Original Article

Increased p85/55/50 Expression and Decreased Phosphotidylinositol 3-Kinase Activity in Insulin-Resistant Human Skeletal Muscle

Gautam K. Bandyopadhyay, Joseph G. Yu, Jachelle Ofrecio, and Jerold M. Olefsky

Insulin resistance is predominantly characterized by decreased insulin-stimulated glucose uptake into skeletal muscle. In the current study, we have assessed various aspects of the phosphatidylinositol (PI) 3-kinase pathway in skeletal muscle biopsies obtained from normal, obese nondiabetic, and type 2 diabetic subjects, before and after a 5-h insulin infusion. We found a highly significant inverse correlation between in vivo insulin sensitivity (as measured by the glucose infusion rate) and increased protein expression of p85/55/50, protein kinase C (PKC)-θ activity, levels of pSer^{307} insulin receptor substrate (IRS)-1 and p-Jun NH_2-terminal kinase (JNK)-1, and myosin heavy chain IIx fibers. Increased basal phosphorylation of Ser^{307} IRS-1 in the obese and type 2 diabetic subjects corresponds with decrease in insulin-stimulated IRS-1 tyrosine phosphorylation, PI 3-kinase activity, and insulin-induced activation of Akt and, more prominently, PKC-ζ/λ. In summary, increased expression of the PI 3-kinase adaptor subunits p85/55/50, as well as increased activity of the proinflammatory kinases JNK-1, PKC-θ, and, to a lesser extent, inhibitor of κB kinase-β, are associated with increased basal Ser^{307} IRS-1 phosphorylation and decreased PI 3-kinase activity and may follow a common pathway to attenuate in vivo insulin sensitivity in insulin-resistant subjects. These findings demonstrate interacting mechanisms that can lead to impaired insulin-stimulated PI 3-kinase activity in skeletal muscle from obese and type 2 diabetic subjects. *Diabetes* 54:2351–2359, 2005

From the [1]Department of Medicine, Division of Endocrinology and Metabolism, University of California, La Jolla, California.

Address correspondence and reprint requests to Jerrold M. Olefsky, MD, University of California at San Diego, Department of Medicine (0673), 225 Stein Clinical Research Bldg., 9500 Gilman Dr., La Jolla, CA 92093. E-mail: jolefsky@ucsd.edu.

Received for publication 7 December 2004 and accepted in revised form 9 May 2005.

ERK, extracellular signal–related kinase; IKKβ, inhibitor of κB kinase-β; IRS, insulin receptor substrate; JNK, Jun NH_2-terminal kinase; MAP, mitogen-activated protein; MHC, myosin heavy chain; PI, phosphatidylinositol; PKC, protein kinase C.

Insulin resistance is a characteristic feature of type 2 diabetes, obesity, syndrome X, and a number of other human disorders (1–3). Insulin resistance refers to a decreased ability of insulin to exert its normal biological effects and a hallmark of this abnormality is decreased insulin-stimulated glucose disposal. Because ~80% of overall in vivo insulin-stimulated glucose disposal in humans is into skeletal muscle, decreased insulin-stimulated glucose transport into muscle is a central feature of human insulin resistance. Although the mechanisms of insulin-stimulated glucose transport are incompletely understood, it is uniformly agreed that normal activation of phosphatidylinositol (PI) 3-kinase plays a central role (4–7).

PI 3-kinase exists is a heterodimer composed of a p110 catalytic subunit and a p85 regulatory subunit. Insulin causes tyrosine phosphorylation of insulin receptor substrate (IRS) proteins, which then associate with PI 3-kinase through the p85 regulatory domain, leading to increased PI 3-kinase activity (1,8). This then leads to activation of Akt2 and protein kinase C (PKC)-ζ/λ, which ultimately can result in insulin-stimulated GLUT-4 translocation and increased glucose transport (9–14). Transcription of the p85 gene leads to the expression of the p85 α protein, as well as two splice variants, p55 and p50; all three of these can bind to p110 and to phosphotyrosine motifs (15–17). Under normal conditions, p85 and its splice variants are in excess compared with p110; therefore, free monomeric regulatory subunits are available to compete with the p110/adaptor subunit heterodimer for binding to target protein phosphotyrosine motifs (18). It could be postulated that increased levels of p85/55/50 subunits might inhibit PI 3-kinase activity by competing with phosphotyrosine targets. By doing this, the presence of these excess subunits could contribute to insulin resistance. Consistent with this idea, a p85 knockout mouse displays increased insulin sensitivity (19–21). In addition, overexpression of the p85 SH2 domains or deletion mutants of p85 that cannot bind to p110 leads to a decrease in PI 3-kinase activity and insulin signaling (22,23). In a model of insulin resistance caused by chronic exposure to placental growth hormone, increased protein expression of p85 was observed (24). Data from obese animal models

TABLE 1
Subject characteristics

	Nondiabetic	Obese	Type 2 diabetic
n	8	8	7
Age (years)	47.25 ± 4.8	43.5 ± 4.0	48.0 ± 5.2
Weight (kg)	75.2 ± 5.4	96.7 ± 4.0	110.0 ± 5.4
BMI (kg/m^2)	24.4 ± 1.2	33.3 ± 1.06*	37.1 ± 2.64*
Fasting plasma glucose (mmol/l)	5.09 ± 0.12	5.1 ± 0.1	11.3 ± 1.5*
Glucose infusion rate (mg · kg^{-1} · min^{-1})	9.9 ± 0.93	7.0 ± 0.37†	2.26 ± 0.76*
Basal insulin (pmol/l)	66.7 ± 10	125.36 ± 26‡	315 ± 78‡
Insulin after 300-min clamping (pmol/l)	1,070 ± 47	1,290 ± 96	1,090 ± 74
Basal plasma free fatty acids (μmol/l)	346 ± 36	400 ± 30§	750 ± 120‡
Free fatty acids after insulin clamping (μmol/l)	19 ± 3.8	16 ± 3.5	270 ± 100
Triacylglycerol (mg/dl)	88.75 ± 6.8	128.6 ± 21‖	390 ± 127†

Data are means ± SE. All *P* values were determined by comparing with nondiabetic subjects. *$P < 0.005$; †$P < 0.05$; ‡$P < 0.02$; §NS; ‖$P = 0.05$.

also suggest an association between obesity and increased combined expression of p85/p55/p50 (25).

The tyrosine phosphorylation of IRS-1/2 is a highly regulated process involving a balance between activation and deactivation (26). Phosphorylation of Ser^{307} and Ser^{1101} of IRS-1/2 can diminish its ability to undergo tyrosine phosphorylation, subsequently causing impaired PI 3-kinase activation (27,28). A number of serine/threonine kinases (most of which are components of the proinflammatory pathway) such as PKC-θ (28), Jun NH_2-terminal kinase (JNK) (29), and inhibitor of κB kinase-β (IKK-β) (30) have been linked to serine phosphorylate IRS-1, leading to inhibition of its activity (27–32). Similarly, the role of PKC-θ activity and localization has been implicated in the development of insulin resistance (31–33)

To quantitatively assess the role of IRS-1/2 and PI 3-kinase function in human insulin resistance, we have performed a number of biochemical analyses on muscle biopsies obtained from lean (nondiabetic), obese, and type 2 diabetic patients before and after insulin infusion. All of these subjects also underwent euglycemic-hyperinsulinemic clamp studies to provide a quantitative measurement of in vivo insulin sensitivity. Our results show that elevated levels of all three splice variants of the PI 3-kinase adaptor subunit (p85, p55, and p50) along with increased basal mitogen-activated protein (MAP) kinases and PKC-θ activities are features of insulin resistance in human skeletal muscle. The degree of increase in these activities is strongly correlated with the magnitude of the decrease in in vivo insulin sensitivity. These human tissues also show impaired IRS-1/2 function. Through increased serine phosphorylation and decreased tyrosine phosphorylation, this IRS-1/2 defect magnifies the decrease in insulin stimulated PI 3-kinase activity. As such, these results demonstrate the critical importance of decreased skeletal muscle PI 3-kinase activity in these insulin-resistant states and provide an underlying mechanism for this effect.

RESEARCH DESIGN AND METHODS

Muscle tissues from human subjects. Eight nondiabetic lean subjects, seven obese subjects with type 2 diabetes, and eight obese subjects without diabetes participated in the study. The clinical characteristics of the subjects are given in Table 1. Studies in subjects with type 2 diabetes were performed after discontinuation of their antihyperglycemic medications (five were taking sulfonylureas and two were taking metformin plus sulfonylurea) for at least 2 weeks. All participants were in good general health and had normal kidney and liver function. None of the control subjects were taking any medications to alter glucose tolerance. During the clamp study, a biopsy from the right leg was performed before the insulin infusion was started (Humulin; Eli Lilly, Indianapolis, IN) at a rate of 80 mU · m^{-2} · min^{-1}. After 300 min of infusion, a second biopsy was performed from the left vastus lateralis. Tissues were blotted free of blood and immediately frozen in liquid nitrogen. To reach a steady state of insulin-stimulated glucose metabolism in the insulin-resistant subjects, 4–5 h of insulin infusion were required. Consequently, the muscle biopsies were taken at time 0 and 5 h. However, previous results have indicated that post–receptor signaling defects are observed as early as 2 h, 3 h, and even 30 min after insulin infusion (34–36). In a previous study, we showed that defects in insulin-stimulated tyrosine phosphorylation were easily detected after 30 min of insulin stimulation and fully sustained throughout the 5-h time course (37).

The study protocol was approved by the Internal Review Board for Human Subjects of the University of California at San Diego. Written informed consent was obtained from each subject. The procedures for hyperinsulinemic-euglycemic clamps and analysis of clinical parameters were described previously in detail (38,39).

Reagents. All buffer components, protease and phosphatase inhibitors, and lipids were purchased from Sigma (St. Louis, MO). Phosphospecific antibodies were from Cell Signaling Technology (Beverly, MA). Rabbit anti-pan p85 (no. 06-496), anti-IRS-1, and p110 were from Upstate Biotechnology (Lake Placid, NY).

Tissue fractionation, immunoprecipitation, and immunoblotting. Muscle samples (50–100 mg) were powdered under liquid nitrogen and homogenized by Polytron (3 × 15 s) in 2 ml ice-cold buffer containing 20 mmol/l Tris/HCl, pH 7.5, 150 mmol/l NaCl, 0.25 mol/l sucrose, 1.2 mmol/l EGTA, 1 mmol/l EDTA, and a cocktail of protease and phosphatase inhibitors as described previously (36). After centrifugation at 1,000*g* for 10 min at 4°C, the supernatants were analyzed as homogenates and fractionated into total membrane and cytosol by centrifugation at 100,000*g* for 1 h at 4°C. For comparison of signal intensity between samples, equal amounts of protein from each sample were loaded per lane during gel electrophoresis. Immunoblotting of the membranes after SDS-PAGE, enhanced chemiluminescence detection, and quantitation of the signals by densitometry were carried out following standard protocols.

For myosin heavy chain (MHC) analysis by SDS-PAGE, muscle homogenates were prepared by following the protocol described by Klitgaard et al. (40) and electrophoresis was carried out at 100 V for 16 h using 6% separating gel and standard running buffer containing 35% glycerol. Proteins were visualized by silver staining with a kit from Pierce (Rockford, IL).

Measurement of PKC-ζ/λ and PKC-θ activities. To measure PKC activity, 0.5–1.0 mg proteins were immunoprecipitated with rabbit polyclonal anti–COOH-terminal PKC-ζ/λ or anti–PKC-θ antisera or preimmune serum in the presence of protein-A/G agarose (all from Santa Cruz Biotechnology, Santa Cruz, CA). The washed immunobeads were assayed for PKC-ζ/λ and PKC-θ activities following previously published methods (12,31,33,36).

PI 3-kinase assay. IRS-1–bound PI 3-kinase activity was assayed in the immunobeads after anti–IRS-1 immunoprecipitation. The agarose beads were washed following a procedure described by Caldwell et al. (41), and the PI 3-kinase activity was assayed as described by Kruszynska et al. (7) and Walsh et al. (42).

Statistical calculations. Results are presented as means ± SE. Significance of a difference between two groups was determined by ANOVA and paired two-tailed *t* tests. The linear regression and multivariate analyses (MINOVA

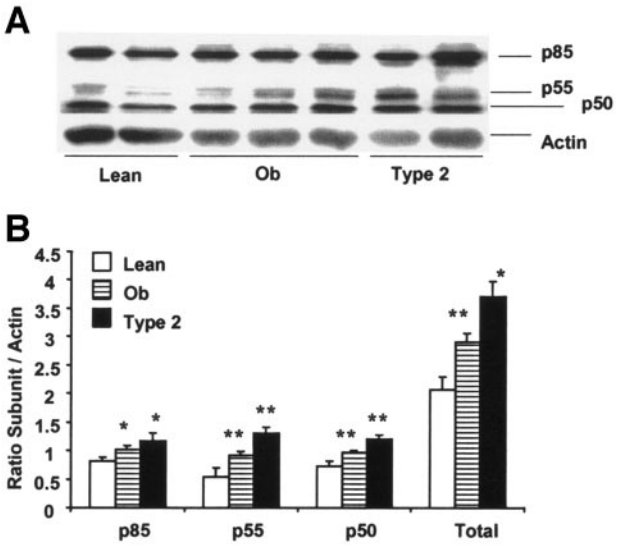

FIG. 1. PI 3-kinase regulatory subunits in human muscle. *A*: A representative Western blot analysis of the basal muscle homogenates with a pan anti-p85 antibody and antiactin antibody. *B*: The ratio subunit to actin in lean, obese (Ob), and type 2 diabetic subjects for individual subunits as well as for combined subunits. *$P < 0.01$, **$P < 0.001$ vs. lean.

and discrimination function tests) were carried out by Minitab statistical software. P values ≤ 0.05 were considered significant.

RESULTS

PI 3-kinase regulatory subunits. Western blotting with a pan-reactive anti-p85 antibody detected all three regulatory subunits (p85, p55, and p50) in the muscle lysates. When normalized against actin, the content of the three regulatory subunits together (p85/p55/p50) was significantly increased (~40–50%) in muscle lysates from obese subjects and even further increased (80–90%) in type 2 diabetic patients compared with lean control subjects (Fig. 1). In terms of individual subunits, the greatest increase was in the level of p55 ($P < 0.01$), followed by p50 ($P < 0.01$) and p85 ($P < 0.05$). The protein expression level of p85/p55/p50 was the same both in the basal state and after the 5-h insulin infusion. Because there was a small but significant ($P < 0.05$) decrease (10–20% compared with lean) in IRS-1 content in the insulin-resistant tissues (Fig. 2*A*), the ratio of regulatory subunits to IRS-1 is even higher than the ratio calculated with respect to actin (results not shown). A similar decrease in IRS-1 level was reported by Brozinick et al. (43) in obese muscle.

Phosphorylation of IRS-1 and PI 3-kinase activity. The phosphorylation of IRS-1 at Ser^{307} was measured in the muscle lysates by Western blotting with a phosphospecific anti-$pSer^{307}$ IRS-1 antibody. As seen in Fig. 2*B*, basal Ser^{307} phosphorylation is low in the lean subjects with a two- to threefold increase after 5 h of in vivo insulin stimulation. In the obese and type 2 diabetic subjects, there is a marked fourfold increase in basal Ser^{307} phosphorylation and insulin stimulation was without further effect. This increase in Ser^{307} phosphorylation would predict impaired insulin-stimulated IRS-1 tyrosine phosphorylation, which is precisely what is shown in Fig. 2*C*. Thus, in lean subjects insulin treatment leads to a fivefold increase in IRS-1 tyrosine phosphorylation; however, there is a marked decrease in the obese group and an even greater degree of impairment in the type 2 diabetic subjects.

The increased concentration of p85-derived regulatory

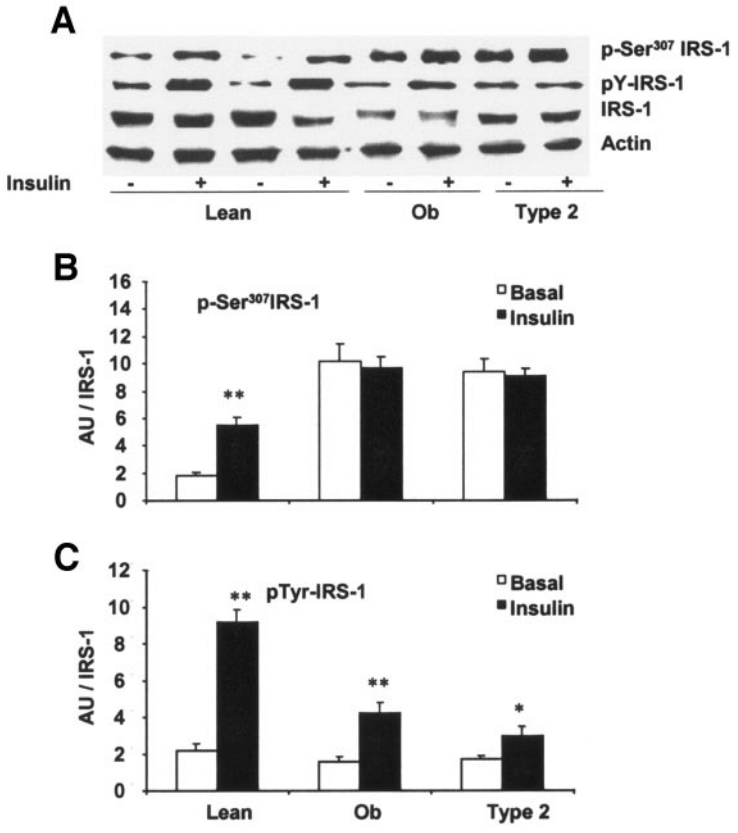

FIG. 2. Phosphorylation of IRS-1. *A*: A representative Western blot for $pSer^{307}$ IRS-1 and pY IRS-1. Areas representing insulin-stimulated levels of $pSer^{307}$ IRS-1/total IRS-1 (*B*) and pY-IRS-1/total IRS-1 (*C*) in lean, obese (Ob), and type 2 diabetic subjects are shown. *$P < 0.001$ vs. lean basal; **$P < 0.001$ vs. lean insulin stimulated.

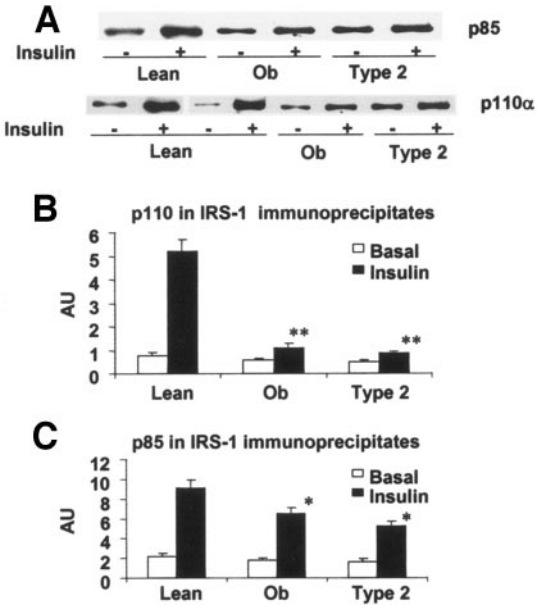

FIG. 3. Interaction of p85 and p110 with IRS-1. *A*: Western blot analysis of IRS-1 bound p85 and p110 in the IRS-1 immunoprecipitates is shown. The signal intensities showing insulin-stimulated binding of p110 to IRS-1 (*B*) and p85 to IRS-1 (*C*) are shown. *$P < 0.05$, **$P < 0.001$ vs. lean. Ob, obese.

subunits, coupled with the decrease in IRS-1 tyrosine phosphorylation, should lead to a substantial decrease in insulin-stimulated IRS-1–associated PI 3-kinase activity. As summarized in Fig. 3, we have measured the amount of p85 and p110 protein recovered in IRS-1 immunoprecipitates before and after insulin stimulation in the three study groups. A very large decrease in p110 association with IRS-1 is observed in the insulin-resistant subjects (Fig. 3*B*), fully consistent with the combination of increased regulatory subunit concentration and decreased IRS-1 tyrosine phosphorylation. Interestingly, as demonstrated in Fig. 3*C*, although a significant decrease in IRS-1–associated p85 protein was quite evident in the insulin-resistant groups, the magnitude of the decrease was much less than the decrease in p110 association. Most likely, this reflects the

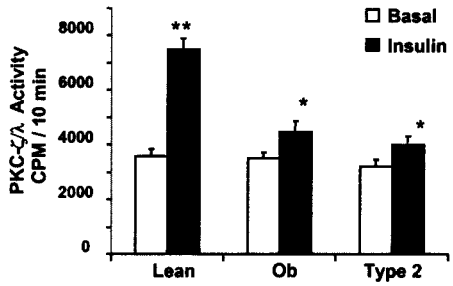

FIG. 5. Insulin-stimulated activation of PKC-ζ/λ. Insulin-stimulated PKC activity was measured in the immune agarose beads obtained after immunoprecipitation of PKC-ζ/λ from the lysates with anti–COOH-terminal PKC-ζ antiserum. *$P < 0.01$ vs. lean basal; **$P < 0.005$ vs. lean insulin stimulated. CPM, counts per minute; Ob, obese.

increased adaptor subunit concentration in the muscle lysates, which leads to competition between monomeric regulatory subunits (p85, p55, and p50) with the heterodimers (p110/p85, p110/p55, and p110/p50) for binding to tyrosine phosphorylated IRS-1. This leads to a greater decrease in formation of the trimeric complex (IRS-1–p110 adaptor protein) compared with the dimeric IRS-1–adaptor protein complex.

We also measured the PI 3-kinase enzymatic activity in the IRS-1 precipitates, and these data show a remarkable decrease in insulin-stimulated IRS-1–associated PI 3-kinase activity (Fig. 4), fully consistent with the decrease in formation of IRS-1/p110 complexes.

Akt and PKCζ/λ activity. Akt and PKC-ζ/λ are two enzymes downstream of PI 3-kinase that mediate PI 3-kinase–directed signals to facilitate GLUT-4 translocation in skeletal muscle and adipocytes. In lean subjects, in vivo insulin stimulation led to a twofold increase in PKC-ζ/λ activity, and this effect was markedly blunted in the insulin-resistant groups (Fig. 5). Insulin also caused a marked stimulation of Akt phosphorylation in the lean subjects, and this effect was only modestly blunted in the insulin-resistant groups (Fig. 6). These results are consistent with previous studies (36,44,45), including an earlier report that showed a dissociation between decreased PI 3-kinase and Akt activity (4,7). It should be noted that Akt2

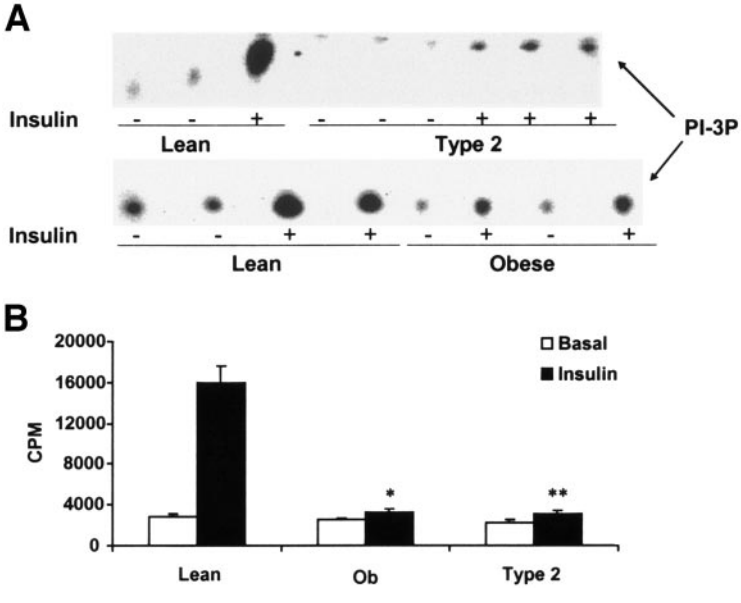

FIG. 4. PI 3-kinase activity. *A*: A representative autoradiogram showing PI 3-phosphate (PI-3P) formed by the kinase activity in the anti-IRS-1 immunoprecipitates. *B*: Quantitative comparison of insulin-stimulated PI 3-kinase activity in human muscle from lean, obese (Ob), and type 2 diabetic subjects. *$P < 0.05$, **$P < 0.01$ vs. lean. CPM, counts per minute.

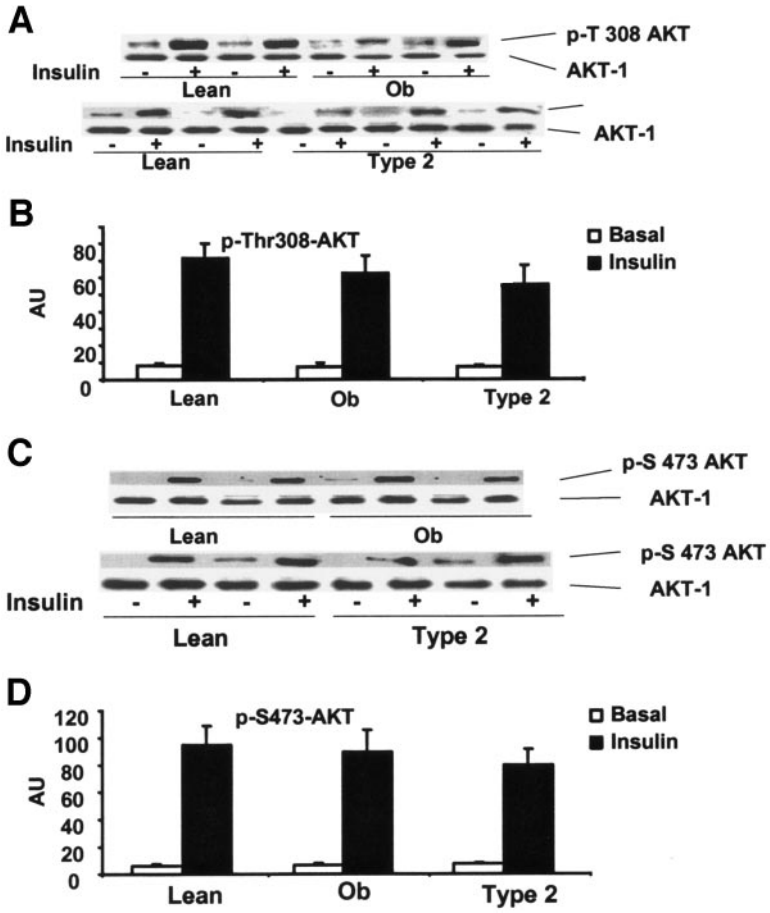

FIG. 6. Insulin-stimulated phosphorylation of Akt in human muscle. Comparison of insulin-stimulated phosphorylation of Thr (308)-Akt (*A* and *B*) and Ser (473)-Akt (*C* and *D*) in human muscle from lean, obese (Ob), and type 2 diabetic subjects.

is more directly related to stimulation of glucose transport (46,47), and the available antibodies do not distinguish between Akt1 and Akt2. Therefore, it is possible that if we were able to measure Akt2 activity separately, a greater defect would be observed.

Proinflammatory kinase activity. As shown earlier, IRS-1 Ser^{307} phosphorylation is elevated in the insulin-resistant groups. It is widely held that activation of the proinflammatory pathway contributes to insulin resistance (27,29–33) and that specific kinases in this pathway can phosphorylate IRS-1 at positions 307 and 1101 (27–30). Three of these candidate kinases were assessed. As seen in Fig. 7*A*, basal PKC-θ activity was elevated in the insulin-resistant groups compared with lean control subjects. More strikingly, when subcellular fractionation was performed (Fig. 7*B*), the appearance of PKC-θ activity in the skeletal muscle membrane fraction is enhanced in the obese subjects and is even further increased in the type 2 diabetic group. These results on PKC-θ activity are consistent with a previously published report (33).

We also assessed JNK-1 activity by measuring the JNK phosphorylation state using a phosphospecific antibody (Fig. 7*C* and *D*). We found a two- to threefold elevation in the level of activated JNK-1 in the insulin-resistant groups. In contrast, levels of phosphorylated IKK-β, which is also a proinflammatory serine/threonine kinase implicated in IRS-1 serine phosphorylation, were only minimally increased in the insulin-resistant subjects (Fig. 7*E* and *F*).

Composition of the muscle fiber types in human vastus lateralis. Electrophoretic analysis of MHC composition showed a significant increase in the percent composition of MHC IIx fibers ($P < 0.005$) in the insulin-resistant muscle (from 5.0 ± 1.2% in lean to 15.9 ± 2.7% and 16.1 ± 1.9% in obese and type 2 muscles, respectively). The increase in MHC IIx was accompanied by a decrease in both MHC I and IIa fibers. MHC IIa decreased from 56.4 ± 1.0% in lean to 46.4 ± 4.9% and 52.5 ± 3.7% in obese and type 2 diabetic muscles, respectively. In comparison to lean subjects, the proportion of MHC I did not change in obese muscle, whereas it fell from 38.5 ± 0.8% in lean to 31.4 ± 3.1% in type 2 diabetic muscle. These observations are consistent with other reports (48–50) showing that insulin resistance is associated with a decrease in oxidative and an increase in glycolytic fiber types in muscle. Although the decreased oxidative and increased glycolytic types of fibers in type 2 diabetic subjects has been previously observed, it is unclear whether this represents a primary or secondary event and further studies into this question seem warranted.

Correlation between in vivo insulin sensitivity and in vitro measurements. Linear regression analyses were carried out using glucose infusion rate as the dependent variable and the ratio of p85/55/50 to actin, basal PKC-θ activity, phosphorylation of JNK and Ser^{307} IRS-1, and the percent composition of MHC IIx fiber type as the independent variables. Pairwise analysis (glucose infusion rate versus a variable) showed significant inverse correlations between glucose infusion rate values and the levels of p85/55/50 ($r = -0.88$, $P < 0.0001$), PKC-θ activity ($r = -0.89$, $P < 0.0001$), pJNK-1 ($r = -0.66$, $P < 0.001$), pSer^{307}

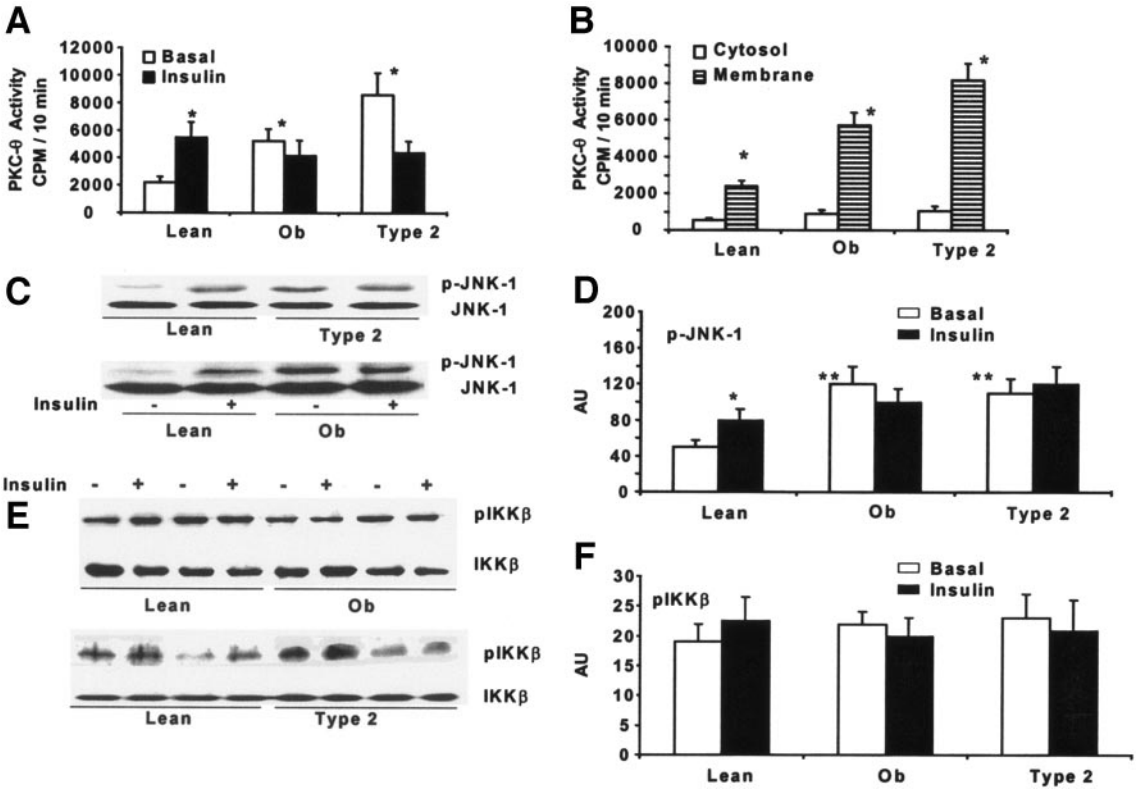

FIG. 7. Insulin-stimulated PKC-θ activity and phosphorylation of JNK-1 and IKK-β. PKC-θ activities in the lysates from muscle obtained before and after insulin infusion (*A*) and the cytosol and membrane fractions (*B*). **P* < 0.01 vs. lean basal. Representative Western blots for phospho-JNK (p-JNK) and total JNK (*C*) and phospho-IKK-β (pIKKβ) and total IKK-β signals (*E*). Results from densitometric scans for pJNK (*D*) and pIKK-β signals (*F*). **P* < 0.05, ***P* < 0.01 vs. lean basal. Ob, obese.

IRS-1 ($r = -0.65$, $P < 0.001$), and the proportion of type IIx fibers ($r = -0.6$, $P < 0.004$). Multilinear regression and multivariate analyses (MANOVA and discrimination function tests) showed that a combination of all five independent variables accounted for 82% of the variation ($r^2 = 0.82$, $F = 14.7$, $P < 0.001$) in the glucose infusion rate value. The residual errors, *t*, and *F* values produced by the regression model suggest that the predictive ability of the variables would be PKC-θ activity > p85/55/50 expression > pJNK > pSer^{307} IRS-1 > MHC IIx fiber content.

Activation of extracellular-related kinase-1 and p38 kinase. Because insulin-resistant patients become hyperinsulinemic, the increased circulating insulin levels could lead to overstimulation of the MAP kinase pathways. We measured extracellular signal–related kinase (ERK)-1 and -2 phosphorylations in our experimental groups, and the results are seen in Fig. 8*A* and *B*. The results show that ERK-2 content and phosphorylation state are relatively constant in the basal and insulin-stimulated states and are the same across the various study groups. In contrast, ERK-1 phosphorylation was markedly stimulated (fivefold) by insulin in the lean subjects, whereas in the hyperinsulinemic insulin-resistant subjects, the basal level of ERK-1 phosphorylation was increased three- to fourfold with little further effect of insulin infusion. Qualitatively similar results were seen when phospho-p38 was measured, with modestly elevated levels seen in the insulin-resistant groups compared with the lean control subjects (Fig. 8*C* and *D*). Interestingly, after insulin stimulation there was a small effect to increase phospho-p38 levels in the lean subjects, whereas insulin led to a paradoxical decrease in phospho-p38 in the insulin-resistant subjects, similar to a previous report (51).

DISCUSSION

Decreased insulin sensitivity is a widely described metabolic characteristic of patients with obesity and type 2 diabetes, and in the current studies we have explored the cellular mechanisms underlying this phenomenon. Because a major manifestation of insulin resistance is decreased insulin-stimulated glucose disposal by skeletal muscle, we have focused our attention on the mechanisms of skeletal muscle insulin resistance. To do this, we analyzed skeletal muscle biopsies taken from normal lean, nondiabetic obese, and obese type 2 diabetic subjects in the basal state and after a 5-h insulin infusion in the course of a euglycemic glucose clamp experiment.

One major finding in this study is increased expression of p85 protein and its splice variants p55 and p50 in the insulin-resistant groups, in the order of p55 > p50 > p85. This finding of increased p85/55/50 expression and the excellent correlation with decreased in vivo insulin sensitivity (glucose infusion rate values) suggest that increased expression of these PI 3-kinase adaptor subunits represents an important cellular mechanism that contributes to skeletal muscle insulin resistance in obesity and type 2 diabetes. A previous report in animals is consistent with the human data, as the combined levels of p85, p55, and p50 in liver of *ob/ob* mice were elevated compared with lean controls (25). Because the published data on the expression of regulatory subunit mRNA in human muscle

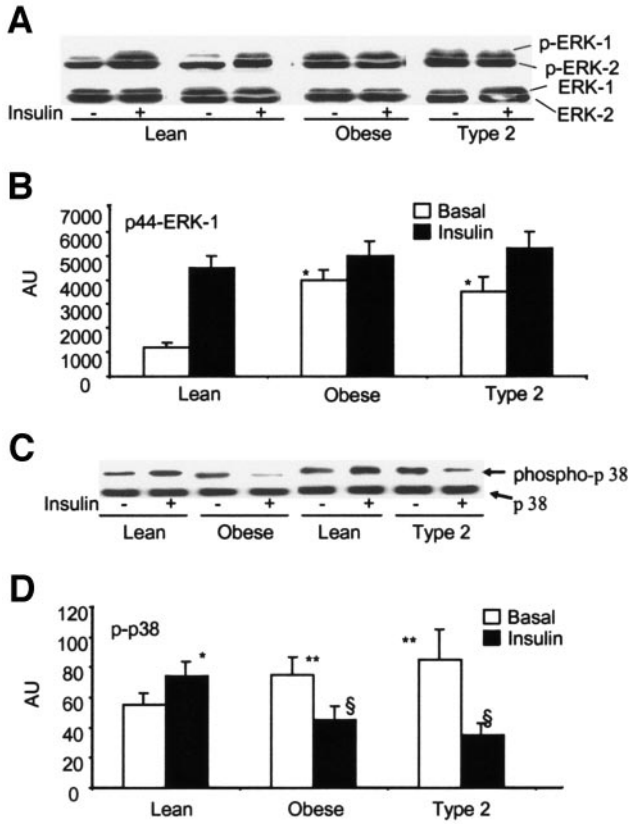

FIG. 8. Insulin-stimulated phosphorylation of ERK-1 and p38 kinase. Representative Western blots for phospho-ERK (*A*) and phospho-p38 (*C*). *B* and *D*: The results after densitometric quantitation. **P* < 0.05 vs. lean basal (*B*) or *P* = 0.05 vs. lean basal (*D*). ***P* < 0.05 vs. lean basal; §*P* < 0.01 vs. lean insulin stimulated.

showed discordant results (52,53) and changes in protein expression are often not reflective of changes in mRNA levels, we have restricted our analysis to protein expression. Furthermore, free p85 subunits are degraded more rapidly than p110-bound p85 (54), creating an additional complicating issue in attempting to correlate p85/55/50 proteins to mRNA levels. In a previous study with control and diabetic groups matched for obesity (BMI values of 26–27 kg/m^2), Tsuchida et al. (52) did not find statistically significant changes in protein expression of the regulatory subunits. Because the obese and diabetic subjects in our study had significantly higher BMI values than the nonobese lean subjects (Table 1), obesity might be a key factor magnifying the differences in subunit protein expression between lean and insulin-resistant subjects. Alternatively, it is possible that other unknown clinical or physiological differences between our patients and those in the earlier report might be responsible.

If competition between the excessive adaptor protein expression and the p85/55/50-p110 heterodimer occurs, then one would predict a greater decrease in IRS-1–associated p110 content compared with IRS-1–associated p85. As seen in Fig. 3, this is precisely what we observed, consistent with the view that excessive adaptor subunits compete with the PI 3-kinase heterodimer for binding to protein targets such as IRS-1, contributing to decreased insulin-stimulated PI 3-kinase activity.

The ability of IRS-1 to interact with the PI 3-kinase heterodimer is even further impaired due to abnormalities in insulin-stimulated IRS-1 tyrosine phosphorylation. Thus, the infused insulin had a four- to fivefold effect to stimulate IRS-1 tyrosine phosphorylation in the lean control subjects, and this effect was blunted by ~50% in the obese subjects and even further impaired in the type 2 diabetic patients. It is well known that phosphorylation of IRS-1 at Ser^{307} can impair subsequent insulin-stimulated tyrosine phosphorylation (26,27). In lean subjects, we found low levels of Ser^{307} phosphorylation in the basal state, which was stimulated approximately threefold by insulin. In the insulin-resistant obese and type 2 diabetic subjects, basal Ser^{307} phosphorylation was markedly increased (fivefold) with no further effect of insulin. Based on the known effects of Ser^{307} phosphorylation to impair tyrosine phosphorylation (26,27), it is likely that this increased basal level of Ser^{307} phosphorylation is responsible for the defect in insulin-stimulated tyrosine phosphorylation of IRS-1.

The results also provide evidence on the mechanism of the increased Ser^{307} phosphorylation. Thus, PKC-θ, JNK-1, and IKK-β are three serine/threonine kinases in the proinflammatory pathway (27,29–33), and all three were assessed in the muscle samples before and after insulin stimulation. There was a threefold increase in basal JNK-1 phosphorylation and three- and fivefold increases in basal activity in the muscle samples from the obese and type 2 diabetic subjects, respectively, compared with the lean control subjects. Interestingly, in the lean control subjects, activation of PKC-θ was stimulated by insulin, whereas basal PKC-θ activity was three- to fivefold higher in the insulin-resistant subjects with no further stimulation by insulin. These results demonstrate that these components of the proinflammatory pathway are hyperactivated in skeletal muscle from insulin-resistant subjects, providing a plausible explanation for the increased phosphorylation of IRS-1 at Ser^{307}. Thus, a combination of two deleterious effects, decreased IRS-1 tyrosine phosphorylation and increased p85/55/50 expression, could coordinately impair insulin stimulation of PI 3-kinase activity.

It is possible that competition with the PI 3-kinase heterodimer is not the only way in which an excessive adaptor protein subunit concentration can lead to impaired insulin action (54). In mice, it has been observed that inactivation of the p85 gene leads to impaired activation of JNK-1 (55) and that restoration of the p85-α level stimulates JNK-1 activity (55), suggesting that p85 can directly regulate JNK-1 activity without requiring PI 3-kinase activity. This may be relevant to our finding that JNK1 was hyperactivated in the insulin-resistant subjects concomitant with the increased expression of the adaptor subunits, despite a decrease in PI 3-kinase activity.

Linear regression analysis indicated that the increased expression of p85/55/50, PKC-θ activity, phosphorylation levels of Ser^{307} IRS-1 and JNK-1, and the proportion of MHC IIx fiber can predict 82% of the decrease in insulin sensitivity (glucose infusion rate values). When the relationship between the five independent variables was examined, a remarkably high correlation (r = 0.92, P < 0.0001) was noted between p85/55/50 and PKC-θ activity. This may suggest a currently unknown biological correlation between these two signaling proteins.

PI 3-kinase mediates insulin-stimulated glucose trans-

port, which results in enhanced glucose disposal into skeletal muscle in vivo (1,4–8). Although the precise mechanisms whereby activation of PI 3-kinase leads to glucose transport stimulation are incompletely understood, stimulation of PI 3-kinase leads to downstream activation of Akt and PKC-λ, and numerous reports exist implicating these kinases in stimulation of GLUT-4 translocation and glucose transport (9–14). The results presented here demonstrate a modest decrease in Akt phosphorylation in insulin-resistant subjects, with a much more dramatic decrease in PKC-λ activation. Although it is clear that inhibition of either Akt2 or PKC-λ will lead to reduced insulin-stimulated glucose transport (9–13), the relative role and importance of these two kinases are unknown. Although our results would suggest a more dominant role for PKC-λ compared with Akt in the insulin-resistant state, it should be noted that the phosphospecific antibody used does not distinguish between Akt1 and Akt2. It has been shown that Akt2 is more important for glucose transport stimulation than Akt1 (46,47). If Akt1 is more abundant in muscle tissue and if no defect in Akt1 exists, then despite the mild decrease in total phospho-Akt, a large decrease in Akt2 phosphorylation and activation might be present. Along these lines, Brozinick et al. (43) demonstrated a decrease in insulin-stimulated Akt2 and Akt3 (but not Akt1) activities in muscle from obese subjects.

Another important result from these studies is the marked increase in basal levels of activated ERK-1 as well as p38 kinase in the obese and type 2 diabetic subjects. Because obese and type 2 diabetic subjects were hyperinsulinemic, it is possible that this contributed to the higher basal phosphorylation of JNK-1, ERK-2, and p38 by hyperstimulation of these pathways. It is also possible that elevated levels of p85/55/50 may inhibit the interaction of p85/p110 complexes with their normal SH2-binding partners, directing a greater proportion of the p85/p110 complexes to p21 Ras, where it will stimulate downstream activation of the ERK pathway.

In summary, we have observed a strong inverse correlation between in vivo insulin sensitivity and increase in the expression of PI 3-kinase adaptor subunits, basal phosphorylation in Ser^{307} IRS-1 and JNK-1, basal PKC-θ activity, and MHC IIx fiber type in skeletal muscle of obese and type 2 diabetic subjects. These increases correspond with a decrease in IRS-1 tyrosine phosphorylation, leading to marked impairment of the ability of insulin to activate PI 3-kinase in skeletal muscle from insulin-resistant subjects, and probably represent an important cellular mechanism for decreased insulin sensitivity in obesity and type 2 diabetes.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health (DK 33651 and DK 16964).

REFERENCES

1. Saltiel AR, Kahn CR: Insulin signaling and the regulation of glucose and lipid metabolism. *Nature* 414:799–806, 2001
2. Leng Y, Karlsson HK, Zierath JR: Insulin signaling defects in type 2 diabetes. *Rev Endocr Metab Disord* 5:111–117, 2004
3. Spiegelman BM, Flier JS: Obesity and the regulation of energy balance. *Cell* 104:531–543, 2001
4. Kim YB, Nikoulina SE, Ciaraldi TP, Henry RR, Kahn BB: Normal insulin-dependent activation of Akt/protein kinase B, with diminished activation of phosphoinositide 3-kinase, in muscle in type 2 diabetes. *J Clin Invest* 104:733–741, 1999
5. Goodyear LJ, Giorgino F, Sherman LA, Carey J, Smith RJ, Dohm GL: Insulin receptor phosphorylation, insulin receptor substrate-1 phosphorylation, and phosphatidylinositol 3-kinase activity are decreased in intact skeletal muscle strips from obese subjects. *J Clin Invest* 95:2195–2204, 1995
6. Bjornholm M, Kawano Y, Lehtihet M, Zierath JR: Insulin receptor substrate-1 phosphorylation and phosphatidylinositol 3-kinase activity in skeletal muscle from NIDDM subjects after in vivo insulin stimulation. *Diabetes* 46:524–527, 1997
7. Kruszynska YT, Worrall DS, Ofrecio J, Frias JP, Macaraeg G, Olefsky JM: Fatty acid-induced insulin resistance: decreased muscle PI3K activation but unchanged Akt phosphorylation. *J Clin Endocrinol Metab* 87:226–234, 2002
8. Shepherd PR, Withers DJ, Siddle K: Phosphoinositide 3-kinase: the key switch mechanism in insulin signaling. *Biochem J* 333:471–490, 1998
9. Kotani K, Ogawa W, Matsumoto M, Kitamura T, Sakaue H, Hino Y, Miyake K, Sano W, Akimoto K, Ohno S, Kasuga M: Requirement of atypical protein kinase Cλ for insulin stimulation of glucose uptake but not for Akt activation in 3T3–L1 adipocytes. *Mol Cell Biol* 18:6971–6982, 1998
10. Bae SS, Cho H, Mu J, Birnbaum MJ: Isoform-specific regulation of insulin-dependent glucose uptake by Akt/protein kinase B. *J Biol Chem* 278:49530–49536, 2003
11. Garofalo RS, Orena SJ, Rafidi K, Torchia AJ, Stock JL, Hildebrandt AL, Coskran T, Black SC, Brees DJ, Wicks JR, McNeish JD, Coleman KG: Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB β. *J Clin Invest* 112:197–208, 2003
12. Bandyopadhyay G, Standaert ML, Zhao L, Yu B, Avignon A, Galloway L, Karnam P, Moscat J, Farese RV: Activation of protein kinase C (α, β, and ζ) by insulin in 3T3/L1 cells: transfection studies suggest a role for PKC-zeta in glucose transport. *J Biol Chem* 272:2551–2558, 1997
13. Imamura T, Huang J, Usui I, Satoh H, Bever J, Olefsky JM: Insulin-induced GLUT4 translocation involves protein kinase C-λ-mediated functional coupling between Rab4 and the motor protein kinesin. *Mol Cell Biol* 23:4892–4900, 2003
14. Arribas M, Valverde AM, Burks D, Klein J, Farese RV, White MF, Benito M: Essential role of protein kinase Cζ in the impairment of insulin-induced glucose transport in IRS-2-deficient brown adipocytes. *FEBS Lett* 536:161–166, 2003
15. Fruman DA, Cantley LC, Carpenter CL: Structural organization and alternative splicing of the murine phosphoinositide 3-kinase p85 α gene. *Genomics* 37:113–121, 1996
16. Inukai K, Funaki M, Ogihara T, Katagiri H, Kanda A, Anai M, Fukushima Y, Hosaka T, Suzuki M, Shin BC, Takata K, Yazaki Y, Kikuchi M, Oka Y, Asano T: p85α gene generates three isoforms of regulatory subunit for phosphatidylinositol 3-kinase (PI 3-Kinase), p50α, p55α, and p85α, with different PI 3-kinase activity elevating responses to insulin. *J Biol Chem* 272:7873–7882, 1997
17. Antonetti DA, Algenstaedt P, Kahn CR: Insulin receptor substrate 1 binds two novel splice variants of the regulatory subunit of phosphatidylinositol 3-kinase in muscle and brain. *Mol Cell Biol* 16:2195–2203, 1996
18. Ueki K, Fruman DA, Brachmann SM, Tseng YH, Cantley LC, Kahn CR: Molecular balance between the regulatory and catalytic subunits of phosphoinositide 3-kinase regulates cell signaling and survival. *Mol Cell Biol* 22:965–977, 2002
19. Chen D, Mauvais-Jarvis F, Bluher M, Fisher SJ, Jozsi A, Goodyear LJ, Ueki K, Kahn CR: p50α/p55α phosphoinositide 3-kinase knockout mice exhibit enhanced insulin sensitivity. *Mol Cell Biol* 24:320–329, 2004
20. Mauvais-Jarvis F, Ueki K, Fruman DA, Hirshman MF, Sakamoto K, Goodyear LJ, Iannacone M, Accili D, Cantley LC, Kahn CR: Reduced expression of the murine p85α subunit of phosphoinositide 3-kinase improves insulin signaling and ameliorates diabetes. *J Clin Invest* 109:141–149, 2002
21. Terauchi Y, Tsuji Y, Satoh S, Minoura H, Murakami K, Okuno A, Inukai K, Asano T, Kaburagi Y, Ueki K, Nakajima H, Hanafusa T, Matsuzawa Y, Sekihara H, Yin Y, Barrett JC, Oda H, Ishikawa T, Akanuma Y, Komuro I, Suzuki M, Yamamura K, Kodama T, Suzuki H, Kadowaki T: Increased insulin sensitivity and hypoglycaemia in mice lacking the p85 α subunit of phosphoinositide 3-kinase. *Nat Genet* 21:230–235, 1999
22. Ueki K, Algenstaedt P, Mauvais-Jarvis F, Kahn CR: Positive and negative regulation of phosphoinositide 3-kinase-dependent signaling pathways by three different gene products of the p85α regulatory subunit. *Mol Cell Biol* 20:8035–8046, 2000

23. Jhun BH, Rose DW, Seely BL, Rameh L, Cantley L, Saltiel AR, Olefsky JM: Microinjection of the SH2 domain of the 85-kilodalton subunit of phosphatidylinositol 3-kinase inhibits insulin-induced DNA synthesis and c-fos expression. *Mol Cell Biol* 14:7466–7475, 1994
24. Barbour LA, Shao J, Qiao L, Leitner W, Anderson M, Friedman JE, Draznin B: Human placental growth hormone increases expression of the p85 regulatory unit of phosphatidylinositol 3-kinase and triggers severe insulin resistance in skeletal muscle. *Endocrinology* 145:1144–1150, 2004
25. Kerouz NJ, Horsch D, Pons S, Kahn CR: Differential regulation of insulin receptor substrates-1 and -2 (IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (*ob/ob*) mouse. *J Clin Invest* 100:3164–3172, 1997
26. Zick Y: Role of Ser/Thr kinases in the uncoupling of insulin signaling. *Int J Obes Relat Metab Disord* 27:S56–S60, 2003
27. Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE, White MF: Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. *J Biol Chem* 277:1531–1537, 2002
28. Li Y, Soos TJ, Li X, Wu J, Degennaro M, Sun X, Littman DR, Birnbaum MJ, Polakiewicz RD: Protein kinase C θ inhibits insulin signaling by phosphorylating IRS1 at Ser^{1101}. *J Biol Chem* 279:45304–45307, 2004
29. Lee YH, Giraud J, Davis RJ, White MF: c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade. *J Biol Chem* 278:2896–2902, 2003
30. Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, Ye J: Serine phosphorylation of insulin receptor substrate 1 by inhibitor κB kinase complex. *J Biol Chem* 277:48115–48121, 2002
31. Bell KS, Schmitz-Peiffer C, Lim-Fraser M, Biden TJ, Cooney GJ, Kraegen EW: Acute reversal of lipid-induced muscle insulin resistance is associated with rapid alteration in PKC-θ localization. *Am J Physiol Endocrinol Metab* 279:E1196–E1201, 2000
32. Kim JK, Fillmore JJ, Sunshine MJ, Albrecht B, Higashimori T, Kim DW, Liu ZX, Soos TJ, Cline GW, O'Brien WR, Littman DR, Shulman GI: PKC-θ knockout mice are protected from fat-induced insulin resistance. *J Clin Invest* 114:823–827, 2004
33. Itani SI, Pories WJ, Macdonald KG, Dohm GL: Increased protein kinase C θ in skeletal muscle of diabetic patients. *Metabolism* 50:553–557, 2001
34. Storgaard H, Jensen CB, Bjornholm M, Song XM, Madsbad S, Zierath JR, Vaag AA: Dissociation between fat-induced in vivo insulin resistance and proximal insulin signaling in skeletal muscle in men at risk for type 2 diabetes. *J Clin Endocrinol Metab* 89:1301–1311, 2004
35. Pratipanawatr W, Pratipanawatr T, Cusi K, Berria R, Adams JM, Jenkinson CP, Maezono K, DeFronzo RA, Mandarino LJ: Skeletal muscle insulin resistance in normoglycemic subjects with a strong family history of type 2 diabetes is associated with decreased insulin-stimulated insulin receptor substrate-1 tyrosine phosphorylation. *Diabetes* 50:2572–2578, 2001
36. Beeson M, Sajan MP, Dizon M, Grebenev D, Gomez-Daspet J, Miura A, Kanoh Y, Powe J, Bandyopadhyay G, Standaert ML, Farese RV: Activation of protein kinase C-ζ by insulin and phosphatidylinositol-3,4,5-$(PO_4)^3$ is defective in muscle in type 2 diabetes and impaired glucose tolerance: amelioration by rosiglitazone and exercise. *Diabetes* 52:1926–1934, 2003
37. Nolan JJ, Ludvik B, Baloga J, Reichart D, Olefsky JM: Mechanisms of the kinetic defect in insulin action in obesity and NIDDM. *Diabetes* 46:994–1000, 1997
38. Frias JP, Yu JG, Kruszynska YT, Olefsky JM: Metabolic effects of troglitazone therapy in type 2 diabetic, obese, and lean normal subjects. *Diabetes Care* 23:64–69, 2000
39. Yu JG, Javorschi S, Hevener AL, Kruszynska YT, Norman RA, Sinha M, Olefsky JM: The effect of thiazolidinediones on plasma adiponectin levels in normal, obese, and type 2 diabetic subjects. *Diabetes* 51:2968–2974, 2002
40. Klitgaard H, Mantoni M, Schiaffino S, Ausoni S, Gorza L, Laurent-Winter C, Schnohr P, Saltin B: Function, morphology and protein expression of ageing skeletal muscle: a cross-sectional study of elderly men with different training backgrounds. *Acta Physiol Scand* 140:41–54, 1990
41. Caldwell KK, Lips DL, Bansal VS, Majerus PW: Isolation and characterization of two 3-phosphatases that hydrolyze both phosphatidylinositol 3-phosphate and inositol 1,3-bisphosphate. *J Biol Chem* 266:18378–18386, 1991
42. Walsh JP, Caldwell KK, Majerus PW: Formation of phosphatidylinositol 3-phosphate by isomerization from phosphatidylinositol 4-phosphate. *Proc Natl Acad Sci U S A* 88:9184–9187, 1991
43. Brozinick JT, Jr, Roberts BR, Dohm GL: Defective signaling through Akt-2 and -3 but not Akt-1 in insulin-resistant human skeletal muscle: potential role in insulin resistance. *Diabetes* 52:935–941, 2003
44. Kim YB, Kotani K, Ciaraldi TP, Henry RR, Kahn BB: Insulin-stimulated protein kinase C λ/ζ activity is reduced in skeletal muscle of humans with obesity and type 2 diabetes: reversal with weight reduction. *Diabetes* 52:1935–1942, 2003
45. Vollenweider P, Menard B, Nicod P: Insulin resistance, defective insulin receptor substrate 2-associated phosphatidylinositol-3′ kinase activation, and impaired atypical protein kinase C (ζ/λ) activation in myotubes from obese patients with impaired glucose tolerance. *Diabetes* 51:1052–1059, 2002
46. Cho H, Thorvaldsen JL, Chu Q, Feng F, Birnbaum MJ: Akt1/PKBα is required for normal growth but dispensable for maintenance of glucose homeostasis in mice. *J Biol Chem* 276:38349–38352, 2001
47. Noda S, Kishi K, Yuasa T, Hayashi H, Ohnishi T, Miyata I, Nishitani H, Ebina Y: Overexpression of wild-type Akt1 promoted insulin-stimulated p70S6 kinase (p70S6K) activity and affected GSK3 β regulation, but did not promote insulin-stimulated GLUT4 translocation or glucose transport in L6 myotubes. *J Med Invest* 47:47–55, 2000
48. Hickey MS, Weidner MD, Gavigan KE, Zheng D, Tyndall GL, Houmard JA: The insulin action-fiber type relationship in humans is muscle group specific. *Am J Physiol* 269:E150–E154, 1995
49. Hedman A, Byberg L, Reneland R, Lithell HO: Muscle morphology, self-reported physical activity and insulin resistance syndrome. *Acta Physiol Scand* 175:325–332, 2002
50. Mathieu-Costello O, Kong A, Ciaraldi TP, Cui L, Ju Y, Chu N, Kim D, Mudaliar S, Henry RR: Regulation of skeletal muscle morphology in type 2 diabetic subjects by troglitazone and metformin: relationship to glucose disposal. *Metabolism* 52:540–546, 2003
51. Koistinen HA, Chibalin AV, Zierath JR: Aberrant p38 mitogen-activated protein kinase signalling in skeletal muscle from type 2 diabetic patients. *Diabetologia* 46:1324–1328, 2003
52. Tsuchida H, Bjornholm M, Fernstrom M, Galuska D, Johansson P, Wallberg-Henriksson H, Zierath JR, Lake S, Krook A: Gene expression of the p85α regulatory subunit of phosphatidylinositol 3-kinase in skeletal muscle from type 2 diabetic subjects. *Pflugers Arch* 445:25–31, 2002
53. Andreelli F, Laville M, Ducluzeau PH, Vega N, Vallier P, Khalfallah Y, Riou JP, Vidal H: Defective regulation of phosphatidylinositol-3-kinase gene expression in skeletal muscle and adipose tissue of non-insulin-dependent diabetes mellitus patients. *Diabetologia* 42:358–364, 1999
54. Brachmann SM, Ueki K, Engelman JA, Kahn RC, Cantley LC: Phosphoinositide 3-kinase catalytic subunit deletion and regulatory subunit deletion have opposite effects on insulin sensitivity in mice. *Mol Cell Biol* 25:1596–1607, 2005
55. Ueki K, Fruman DA, Yballe CM, Fasshauer M, Klein J, Asano T, Cantley LC, Kahn CR: Positive and negative roles of p85 α and p85 β regulatory subunits of phosphoinositide 3-kinase in insulin signaling. *J Biol Chem* 278:48453–48466, 2003