver of ob/ob mice, IRS-2 does not compensate for the reduced IRS-1 signaling, but also contributes to insulin resistance. In contrast, in ob/ob muscle, IRS-1 is the dominant IRS and its downregulation appears to be mainly responsible for insulin resistance. Again, this is not compensated for by IRS-2 signaling, which is relatively minor and also reduced to some extent. Although it was not possible to evaluate the contribution of other substrates, such as IRS-3 (p60) (32, 33) or IRS-4 (41) to PI 3-kinase activation, our data emphasize the importance of coordinate changes in IRS-1 and IRS-2 in insulin signaling pathways in liver and muscle.

In addition to differential regulation of the IRS proteins, this study also revealed unexpected alterations in the levels of regulatory subunits of PI 3-kinase in liver of the ob/ob mouse. Previous studies in normal animals have suggested that p85α is the major regulatory subunit of PI 3-kinase in liver, whereas AS53 is the dominant isomer in human skeletal muscle (24). In the present study, we find almost equal levels of p85α and p50α liver by Western blotting in liver of thin ob/+ mice. In the livers of the ob/ob mouse, the spliced isoforms of the p85α subunit of PI 3-kinase were preferentially overexpressed, and the expression of the normal p85α isoform was reduced. This was especially true for the AS53/p55α form which increased ninefold in obesity. Northern blot analysis demonstrated a downregulation of p85α mRNA and increases in AS53 and p50α mRNA that paralleled these changes at the protein level. In contrast, the levels of p85β, p55IR, and p110α were not significantly altered in ob/ob liver.

The upregulation of alternatively spliced isoforms of p85α in liver of the diabetic ob/ob mice raises the question whether alternative splicing of p85α is caused by the hyperglycemia, hyperinsulinemia, or some other component of the diabetes and obesity pathogenesis. In a genetically derived, nonobese NIDDM mouse (29), and in the streptozotocin diabetic rat, no alterations in the level of p85α or its isoforms are observed (Kerouz, N., D. Hörsch, and C.R. Kahn, unpublished data). Therefore, it is unlikely that upregulation of alternatively spliced isoforms in liver of ob/ob mice is caused simply by high glucose and high insulin levels. On the other hand, a similar pattern of upregulation of AS53 and p50α isoforms was ob-
served in the liver of other models of obesity, like the db/db mouse and Agouti mouse (our unpublished observations).

To what extent these changes in p85α and its isoforms contribute to changes in metabolic pathways in the liver is unknown, however PI 3-kinase activity is essential for differentiation and function of many cell types, including adipocytes (42), skeletal muscle cells (43), and hepatocytes (44). Whether these changes in PI 3-kinase isoform expression might also contribute to alterations in specific insulin pathways is not known. Preliminary studies in our laboratory have indicated that the AS53 regulatory subunit binds less well to IRS-1 than does p85α. On the other hand, it has been reported recently that p50α has a higher affinity for phosphorylated IRS-1 than the other p85α isoforms (26). However, in regard to overall insulin signaling to PI 3-kinase, it is obvious that the increased levels of AS53 and p50α isoforms in liver of ob/ob mice do not compensate for the reduction in p85α and the observed decrease in IRS-associated PI 3-kinase activity. Also, it has been reported that alternative splicing of the insulin receptor is increased in muscle tissue of NIDDM subjects (45–47). Thus, it is tempting to speculate that these changes in PI 3-kinase isoforms might contribute to alterations in metabolic pathways in the liver of these models of obesity.

Figure 5. Expression of p85α isoforms in liver of ob/ob and ob/+ control mice. Equal amounts of protein were subjected to immunoprecipitation with polyclonal p85α antibody, separated by SDS-PAGE, and Western immunoblotted with anti-p85α antibody. The bar graphs show data quantification by ImageQuant (Molecular Dynamics). The migration of bands of 85, 53, and 50 kD is indicated. Data are the mean±SEM of 12 independent experiments. They are expressed as relative to control, assigning a value of 100% to the control mean of p85α expression.

Figure 6. Differential expression of PI 3-kinase isoform mRNA in liver of ob/ob mice. RNA samples of ob/+ and ob/ob mice were subjected to Northern blotting using the indicated probes as described in Methods. A, B, E, F, and G show total RNA blots (40 μg loaded/lane). C and D depict poly(A)+ RNA blots (2 μg loaded/lane). Markers on the left denote transcript sizes.
to speculate that changes in the alternative splicing of various signaling proteins may contribute to a range of alterations observed in the pathogenesis of diabetes and obesity.

In summary, we have shown that insulin resistance of obesity is associated with differential regulation of IRS-1, IRS-2, and several isoforms of PI 3-kinase regulatory subunits. The nature of these changes in liver and muscle is distinct and complex. These differential changes may produce differential alterations in insulin signaling in these two tissues and may contribute to the hepatic and muscular insulin resistance observed in obesity and diabetes.

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References


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