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

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Impaired glucose tolerance (IGT) is characterized by insulin resistance. Recently, defects in the insulin-signaling cascade have been implicated in the pathogenesis of insulin resistance. To study insulin signaling in IGT, we used human skeletal muscle cells in primary culture from patients with IGT and control subjects. In these cultured myotubes, we assessed insulin-induced 2-deoxyglucose uptake and early steps of the metabolic insulin-signaling cascade. Myotubes in culture from patients with IGT had insulin-induced glucose uptake that was roughly 30–50% less than that from control subjects. This insulin resistance was associated with impaired insulin receptor substrate (IRS)-2–associated phosphatidylinositol 3′ (PI3) kinase activation and IRS-2 tyrosine phosphorylation as well as significantly decreased protein kinase C (PKC)-ζ/λ activation in response to insulin. IRS-1–associated PI3 kinase activation and insulin receptor autophosphorylation were comparable in the two groups. Protein expression levels for the insulin receptor, IRS-1, IRS-2, the p85 regulatory subunit of PI3 kinase, Akt, PKC-ζ/λ, GLUT1, and GLUT4 were also similar in the two groups. In conclusion, myotubes from patients with IGT have impaired insulin-induced glucose uptake. This is associated with impaired IRS-2–associated PI3 kinase activation and PKC-ζ/λ activation. Our results suggest that these defects may contribute to insulin resistance in IGT patients. ***Diabetes*** **51:1052–1059, 2002**

Insulin resistance is a key feature of impaired glucose tolerance (IGT) and type 2 diabetes (1). It is characterized by a diminished ability of insulin-sensitive tissues to take up and metabolize glucose in response to insulin.

Skeletal muscle is the primary site of insulin-mediated glucose disposal and contributes significantly to decreased glucose uptake, as seen in states of insulin resistance (1). Defects in the early insulin-signaling cascade leading to glucose uptake have been shown to play a key role in the pathogenesis of insulin resistance (2).

In target tissues, such as skeletal muscle, insulin promotes glucose uptake through the translocation of the GLUT4 glucose transporter from an intracellular vesicular pool to the plasma membrane (3). Insulin binding to the extracellular α-subunit of its receptor results in autophosphorylation of tyrosine residues in the receptor β-subunit and activation of a tyrosine kinase intrinsic to the β-subunit. This leads to the recruitment and tyrosine phosphorylation of intracellular substrates such as insulin receptor substrates (IRSs) 1–4. Phosphotyrosines on the IRS proteins bind the p85 regulatory subunit of phosphatidylinositol 3′ (PI3) kinase. PI3 kinase is a heterodimer of a regulatory subunit (p85) and a catalytic subunit (p110), and its activation in response to insulin results primarily through its association with the IRS proteins (4). PI3 kinase activation is required for GLUT4 translocation and subsequent glucose uptake. Current data indicate that PI3 kinase activates the serine/threonine kinase Akt/PKB, which may be involved in insulin-induced GLUT4 translocation (5), even though a definitive role has not been proven (6). Indeed, in rat adipocytes, only a relatively small fraction of insulin-stimulated GLUT4 translocation is inhibited by a kinase inactive form of Akt (7). Furthermore, in 3T3-L1 adipocytes, studies with a dominant-negative mutant of Akt suggest that Akt is not required for insulin-stimulated GLUT4 translocation and glucose transport (8). In adipocytes and muscle tissue, insulin also activates, in a PI3 kinase–dependent manner, atypical forms of protein kinase C (PKC) (λ and ζ), which have also been suggested to mediate glucose transport (6,9,10).

IRS-1 and IRS-2 are important in transmitting the metabolic actions of insulin. They are strongly expressed in insulin-sensitive tissues (4). Mice with targeted disruption of the IRS-1 or IRS-2 gene show either insulin resistance (11,12) or type 2 diabetes (13), respectively, whereas IRS-3 and IRS-4 knockout mice have only minimal metabolic abnormalities (14,15).

There is increasing evidence that defects in the insulin-signaling pathway contribute to insulin resistance in humans and in tissues like the skeletal muscle. For example,

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IGT, impaired glucose tolerance; IRS, insulin receptor substrate; MEM, modified Eagle's medium; PI3, phosphatidylinositol 3′; PKC, protein kinase C; PTPase, protein tyrosine phosphatase.

in patients with type 2 diabetes or morbid obesity, several investigators have shown, in muscle biopsies, decreased autophosphorylation of the insulin receptor (16,17), decreased IRS-1 tyrosine phosphorylation (18), and decreased PI3 kinase activation (18,19), which could all contribute to the observed insulin resistance. But patients with type 2 diabetes often have complex metabolic abnormalities such as hyperinsulinemia, hyperglycemia, and dyslipidemia, which can induce insulin resistance per se (20) and introduce confounding factors in the study of potential signaling defects.

Therefore, we chose to study patients with IGT. Patients with IGT are insulin resistant (1) and at increased risk to develop type 2 diabetes (21,22). They do not have fasting hyperglycemia. To minimize the effects of environmental factors, we used human skeletal muscle cells in primary culture. Human skeletal muscle cells in primary culture display numerous features of mature skeletal muscle (23–25), and muscle cells from different individuals can be studied under strictly controlled experimental conditions. Therefore, these cells are a useful tool to search for primary defects in skeletal muscle. This system has been used previously to study muscle metabolism in patients with type 2 diabetes or in Pima Indians (23,26–28).

Here, we show that muscle cells in culture from obese patients with IGT have decreased insulin-mediated glucose uptake. Furthermore, we demonstrate that IRS-2–associated PI3 kinase activation and PKC-ζ/λ activation in response to insulin are decreased. This result suggests that these defects might be involved in the pathogenesis of insulin resistance in skeletal muscle.

RESEARCH DESIGN AND METHODS

Materials. Culture growth media (SkGM Bulletkit) was obtained from Clonetics (San Diego, CA). α-Modified Eagle's medium (MEM) was purchased from Irvine Scientific (Santa Ana, CA). FCS was from Life Technologies (Rockville, MD). Anti–IRS-1, anti–IRS-2, anti-p85, anti-insulin receptor, and β-subunit were from Upstate Biotechnology (Lake Placid, NY); anti-phosphotyrosine antibody (PY20) was from Transduction Laboratories (Lexington, KY); anti–Akt 1/2 and polyclonal anti–PKC-ζ/λ were from Santa Cruz Biotechnology (Santa Cruz, CA); anti–phospho-Akt was from New England BioLabs (Beverly, MA); and anti-GLUT1 and anti-GLUT4 were from Chemicon International (Temecula, CA). ^{159}Ser-PKC-ϵ (amino acids 153–164)-NH_2 substrate was from Biosource International (Camarillo, CA). [2-^{3}H]deoxyglucose and L-[1-^{3}H(N)]glucose were from DuPont-NEN (Boston, MA), and [γ-^{32}P]ATP was from Amersham Pharmacia (Piscataway, NJ). All other reagents or chemicals were purchased from Sigma (St. Louis, MO).

Human subjects. Seven healthy control subjects and six patients with IGT participated in these studies. All the subjects underwent total hip or knee joint replacement during which muscle tissue was taken either from the gluteus maximus or the vastus lateralis. All experimental protocols were approved by the local Ethics Committee of the University of Lausanne. Informed written consent was obtained from all subjects after explanation of the protocol. No subject was taking pharmacological agents known to affect carbohydrate metabolism. All subjects underwent, before surgery, an oral glucose tolerance test (75 g glucose) after an overnight fast, and blood glucose was determined at basal and after 2 h. Percentage of body fat was determined with the skinfold method.

Primary human muscle cell cultures. During surgery, muscle tissue was taken, and part of the biopsy was immediately frozen in liquid nitrogen. Muscle cell isolation and subculture were carried out as previously described (23). Briefly, muscle tissue was placed in 15 ml Ham's F-10 media at 4°C and carefully dissected, minced, and washed three times with F-10 media at 4°C. The tissue was then dissociated by three successive treatments of 20 min each in 25 ml of 0.05% trypsin/EDTA at room temperature. After trypsinization, cells were resuspended in SkGM Bulletkit without added insulin, supplemented with 2% FCS, plated, and grown at 37°C in an incubator containing 95% air/5% CO_2. At confluence, cells were passed to multiple dishes (for studies on glucose transport, cells were plated in 12-well tissue culture dishes at a density of 6,000 cells/well, and for other studies, 15,000–20,000 cells were plated on 100-mm dishes) and grown to 80% confluence. They were then fused for 4 days in α-MEM containing 2% FCS, penicillin (100 units/ml), and streptomycin (100 μg/ml). All studies were carried out on cultures after first passage.

2-Deoxyglucose transport. The procedure for glucose uptake has been described previously (23). Cells were incubated in serum-free media containing increasing concentrations of insulin (basal, 0.17 nmol/l [1 ng/ml], 1.7 nmol/l [10 ng/ml], and 17 nmol/l [100 ng/ml]) for 60 min in a 95% O_2/5% CO_2 incubator at 37°C. Glucose uptake was determined in triplicate at each point after the addition of 10 μl substrate ([2-^{3}H]deoxyglucose/L-[1-^{3}H(N)]glucose, 0.1 mCi, to a final concentration of 0.01 mmol/l) to provide a concentration at which cell membrane transport is rate limiting. The value for L-glucose uptake was subtracted to correct each sample for the contributions of diffusion and trapping. Protein content was determined on a 50-μl aliquot of the cell suspension using the Bradford method.

Immunoblotting analysis

Sample preparation. All samples for Western blotting were prepared from confluent monolayers of fused myoblasts. After insulin stimulation (at the indicated time and concentration), cells were washed with ice-cold PBS buffer, and lysis buffer (50 mmol/l HEPES, 150 mmol/l NaCl, 1% Triton X-100, 4 mmol/l sodium orthovanadate, 20 mmol/l sodium pyrophosphate, 200 mmol/l sodium fluoride, 10 mmol/l EDTA, 2 mmol/l phenylmethylsulfoxide, and 10% glycerol, pH 7.4) was added to each plate and the cells scraped with a rubber policeman into Eppendorf tubes. After centrifugation at 20,000*g* for 10 min, supernatants were collected, and protein content was determined using a Bradford method with BSA as a standard.

SDS-PAGE. SDS-PAGE was carried out as previously described. For GLUT1, GLUT4, and insulin receptor, samples were not boiled. To compare and quantify expression levels between the different subjects, in all the experiments, an internal standard consisting of the same cell lysate of a control subject was run in parallel. After electrotransfer to either nitrocellulose or PVDF (polyvinylidine fluoride), membranes were blocked with 5% (wt/vol) nonfat dry milk or 2.5% BSA. After blocking, membranes were blotted with specific monopolyclonal or polyclonal antibodies, washed, and incubated with anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase. Immune complexes were detected using enhanced chemiluminescence.

Insulin receptor autophosphorylation. Cells were starved overnight, stimulated with insulin (17 nmol/l) for 30 min, and lysed. Insulin receptor was immunoprecipitated with a specific antibody against the β-subunit of the receptor and then separated by SDS-PAGE. After transfer, the membranes were blotted first with a specific anti-phosphotyrosine antibody. The same membrane was then stripped and blotted with an insulin receptor β-subunit antibody to check for equal loading.

IRS-1 and IRS-2 tyrosine phosphorylation. Myotubes were starved overnight and stimulated with 17 nmol/l insulin for 3, 10, 15, 30, and 60 min. Cells were lysed, and IRS-1 and IRS-2 were immunoprecipitated with specific antibodies and then separated by SDS-PAGE. After transfer, membranes were first probed with an anti-phosphotyrosine antibody and then stripped and probed with either an anti–IRS-1 or –IRS-2 antibody to check for equal loading.

Specificity of the IRS-1 and IRS-2 antibodies was confirmed in immunoprecipitation experiments after transient overexpression of tagged full-length IRS-1 and IRS-2 proteins (data not shown).

Quantification. Quantification was performed using scanning densitometry with a Fluor-S Imager from Biorad (Hercules, CA) with Quantity One software (Biorad). Protein expression levels are expressed as arbitrary units.

PI3 kinase activity. After stimulation with insulin for the indicated time periods, skeletal muscle cells were lysed at 4°C in a lysis buffer containing 50 mmol/l HEPES, 150 mmol/l NaCl, 1% Triton X-100, 4 mmol/l sodium orthovanadate, 20 mmol/l sodium pyrophosphate, 200 mmol/l sodium fluoride, 10 mmol/l EDTA, 2 mmol/l phenylmethylsulfoxide, and 10% glycerol, pH 7.4. Lysates were centrifuged at 20,000*g* for 10 min at 4°C. Supernatants (50 μg total protein) were then incubated in anti–IRS-1, anti–IRS-2, or anti-phosphotyrosine antibodies and recombinant protein A-agarose overnight at 4°C. Beads were pelleted by a 10-min spin at 20,000*g* and washed three times with buffer A (Tris-buffered saline pH 7.5, 1% NP-40, and 100 μmol/l Na_3VO_4), buffer B (100 mmol/l Tris, pH 7.5, 500 mmol/l $LiCl_2$, and 100 μmol/l Na_3VO_4), and buffer C (10 mmol/l Tris, pH 7.5, 100 mmol/l NaCl, 1 mmol/l EDTA, and 100 μmol/l Na_3VO_4). Pellets were then resuspended in 50 μl buffer C without Na_3VO_4. As previously described, PI3 kinase activity was assessed by the phosphorylation of phosphatidylinositol in the presence of 20 μCi [γ-^{32}P]ATP. After the separation of lipids by thin-layer chromatography using a chloroform/methanol buffer, radiolabeled phosphoinositides were visualized and quantified using an FX Personal Imager phosphorimager (Biorad).

Akt activation. After insulin stimulation for the indicated time periods, Akt activity in immunoprecipitates was measured using a kit from Upstate

TABLE 1
Patients characteristics

	Control subjects	IGT subjects	*P*
n	7	6	
Age (years)	46 ± 3	51 ± 4	NS
Weight (kg)	72 ± 6	87 ± 3	<0.05
Height (cm)	171 ± 4	165 ± 5	NS
BMI (kg/m^2)	24.1 ± 1.0	32.5 ± 1.6	<0.05
Percent fat	27 ± 1	38 ± 3	<0.05
Systolic blood pressure (mmHg)	129 ± 4	142 ± 5	NS
Diastolic blood pressure (mmHg)	79 ± 3	85 ± 2	NS
Fasting blood glucose (mmol/l)	6.1 ± 0.4	6.3 ± 0.3	NS
2-h blood glucose (mmol/l)	6.0 ± 1.2	8.9 ± 1.2	<0.05
Fasting insulin (pmol/l)	25 ± 6	52 ± 16	<0.05

Data are means ± SE.

Biotechnology (Lake Placid, NY) according to the manufacturer's instructions. Akt serine phosphorylation (Ser 473) was assessed with a specific phospho-antibody.

PKC-ζ/λ activation. Activation of immunoprecipitable PKC-ζ/λ was determined as described (29,30). In brief, myotubes were incubated without or with insulin (17 nmol/l) for 10 min, lysed, and subjected to immunoprecipitation with a polyclonal antiserum that recognizes the COOH-terminal sequences of both PKC-ζ and PKC-λ. After overnight incubation at 4°C, proteins were collected on protein A-agarose beads, washed, and incubated for 8 min at 30°C in 50 μl buffer containing 50 mmol/l Tris/HCl (pH 7.5), 5 mmol/l $MgCl_2$, 100 μmol/l Na_3VO_4, 100 μmol/l $Na_4P_2O_7$, 1 mmol/l NaF, 100 μmol/l phenylmethylsulfonyl fluoride, 3–5 μCi [γ-^{32}P]ATP, 50 μg ATP, 4 μg phosphatidylserine, and 40 μmol/l ^{159}Ser-PKC-ε (amino acids 153–164)-NH_2 substrate. After incubation, aliquots of the reaction mixtures were spotted on P81 filter paper, washed in 5% acetic acid, and counted for ^{32}P.

Statistical analysis. All results are given as means ± SE. Differences between the control and IGT groups were determined using an unpaired Student's *t* test, and changes over time were compared with ANOVA. Statistical significance was determined with a *P* value <0.05.

RESULTS

Subjects. Summary data for the subjects are given in Table 1. The subjects were matched for age. Patients with IGT had a significantly higher BMI and body fat than control subjects. Basal blood glucose values were comparable between the two groups, whereas the 2-h glucose value after the oral glucose tolerance test was significantly different.

Human skeletal muscle cells. In culture, after differentiation, most of the human skeletal muscle myoblasts fused into multinucleated myotubes, as shown in Fig. 1. We performed immunostaining with an antibody against human sarcomeric actin, which is expressed in differentiated skeletal muscle, and found that over 80–90% of cells displayed positive staining (data not shown), confirming differentiation. There was no variance in the differentiation among myotubes from both groups.

2-Deoxyglucose uptake. 2-Deoxyglucose uptake was measured in myotubes after 4 days of fusion. Basal values of 2-deoxyglucose uptake were comparable in both groups. Myotubes from control subjects displayed insulin-mediated glucose uptake in a dose-dependent manner. At maximal insulin stimulation, about a twofold increase over basal was observed, which is comparable with previously published data (23). Myotubes from patients with IGT also increased insulin-mediated glucose uptake in a dose-dependent manner. For each concentration of insulin, 2-deoxyglucose uptake was decreased in the myotubes of IGT patients (Fig. 2).

Protein expression. To examine whether the impaired 2-deoxyglucose uptake is due to differences in expression levels of proteins of the insulin-signaling pathway, we compared protein expression levels by Western blot for the insulin receptor, IRS-1, IRS-2, the p85 regulatory subunit of PI3 kinase, Akt, PKC-ζ/λ, GLUT1, and GLUT4 with specific antibodies directed against these proteins. Western blots were then quantified by scanning densitometry. Figure 3 shows that all these proteins were expressed in the cultured myotubes, with no significant differences between the two groups (*P* = NS for all results). In the cultured cells, GLUT1 expression levels were roughly two times higher than GLUT4 levels per milligram of protein loaded, similar to previously published reports (23). We also compared expression levels for these same proteins directly in the muscle biopsy material and found no differences in expression between control subjects and patients with IGT. When comparing protein expression between the muscle biopsy and the cells in culture, IRS-1 and IRS-2 expression levels were 10–15% higher in the cells per milligram of protein loaded (data not shown).

Insulin receptor autophosphorylation. The first step in the insulin-signaling cascade is autophosphorylation of the β-subunit of the insulin receptor in response to hormone binding. Insulin receptor autophosphorylation was determined by tyrosine phosphorylation of the immunoprecipitated β-subunit of the receptor. Tyrosine phosphorylation in the basal state and autophosphorylation in response to insulin were comparable in the two groups (Fig. 4).

IRS-1/IRS-2 tyrosine phosphorylation. IRS-1 and IRS-2 are phosphorylated on tyrosine residues by the insulin receptor after insulin stimulation. Basal levels of IRS-1 and IRS-2 tyrosine phosphorylation were comparable in the two groups. Figure 5 shows that, in myotubes, IRS-1 and IRS-2 are strongly tyrosine phosphorylated in response to insulin. IRS-1 tyrosine phosphorylation had a tendency to be lower in myotubes from IGT patients, but this did not reach statistical significance (Fig. 5). Alternatively, insulin-stimulated IRS-2 tyrosine phosphorylation was significantly impaired in myotubes from patients with IGT, as shown in Fig. 5. This result was statistically significant after 3 and 10 min of insulin stimulation.

PI3 kinase activation. PI3 kinase is a key enzyme stimulated by insulin that leads to GLUT4 translocation and glucose uptake. PI3 kinase activity was determined in cells lysates of myotubes starved overnight and stimulated with insulin (17 nmol/l) for 3, 10, 15, 30, and 60 min. Basal levels of PI3 kinase activity associated with tyrosine phosphorylated proteins, IRS-1, and IRS-2 were comparable in the two groups. Figure 6 shows that the pattern and amplitude of the increase in PI3 kinase activity associated with tyrosine phosphorylated proteins and IRS-1 (Fig. 6*A* and *B*) were comparable between

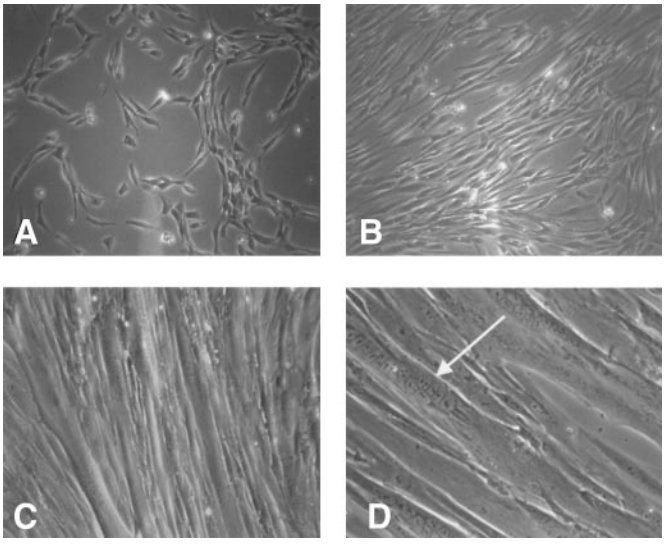

FIG. 1. Photomicrographs of myoblasts obtained from a human muscle biopsy. *A* and *B* show myoblasts during the growing phase. When the cells were almost confluent, they were changed to differentiation media, and after 4 days, most of these myoblasts fused to multinucleated myotubes (*C*). *D* shows a myotube at higher magnification that contains numerous nuclei (arrow).

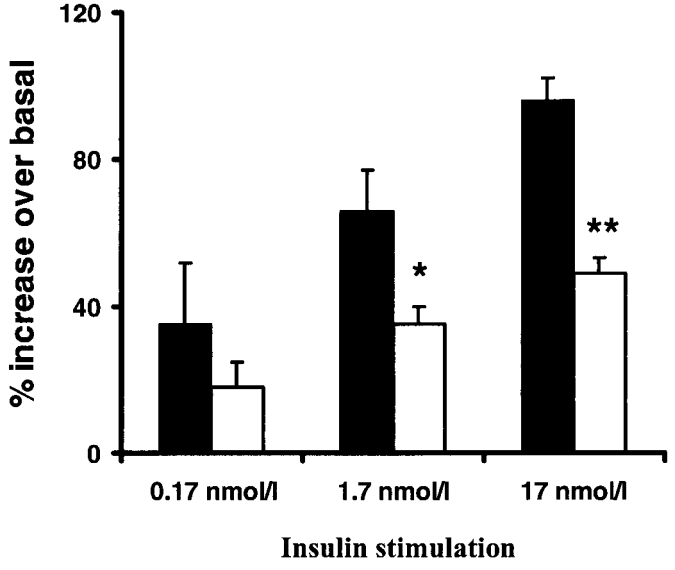

FIG. 2. 2-Deoxyglucose uptake in response to insulin. Myotubes from control and IGT subjects were stimulated with increasing concentrations of insulin, as indicated, and 2-deoxyglucose uptake was measured. There was a dose-dependent increase in 2-deoxyglucose uptake in myotubes from control subjects (■) and patients with IGT (□), but it was significantly less in IGT patients. *$P < 0.05$; **$P < 0.005$.

control and IGT subjects. In contrast, PI3 kinase activation associated with IRS-2 was significantly impaired in patients with IGT (Fig. 6*C*). IRS-2–associated PI3 kinase activation increased after 3 min of insulin stimulation and decreased more rapidly over time than IRS-1–associated PI3 kinase activity.

Akt activation. Akt has been suggested to play a role in insulin-induced glucose uptake and also GLUT4 translocation. Insulin stimulation of Akt, assessed either by an in vitro kinase assay or by phosphorylation at serine 473, was comparable in both groups (Fig. 7).

PKC-ζ/λ activation. PKC-ζ/λ activation has been suggested to play a role in insulin-mediated glucose uptake and GLUT4 translocation. In the basal state, immunoprecipitable PKC-ζ/λ activity was comparable in the two groups. Insulin-mediated PKC-ζ/λ activation was decreased by ~40% in patients with IGT ($P < 0.05$) (Fig. 8).

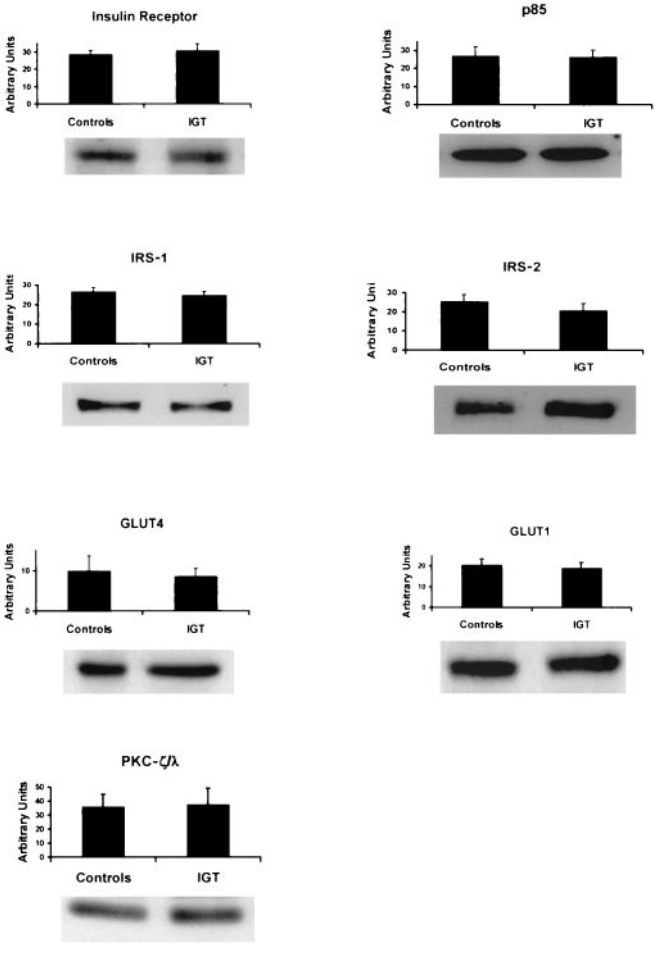

FIG. 3. Protein expression levels. Myotubes from control subjects and patients with IGT were lysed, and the expression of the indicated proteins was determined by Western blotting using specific antibodies. Quantification was performed by scanning densitometry. For each panel, a representative Western blot for a control subject and IGT patient is shown. Bar graphs represent means ± SE data expressed as arbitrary units for each group. There was no difference in protein expression levels between the two groups.

DISCUSSION

Defects in the insulin-signaling cascade leading to GLUT4 translocation and glucose uptake play an important role in the pathogenesis of insulin resistance in skeletal muscle (2,31). Most of the data have been gathered in patients with full-blown type 2 diabetes. However, little is known regarding signaling defects in prediabetic stages such as IGT. The study of insulin signaling in patients with IGT could give insight into the early defects involved in the pathogenesis of insulin resistance.

To study insulin action in a relevant target tissue, we used an in vitro model of skeletal muscle cells in primary culture from patients with IGT. Myotubes in culture express proteins characteristic of differentiated muscle cells (23) and are insulin responsive, with expression of molecules involved in the insulin-signaling pathway (23,26,32). Cells from different patients can be studied under strictly controlled conditions, minimizing the influence of environmental factors such as hyperinsulinemia and hyperglycemia, which could have unforeseen effects on skeletal muscle during in vivo experiments.

The main finding of this study was that insulin-mediated glucose uptake in cultured myotubes from patients with IGT was roughly 30–50% less than that in myotubes obtained from control subjects. This defect in glucose uptake was associated with impaired IRS-2–associated PI3 kinase activation and PKC-ζ/λ activation in response to insulin.

To study underlying defects that may lead to the observed insulin resistance in vitro, we first analyzed expression levels of relevant proteins of the insulin-signaling pathways. In the Zucker fatty rat model, decreased levels of IRS proteins are thought to contribute to insulin resistance (33), and in adipose tissue of patients with type 2 diabetes, decreased levels of IRS-1 have been found (34). In the present studies, expression levels of insulin receptor, IRS-1, IRS-2, p85, Akt, PKC-ζ/λ, GLUT1, and GLUT4 were comparable in both groups.

We next turned our attention to insulin-signaling steps. The major findings were that IRS-2–associated activation of PI3 kinase and PKC-ζ/λ activation were impaired in myotubes of patients with IGT. This impairment was not related to a decreased insulin receptor β-subunit autophosphorylation in response to insulin stimulation but was associated with decreased IRS-2 tyrosine phosphorylation. PI3 kinase activation in response to insulin is key to induce the translocation of the GLUT4 glucose transporter

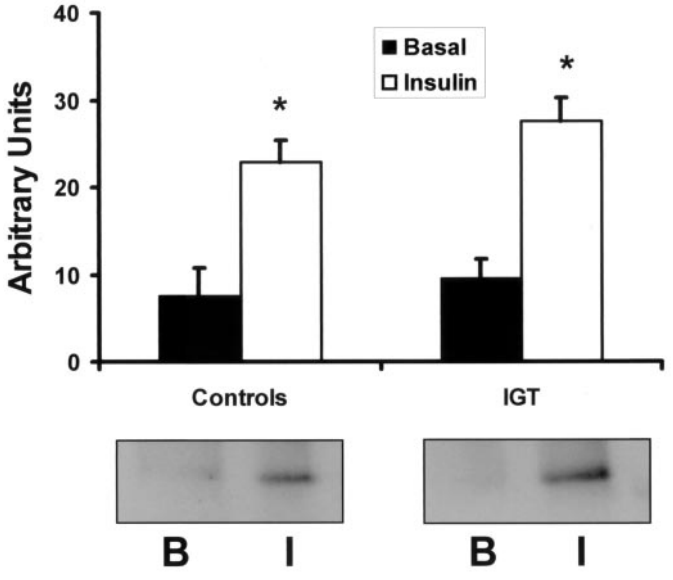

FIG. 4. Insulin receptor autophosphorylation. Starved myotubes from control subjects or IGT patients were either treated without (basal) or with 17 nmol/l insulin for 30 min. The insulin receptor β-subunit was then immunoprecipitated from equal amounts of total proteins. Autophosphorylation was determined with anti-phosphotyrosine antibodies. Representative Western blots in basal conditions (B) or after insulin stimulation (I) are shown for one subject of each group. The bar graphs represent summary data (means ± SE) for each group. There was no difference in the basal or insulin-induced autophosphorylation of the insulin receptor between the two groups. *$P < 0.05$.

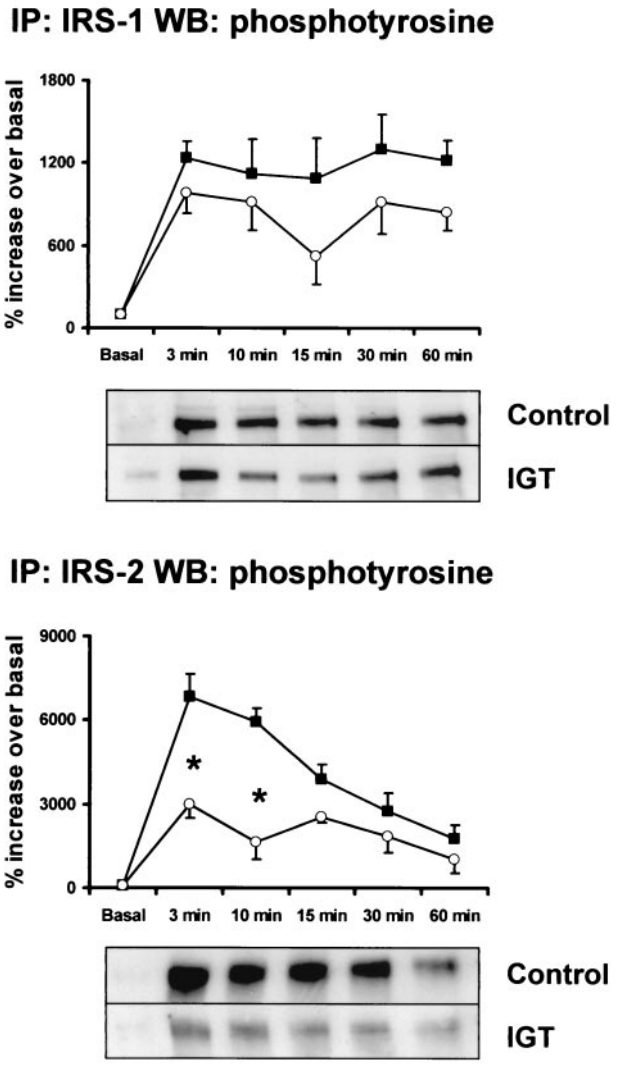

FIG. 5. IRS-1 and IRS-2 tyrosine phosphorylation. Myotubes were starved overnight and then stimulated with insulin (17 nmol/l) for the indicated times. Cells were lysed and IRS-1 and IRS-2 immunoprecipitated from an equal amount of total proteins and separated by SDS-PAGE. Tyrosine phosphorylation was determined with anti-phosphotyrosine antibodies. Line graphs represent summary data (means ± SE) at each time for control subjects (■) and IGT patients (○). Representative Western blots are shown for a control subject and an IGT subject. IRS-2 tyrosine phosphorylation was decreased in IGT patients. *$P < 0.05$. IP, immunoprecipitation; WB, Western blot.

from an intracellular pool to the cell membrane leading to increased glucose uptake (35,36). Even though IRS-1 and IRS-2 are homologous, it has become clear that they have distinct functions, but their specific role in transmitting the insulin signal is not completely understood. IRS-2 has been shown to mediate metabolic responses and GLUT4 translocation in response to insulin (37–39). In addition, it was recently shown that in muscle biopsies from patients with type 2 diabetes, there was a decreased IRS-2–associated PI3 kinase activation in addition to defects of signaling through IRS-1 (19). In the IRS-1 knockout mouse, which is insulin resistant, IRS-2 expression is not increased, but there is increased activation of PI3 kinase associated with IRS-2, which could explain why these mice are only mildly insulin resistant (12). IRS-2 knockout mice display a phenotype that resembles type 2 diabetes in humans; this is due to the simultaneous presence of insulin resistance in skeletal muscle liver and adipose tissue (40) and the decreased insulin secretion related to lower β-cell mass in the pancreas (13). In contrast to our findings in humans, Higaki et al. (41) found normal glucose uptake in response to insulin in isolated muscles of IRS-2 knockout mice. One potential explanation for this discrepancy could be that in the absence of IRS-2, compensatory mechanisms may be involved. Thus, whereas the exact role of IRS-2 in metabolic insulin signaling in skeletal muscle is not fully understood, our data could be consistent with the concept that defects in signaling through IRS-2 may contribute to insulin resistance.

In the present study, the decreased IRS-2–associated PI3 kinase activation was associated with a decreased IRS-2 tyrosine phosphorylation in myotubes in response to insulin. It is well established that tyrosine phosphorylation of IRS proteins by the insulin receptor is key for their association with and activation of PI3 kinase. In the present experiments, however, the ability of the insulin receptor to be autophosphorylated was comparable in both groups. There could be at least two potential explanations for the impaired IRS-2 tyrosine phosphorylation. First, IRS-2 in myotubes from IGT patients could be phosphorylated on serine of threonine residues. Increased serine/threonine phosphorylation of IRS-1 is known to impair its tyrosine phosphorylation in response to insulin and to hamper intracellular signaling (42). Second, IRS-2 could be more rapidly dephosphorylated in myotubes from patients with IGT. For example, overexpression of protein tyrosine phosphatase (PTPase) 1 in L6 cells negatively regulates insulin signaling by decreasing IRS-1 tyrosine phosphorylation (43). Consistent with this hypothesis, PTPase activity is augmented in skeletal muscle and adipose tissue of obese subjects (44,45).

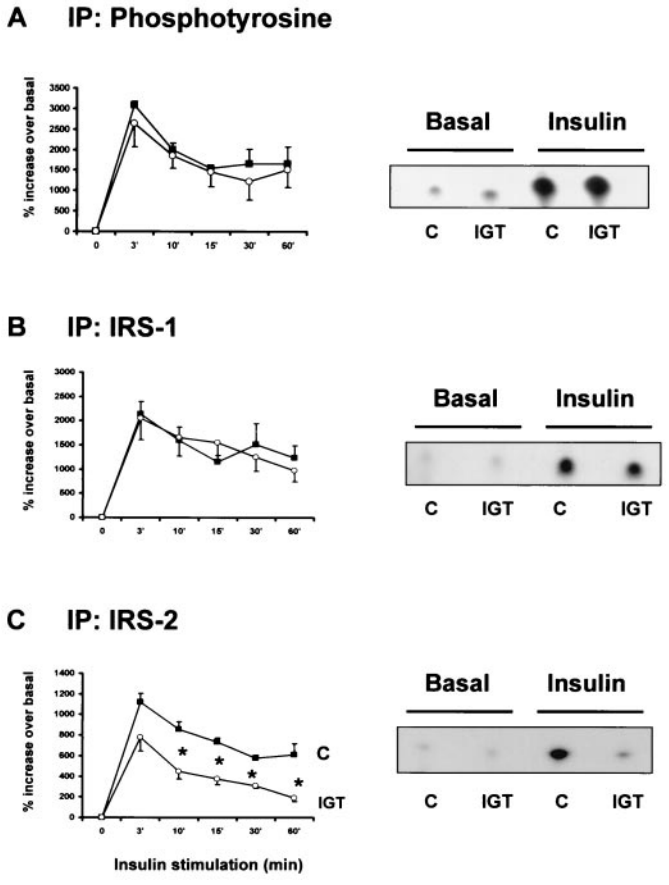

FIG. 6. PI3 kinase activation in response to insulin. Myotubes were starved overnight and then stimulated with insulin (17 nmol/l) for the indicated times. Cells were lysed and tyrosine phosphoprotein (*A*), IRS-1 (*B*), and IRS-2 (*C*) immunoprecipitated with specific antibodies from equal amount of proteins, and PI3 kinase activity was determined in the pellets by an in vitro kinase assay. Line graphs show summary data (means ± SE) of increased activation over basal for control subjects (■) and IGT patients (○). Inserts show characteristic PI3 kinase assays for a control subject (C) and an IGT patient at basal and 30 min of insulin stimulation. PI3 kinase activity associated with IRS-2 was significantly decreased in patients with IGT. *$P < 0.005$. IP, immunoprecipitation.

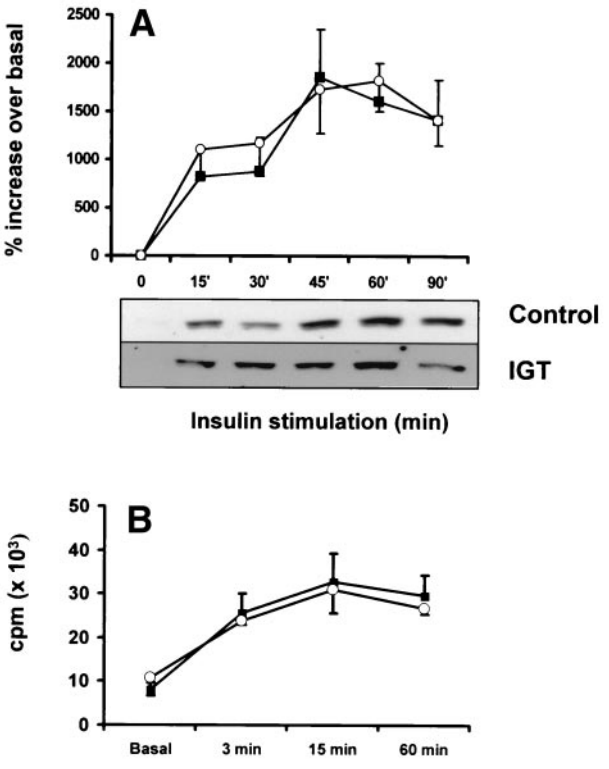

FIG. 7. Akt activation in response to insulin. Myotubes were starved overnight and stimulated with insulin (17 nmol/l) for the indicated times. Phospho-Akt was determined in cell lysates with specific phospho-Akt antibodies (ser 473) (*A*), and Akt activity was determined in immunoprecipitates using an in vitro kinase assay (*B*). Representative Western blots are shown for a control subjects and an IGT patient. Line graphs show summary data (means ± SE) for control subjects (■) and IGT patients (○). Activation and phosphorylation of Akt were similar in both groups.

To further analyze potential insulin-signaling defects downstream of PI3 kinase, we assessed Akt and PKC-ζ/λ activation. The main finding was that in myotubes of obese patients with IGT, PKC-ζ/λ activation in response to insulin is markedly impaired, whereas Akt activation is comparable in the two groups. Atypical PKCs (ζ/λ) are activated by insulin in a PI3 kinase–dependent manner and have been suggested to be involved in insulin-mediated glucose uptake (6,10,46). Indeed, in L6 myotubes, overexpression of a kinase-defective PKC-λ mutant almost completely blocked insulin-mediated glucose uptake (10). Furthermore, in vivo adenoviral delivery of recombinant PKC-ζ stimulated glucose transport activity in rat skeletal muscle (47). These data would suggest that impaired PKC-ζ/λ activation in myotubes of our IGT patients may be involved in the observed insulin resistance. The defect in PKC-ζ/λ activation is unlikely to be related to decreased PI3 kinase activity associated with tyrosine phosphoproteins or IRS-1 (because they were comparable in the two groups) but rather with the decreased IRS-2–associated PI3 kinase activation. Consistent with this possibility, Oriente et al. (48) showed that in L6 myotubes, overexpression of the kinase regulatory loop binding domain of IRS-2, which decreased IRS-2 but not IRS-1, tyrosine phosphorylation, and PI3 kinase activation, inhibited PKC-ζ activation in response to insulin. These findings may also suggest that IRS-2–associated PI3 kinase activation may occur in a different intracellular compartment from where IRS-1–associated PI3-kinase is activated. Consistent with this possibility, tyrosine-phosphorylated IRS-1 and IRS-2 are differentially located inside the cell (49,50).

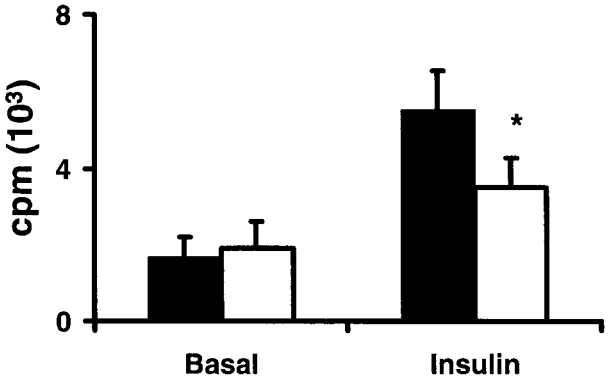

FIG. 8. PKC-ζ/λ activation in response to insulin. Myotubes were starved overnight and treated without or with insulin (17 nmol/l) for 10 min. After incubation, PKC-ζ/λ was immunoprecipitated with anti–COOH-terminal antiserum and assayed. Bar graphs are summary data (means ± SE) for control subjects (■) and IGT patients (□). PKC-ζ/λ activation in patients with IGT was significantly decreased (*$P < 0.05$).

In vitro, Akt has been suggested to participate to insulin-induced GLUT4 translocation. Akt activation, assessed by an in vitro kinase assay and phosphorylation of serine 473, was comparable in the two groups. These results suggest that Akt activation is not a key step downstream of PI3 kinase that has to be activated in response to insulin for glucose uptake. These findings can be related to a recent report in which Akt activation measured in muscle biopsies during a euglycemic-hyperinsulinemic clamp in patients with type 2 diabetes and control subjects was comparable (19). Finally, we cannot exclude that a defect in Akt activation in a specific subcellular compartment associated with decreased IRS-2–associated PI3 kinase activity may contribute to the observed insulin resistance in myotubes from patients with IGT.

Our results show for the first time that myotubes from obese patients with IGT are insulin resistant in vitro. It has previously been reported that myotubes from patients with type 2 diabetes are insulin resistant in vitro (23,28). In these studies, the defect observed in vitro was well correlated with in vivo measurements of insulin sensitivity. In another report, Krüzfeldt et al. (32) measured insulin sensitivity of myotubes from normoglycemic first-degree relatives of patients with type 2 diabetes and found no difference in glucose metabolism compared with control subjects. In their study, insulin-resistant subjects were not glucose intolerant.

In contrast to earlier reports, in our system, we did not observe decreased IRS-1 tyrosine phosphorylation and IRS-1–associated PI3 kinase activation (18,19,51). There could be several explanations for this. First, most of these studies were performed in muscle biopsies from patients with type 2 diabetes, whereas we studied patients with IGT. Some of the studies using muscle biopsies were performed in subjects who were hyperglycemic, which is known to induce insulin resistance per se (glucotoxicity). In the study by Goodyear et al. (18), the subjects were morbidly obese (BMI >50 kg/m^2), whereas we studied patients with a BMI of 34 kg/m^2. Insulin signaling was studied in muscle strips where there also could still have been the influence of environmental factors such as increased free fatty acids. In the myotube culture system, glycemia and insulinemia were well controlled.

In conclusion, myotubes from patients with IGT display insulin resistance in vitro, and this defect is associated with impaired IRS-2–associated PI3 kinase activation and PKC-ζ/λ activation in response to insulin. Finally, although our patients clearly had IGT, they were also obese, and we cannot exclude that some of the observed effects may be due to obesity per se.

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REFERENCES

1. DeFronzo RA: Pathogenesis of type 2 (non-insulin dependent) diabetes mellitus: a balanced overview. *Diabetologia* 35:389–397, 1992
2. Shulman GI: Cellular mechanisms of insulin resistance. *J Clin Invest* 106:171–176, 2000
3. Guma A, Zierath JR, Wallberg-Henriksson H, Klip A: Insulin induces translocation of GLUT-4 glucose transporters in human skeletal muscle. *Am J Physiol* 268:E613–E622, 1995
4. White MF: The insulin signalling system and the IRS proteins. *Diabetologia* 40 (Suppl. 2):S2–S17, 1997
5. Kohn AD, Summers SA, Birnbaum MJ, Roth RA: Expression of a constitutively active Akt Ser/Thr kinase in 3T3–L1 adipocytes stimulates glucose uptake and glucose transporter 4 translocation. *J Biol Chem* 271:31372–31378, 1996
6. 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 clambda for insulin stimulation of glucose uptake but not for Akt activation in 3T3–L1 adipocytes. *Mol Cell Biol* 18:6971–6982, 1998
7. Cong LN, Chen H, Li Y, Zhou L, McGibbon MA, Taylor SI, Quon MJ: Physiological role of Akt in insulin-stimulated translocation of GLUT4 in transfected rat adipose cells. *Mol Endocrinol* 11:1881–1890, 1997
8. Kitamura T, Ogawa W, Sakaue H, Hino Y, Kuroda S, Takata M, Matsumoto M, Maeda T, Konishi H, Kikkawa U, Kasuga M: Requirement for activation of the serine-threonine kinase Akt (protein kinase B) in insulin stimulation of protein synthesis but not of glucose transport. *Mol Cell Biol* 18:3708–3717, 1998
9. Standaert ML, Galloway L, Karnam P, Bandyopadhyay G, Moscat J, Farese RV: Protein kinase C-zeta as a downstream effector of phosphatidylinositol 3-kinase during insulin stimulation in rat adipocytes: potential role in glucose transport. *J Biol Chem* 272:30075–30082, 1997
10. Bandyopadhyay G, Kanoh Y, Sajan MP, Standaert ML, Farese RV: Effects of adenoviral gene transfer of wild-type, constitutively active, and kinase-defective protein kinase C-lambda on insulin-stimulated glucose transport in L6 myotubes. *Endocrinology* 141:4120–4127, 2000
11. Tamemoto H, Kadowaki T, Tobe K, Yagi T, Sakura H, Hayakawa T, Terauchi Y, Ueki K, Kaburagi Y, Satoh S: Insulin resistance and growth retardation in mice lacking insulin receptor substrate-1. *Nature* 372:182–186, 1994
12. Araki E, Lipes MA, Patti ME, Bruning JC, Haag B, Johnson RS, Kahn CR: Alternative pathway of insulin signalling in mice with targeted disruption of the IRS-1 gene. *Nature* 372:186–190, 1994
13. Withers DJ, Gutierrez JS, Towery H, Burks DJ, Ren JM, Previs S, Zhang Y, Bernal D, Pons S, Shulman GI, Bonner-Weir S, White MF: Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* 391:900–904, 1998
14. Liu SC, Wang Q, Lienhard GE, Keller SR: Insulin receptor substrate 3 is not essential for growth or glucose homeostasis. *J Biol Chem* 274:18093–18099, 1999
15. Fantin VR, Lavan BE, Wang Q, Jenkins NA, Gilbert DJ, Copeland NG, Keller SR, Lienhard GE: Cloning, tissue expression, and chromosomal location of the mouse insulin receptor substrate 4 gene. *Endocrinology* 140:1329–1337, 1999
16. Nolan JJ, Freidenberg G, Henry R, Reichart D, Olefsky JM: Role of human skeletal muscle insulin receptor kinase in the in vivo insulin resistance of noninsulin-dependent diabetes mellitus and obesity. *J Clin Endocrinol Metab* 78:471–477, 1994
17. Maegawa H, Shigeta Y, Egawa K, Kobayashi M: Impaired autophosphorylation of insulin receptors from abdominal skeletal muscles in nonobese subjects with NIDDM. *Diabetes* 40:815–819, 1991
18. 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
19. 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
20. Dresner A, Laurent D, Marcucci M, Griffin ME, Dufour S, Cline GW, Slezak LA, Andersen DK, Hundal RS, Rothman DL, Petersen KF, Shulman GI: Effects of free fatty acids on glucose transport and IRS-1-associated phosphatidylinositol 3-kinase activity. *J Clin Invest* 103:253–259, 1999

21. Jarrett RJ, Keen H, McCartney P: The Whitehall Study: ten year follow-up report on men with impaired glucose tolerance with reference to worsening to diabetes and predictors of death. *Diabet Med* 1:279–283, 1984
22. Keen H, Jarrett RJ, McCartney P: The ten-year follow-up of the Bedford survey (1962–1972): glucose tolerance and diabetes. *Diabetologia* 22:73–78, 1982
23. Henry RR, Abrams L, Nikoulina S, Ciaraldi TP: Insulin action and glucose metabolism in nondiabetic control and NIDDM subjects: comparison using human skeletal muscle cell cultures. *Diabetes* 44:936–946, 1995
24. Blau HM, Webster C: Isolation and characterization of human muscle cells. *Proc Natl Acad Sci U S A* 78:5623–5627, 1981
25. Sarabia V, Lam L, Burdett E, Leiter LA, Klip A: Glucose transport in human skeletal muscle cells in culture: stimulation by insulin and metformin. *J Clin Invest* 90:1386–1395, 1992
26. Henry RR, Ciaraldi TP, Abrams-Carter L, Mudaliar S, Park KS, Nikoulina SE: Glycogen synthase activity is reduced in cultured skeletal muscle cells of non-insulin-dependent diabetes mellitus subjects: biochemical and molecular mechanisms. *J Clin Invest* 98:1231–1236, 1996
27. Henry RR, Ciaraldi TP, Mudaliar S, Abrams L, Nikoulina SE: Acquired defects of glycogen synthase activity in cultured human skeletal muscle cells: influence of high glucose and insulin levels. *Diabetes* 45:400–407, 1996
28. Thompson DB, Pratley R, Ossowski V: Human primary myoblast cell cultures from non-diabetic insulin resistant subjects retain defects in insulin action. *J Clin Invest* 98:2346–2350, 1996
29. Standaert ML, Bandyopadhyay G, Sajan MP, Cong L, Quon MJ, Farese RV: Okadaic acid activates atypical protein kinase C (zeta/lambda) in rat and 3T3/L1 adipocytes: an apparent requirement for activation of Glut4 translocation and glucose transport. *J Biol Chem* 274:14074–14078, 1999
30. Standaert ML, Bandyopadhyay G, Perez L, Price D, Galloway L, Poklepovic A, Sajan MP, Cenni V, Sirri A, Moscat J, Toker A, Farese RV: Insulin activates protein kinases C-zeta and C-lambda by an autophosphorylation-dependent mechanism and stimulates their translocation to GLUT4 vesicles and other membrane fractions in rat adipocytes. *J Biol Chem* 274:25308–25316, 1999
31. Garvey WT, Maianu L, Zhu JH, Brechtel-Hook G, Wallace P, Baron AD: Evidence for defects in the trafficking and translocation of GLUT4 glucose transporters in skeletal muscle as a cause of human insulin resistance. *J Clin Invest* 101:2377–2386, 1998
32. Krutzfeldt J, Kausch C, Volk A, Klein HH, Rett K, Haring HU, Stumvoll M: Insulin signaling and action in cultured skeletal muscle cells from lean healthy humans with high and low insulin sensitivity. *Diabetes* 49:992–998, 2000
33. Anai M, Funaki M, Ogihara T, Terasaki J, Inukai K, Katagiri H, Fukushima Y, Yazaki Y, Kikuchi M, Oka Y, Asano T: Altered expression levels and impaired steps in the pathway to phosphatidylinositol 3-kinase activation via insulin receptor substrates 1 and 2 in Zucker fatty rats. *Diabetes* 47:13–23, 1998
34. Rondinone CM, Wang LM, Lonnroth P, Wesslau C, Pierce JH, Smith U: Insulin receptor substrate (IRS) 1 is reduced and IRS-2 is the main docking protein for phosphatidylinositol 3-kinase in adipocytes from subjects with non-insulin-dependent diabetes mellitus. *Proc Natl Acad Sci U S A* 94:4171–4175, 1997
35. Haruta T, Morris AJ, Rose DW, Nelson JG, Mueckler M, Olefsky JM: Insulin-stimulated GLUT4 translocation is mediated by a divergent intracellular signaling pathway. *J Biol Chem* 270:27991–27994, 1995
36. Martin SS, Haruta T, Morris AJ, Klippel A, Williams LT, Olefsky JM: Activated phosphatidylinositol 3-kinase is sufficient to mediate actin rearrangement and GLUT4 translocation in 3T3–L1 adipocytes. *J Biol Chem* 271:17605–17608, 1996
37. Fasshauer M, Klein J, Ueki K, Kriauciunas KM, Benito M, White MF, Kahn CR: Essential role of insulin receptor substrate-2 in insulin stimulation of Glut4 translocation and glucose uptake in brown adipocytes. *J Biol Chem* 275:25494–25501, 2000
38. Rother KI, Imai Y, Caruso M, Beguinot F, Formisano P, Accili D: Evidence that IRS-2 phosphorylation is required for insulin action in hepatocytes. *J Biol Chem* 273:17491–17497, 1998
39. Zhou L, Chen H, Lin CH, Cong LN, McGibbon MA, Sciacchitano S, Lesniak MA, Quon MJ, Taylor SI: Insulin receptor substrate-2 (IRS-2) can mediate the action of insulin to stimulate translocation of GLUT4 to the cell surface in rat adipose cells. *J Biol Chem* 272:29829–29833, 1997
40. Previs SF, Withers DJ, Ren JM, White MF, Shulman GI: Contrasting effects of IRS-1 versus IRS-2 gene disruption on carbohydrate and lipid metabolism in vivo. *J Biol Chem* 275:38990–38994, 2000
41. Higaki Y, Wojtaszewski JF, Hirshman MF, Withers DJ, Towery H, White MF, Goodyear LJ: Insulin receptor substrate-2 is not necessary for insulin- and exercise-stimulated glucose transport in skeletal muscle. *J Biol Chem* 274:20791–20795, 1999
42. Paz K, Hemi R, LeRoith D, Karasik A, Elhanany E, Kanety H, Zick Y: A molecular basis for insulin resistance: elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. *J Biol Chem* 272:29911–29918, 1997
43. Egawa K, Maegawa H, Shimizu S, Morino K, Nishio Y, Bryer-Ash M, Cheung A, Kolls J, Kikkawa R, Kashiwagi A: Protein tyrosine phosphatase-1B negatively regulates insulin signaling in L6 myocytes and Fao heptaoma cells. *J Biol Chem* 276:10207–10211, 2001
44. Ahmad F, Azevedo JL, Cortright R, Dohm GL, Goldstein BJ: Alterations in skeletal muscle protein-tyrosine phosphatase activity and expression in insulin-resistant human obesity and diabetes. *J Clin Invest* 100:449–458, 1997
45. McGuire MC, Fields RM, Nyomba BL, Raz I, Bogardus C, Tonks NK, Sommercorn J: Abnormal regulation of protein tyrosine phosphatase activities in skeletal muscle of insulin-resistant humans. *Diabetes* 40:939–942, 1991
46. Bandyopadhyay G, Standaert ML, Zhao L, Yu B, Avignon A, Galloway L, Karnam P, Moscat J, Farese RV: Activation of protein kinase C (alpha, beta, and zeta) by insulin in 3T3/L1 cells: transfection studies suggest a role for PKC-zeta in glucose transport. *J Biol Chem* 272:2551–2558, 1997
47. Etgen GJ, Valasek KM, Broderick CL, Miller AR: In vivo adenoviral delivery of recombinant human protein kinase C-zeta stimulates glucose transport activity in rat skeletal muscle. *J Biol Chem* 274:22139–22142, 1999
48. Oriente F, Formisano P, Miele C, Fiory F, Maitan MA, Vigliotta G, Trencia A, Santopietro S, Caruso M, Van Obberghen E, Beguinot F: Insulin receptor substrate-2 phosphorylation is necessary for protein kinase C zeta activation by insulin in L6hIR cells. *J Biol Chem* 276:37109–37119, 2001
49. Inoue G, Cheatham B, Emkey R, Kahn CR: Dynamics of insulin signaling in 3T3–L1 adipocytes: differential compartmentalization and trafficking of insulin receptor substrate (IRS)-1 and IRS-2. *J Biol Chem* 273:11548–11555, 1998
50. Caruso M, Miele C, Oliva A, Condorelli G, Oriente F, Riccardi G, Capaldo B, Fiory F, Accili D, Formisano P, Beguinot F: The IR1152 mutant insulin receptor selectively impairs insulin action in skeletal muscle but not in liver. *Diabetes* 49:1194–1202, 2000
51. 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