

Characterization of Signal Transduction and Glucose Transport in Skeletal Muscle From Type 2 Diabetic Patients

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We characterized metabolic and mitogenic signaling pathways in isolated skeletal muscle from well-matched type 2 diabetic and control subjects. Time course studies of the insulin receptor, insulin receptor substrate (IRS)-1/2, and phosphatidylinositol (PI) 3-kinase revealed that signal transduction through this pathway was engaged between 4 and 40 min. Insulin-stimulated (0.6–60 nmol/l) tyrosine phosphorylation of the insulin receptor β -subunit, mitogen-activated protein (MAP) kinase phosphorylation, and glycogen synthase activity were not altered in type 2 diabetic subjects. In contrast, insulin-stimulated tyrosine phosphorylation of IRS-1 and anti-phosphotyrosine-associated PI 3-kinase activity were reduced 40–55% in type 2 diabetic subjects at high insulin concentrations (2.4 and 60 nmol/l, respectively). Impaired glucose transport activity was noted at all insulin concentrations (0.6–60 nmol/l). Aberrant protein expression cannot account for these insulin-signaling defects because expression of insulin receptor, IRS-1, IRS-2, MAP kinase, or glycogen synthase was similar between type 2 diabetic and control subjects. In skeletal muscle from type 2 diabetic subjects, IRS-1 phosphorylation, PI 3-kinase activity, and glucose transport activity were impaired, whereas insulin receptor tyrosine phosphorylation, MAP kinase phosphorylation, and glycogen synthase activity were normal. Impaired insulin signal transduction in skeletal muscle from type 2 diabetic patients may partly account for reduced insulin-stimulated glucose transport; however, additional defects are likely to play a role. *Diabetes* 49:284–292, 2000

Skeletal muscle is a primary site of insulin-stimulated glucose disposal, which accounts for 70–80% of postprandial glucose disposal (1). In vivo studies reveal that insulin resistance in skeletal muscle is one of the first measurable defects associated with type 2 dia-

betes (2,3). The molecular basis for the development of whole-body insulin resistance remains unclear, although decreased insulin-stimulated glucose transport activity has been observed in isolated skeletal muscle from lean and obese people with type 2 diabetes (4–8). Because glucose transport is an early step in peripheral glucose utilization, a defect in glucose transport most likely plays a major role in the pathogenesis of peripheral insulin resistance (9). Thus, an understanding of the mechanisms that control glucose transport into insulin-sensitive tissues is essential to develop strategies for reestablishing normal glucose homeostasis in people with type 2 diabetes.

Insulin-stimulated glucose transport is achieved by translocation of the major insulin-responsive glucose transporter, GLUT4, from an intracellular vesicle storage site to the plasma membrane and transverse tubules (10–12). Reduced glucose transport activity in skeletal muscle from people with type 2 diabetes may be a consequence of impaired insulin signal transduction (13,14) and/or alterations in the traffic and translocation of GLUT4 to the plasma membrane (8,15,16). Insulin binding to the insulin receptor initiates a cascade of intracellular signaling events, which include receptor autophosphorylation and subsequent phosphorylation of downstream target molecules, such as insulin receptor substrates and Shc (17). Of the four different IRS proteins cloned (18–21) to date, IRS-1 and IRS-2 are the major IRSs in skeletal muscle. IRS proteins are docking proteins for downstream signaling molecules that contain Src homology 2 domains, including the 85-kDa regulatory subunit of phosphatidylinositol (PI) 3-kinase (17). PI 3-kinase is a key signaling transducer that mediates the metabolic effects of insulin, such as glucose transport (22). Signal transduction defects at the level of the insulin receptor, IRS-1, IRS-2, and/or PI 3-kinase may lead to impaired glucose transport and impaired whole-body glucose uptake in people with type 2 diabetes.

Evidence from animal studies suggests that insulin-signaling defects at the level of IRS-1 and PI 3-kinase are associated with altered whole-body glucose homeostasis (23–26). However, the majority of these studies have been performed with the use of severely obese monogenic animal models of insulin resistance, which makes it difficult to predict the extent of signal transduction defects in lean to moderately overweight type 2 diabetic patients. We have shown that insulin-stimulated tyrosine phosphorylation of IRS-1 and PI 3-kinase activity are impaired in muscle from moderately overweight people with type 2 diabetes (13). Nevertheless, the

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ANOVA, analysis of variance; BSA, bovine serum albumin; DTT, dithiothreitol; ECL, enhanced chemiluminescence; ERK, extracellular-regulated kinase; FFA, free fatty acid; IRS, insulin receptor substrate; KHB, Krebs-Henseleit buffer; MAP, mitogen-activated protein; PI, phosphatidylinositol; PVDF, polyvinylidene difluoride; TNF- α , tumor necrosis factor- α .

full extent of these signaling defects has not been determined. For example, it is not yet known whether insulin-signaling defects in skeletal muscle from people with type 2 diabetes are results of impaired insulin responsiveness or sensitivity or aberrant protein expression of the signal transduction intermediates.

Our aim was to characterize metabolic and mitogenic signal transduction networks in isolated skeletal muscle from people with type 2 diabetes and well-matched control subjects. To achieve our aim, we initially established the time course for the activation of key components of the insulin-signaling cascade and then established insulin dose-response curves for the activation of the insulin receptor, IRS-1/2, PI 3-kinase, glucose transport, glycogen synthase, and MAP kinase. We then determined whether changes in expression of key proteins in the insulin-signaling pathway account for impaired insulin action in skeletal muscle from type 2 diabetic patients.

RESEARCH DESIGN AND METHODS

Subject characteristics. The study protocol was reviewed and approved by the institutional ethical committee of the Karolinska Institute, and informed consent was received from all subjects before participation. The clinical characteristics of the subjects are presented in Table 1. The diabetic group comprised 13 type 2 diabetic men with a mean duration of disease of 6 years (range 0–12). Glycemic control, which was evaluated by measuring levels of HbA_{1c}, was moderate (6.7 ± 0.4%). The normal range for HbA_{1c} concentration in our laboratory was <5.2%. Of the 13 patients in the diabetic group, 3 subjects were treated with insulin alone, 2 subjects were treated with a combination of sulfonylureas and insulin, 5 subjects were treated with sulfonylureas, and 3 subjects were treated with diet alone. The control group comprised 18 healthy men. Skeletal muscle biopsies were obtained from a subgroup of five healthy men and were used for the initial determination of the time course of insulin action in skeletal muscle. Of the study participants, none were smokers or were taking any other medication. The subjects were instructed to abstain from any form of strenuous physical activity for a period of 48 h before the experiment. On the day of the test, the subjects reported to the laboratory after an overnight fast and, in the case of the type 2 diabetic patients, before administration of any antidiabetic medication.

Euglycemic-hyperinsulinemic clamp procedure. Peripheral insulin sensitivity was evaluated by the euglycemic-hyperinsulinemic clamp procedure (27). A polyethylene catheter was placed in the brachial artery for blood determinations. A second catheter was placed in an antecubital vein for glucose and insulin infusions. A bolus infusion of insulin was administered for 5 min (18.4 pmol · kg⁻¹ · min⁻¹). The infusion rate was reduced for 5 min (9.2 pmol · kg⁻¹ · min⁻¹) followed by a continuous infusion of insulin (6.9 pmol · kg⁻¹ · min⁻¹) for 2 h to obtain physiological hyperinsulinemia. The fasting plasma glucose concentration was maintained throughout the insulin infusion by means of a variable glucose infusion and blood glucose determinations every 5 min. Whole-body peripheral glucose utilization was calculated during the last 100-min period of the steady-state insulin infusion.

Blood chemistry. Plasma glucose levels were measured by a glucose-oxidase method. Serum immunoreactive insulin was assayed by using the Phadesept Insulin (RIA) method (Pharmacia, Uppsala, Sweden); this method's lower limit of sensitivity is 18 pmol/l of insulin. HbA_{1c} concentration was determined by using a specific ion-exchange chromatography kit (mono S HR 5/5; Pharmacia). Plasma free fatty acid (FFA) levels were determined by using a microfluorometric method (28). Levels of serum triglycerides, HDL cholesterol, and LDL cholesterol were assessed by reflectance spectrometry by using Kodak Ektachem clinical chemistry slides (Eastman Kodak, Rochester, NY).

Maximal oxygen uptake determination. On a separate occasion, maximal oxygen uptake ($\dot{V}O_{2max}$) was determined on a bicycle ergometer as previously described (29). $\dot{V}O_{2max}$ was measured continuously with a breath-by-breath data collection technique (Erich Jaeger, Hoechst, Germany) and calculated at each 20-s interval.

Body composition measurements. Regional body analysis of lean body mass, body fat, and bone mineral content was performed by dual-energy X-ray absorptiometry (Lunar, Madison, WI) (30) as reported earlier (31).

Open muscle biopsy procedure and in vitro incubation of human skeletal muscle. Open muscle biopsies were performed as previously described (32). Muscle biopsies were obtained from a healthy control subject and a type 2 diabetic patient on the same day and were processed in parallel. Biopsies were obtained under local anesthesia from the vastus lateralis portion of the quadriceps femoris muscle and were placed in oxygenated Krebs-Henseleit buffer (KHB), which

contained 5 mmol/l HEPES and 0.1% bovine serum albumin (BSA) (RIA Grade; Sigma, St. Louis, MO). Smaller muscle specimens were dissected, mounted on Plexiglass clamps, and incubated in vitro in pre-gassed (95% O₂ and 5% CO₂) KHB as previously described (32). The gas phase in the vials was maintained at 95% O₂ and 5% CO₂ during each incubation. Muscles were incubated at 35°C for 30 min in KHB containing 5 mmol/l glucose and 15 mmol/l mannitol and for 20 min without or with insulin (0.6, 2.4, or 60 nmol/l as indicated).

Glucose transport activity. After the initial 20-min incubation described above, muscles were incubated at 35°C for an additional 20 min without or with insulin (0.6, 2.4, or 60 nmol/l as indicated) in KHB containing 5 mmol/l 3-O-methyl[³H]glucose (2.5 μCi/mmol) and 15 mmol/l [¹⁴C]mannitol (26.3 μCi/mmol). Thus, the total insulin exposure was 40 min. After incubation, the muscles were snap frozen in liquid nitrogen and stored at -80°C until processing. Thereafter, the incubated muscle specimens were homogenized in ice cold homogenizing buffer (50 mmol/l HEPES pH 7.6, 150 mmol/l NaCl, 1% Triton X 100, 1 mmol/l Na₃VO₄, 10 mmol/l NaF, 30 mmol/l Na₂P₂O₇, 10% [vol/vol] glycerol, 1 mmol/l benzamidine, 1 mmol/l dithiothreitol [DTT], 10 μg/ml leupeptin, 1 mmol/l phenylmethylsulfonyl fluoride, and 1 μmol/l microcystin) by using a glass-on-glass rotating homogenizer and were subjected to centrifugation (12,000g for 10 min at 4°C). Protein was determined in the supernatant by using a commercial kit (Bio-Rad, Richmond, CA). Glucose transport was assessed as described by Wallberg-Henriksson et al. (33), and data were expressed as 3-O-methylglucose mg · protein⁻¹ · h⁻¹.

Tyrosine phosphorylation of insulin receptor and IRS-1. From the remaining homogenate described above, an aliquot of the supernatant (800 μg) was immunoprecipitated as specified in the figure legends with the appropriate antibodies coupled to protein A Sepharose. The immunoprecipitates were washed four times in ice-cold homogenizing buffer, resuspended in Laemmli sample buffer with 100 mmol/l DTT, and heated at 95°C for 4 min. Proteins were separated by SDS-PAGE, transferred to polyvinylidene difluoride (PVDF) membranes incubated with anti-phosphotyrosine antibodies, washed, and incubated with appropriate secondary antibodies. The reactions were visualized by enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL) and quantified by densitometry.

PI 3-kinase activity. Muscle specimens were homogenized as described above, and an aliquot of the supernatant was immunoprecipitated overnight (4°C) with either anti-phosphotyrosine antibody, anti-IRS-1, or anti-IRS-2 antibody coupled to protein A-Sepharose (Sigma). PI 3-kinase activity was assessed directly on the protein A-Sepharose beads as previously reported (34). Reaction products were resolved by thin layer chromatography and were quantitated by using a PhosphorImager.

Glycogen synthase activity. Glycogen synthase activity was assessed in isolated skeletal muscle as previously described (35). Muscle was homogenized (1:50 dilution) in 50 mmol/l 4-MOPS, 25 mmol/l NaF, 20 mmol/l EDTA, and 0.1% BSA. Glycogen synthase activity was measured in the presence of 0.17 mmol/l UDP-glucose and either 0.2 mmol/l (active form) or 4 mmol/l (total activity) glucose-6-phosphate. Glycogen synthase activity is reported as fractional activity of the enzyme (active/total).

MAP kinase phosphorylation. Aliquots (30 μg) of the supernatant were separated by SDS-PAGE (10% resolving gel), blocked overnight (5% milk in Tris-buffered saline with 0.1% Tween 20), and subjected to immunoblot analysis with phosphospecific MAP kinase (extracellular-regulated kinase [ERK]-1/ERK2) rabbit polyclonal antibody. Membranes were washed in Tris-buffered saline with 0.1% Tween 20. Bound antibodies were detected with peroxidase-linked anti-rabbit IgG (1:2,000) (Amersham, Buckinghamshire, England) and were incubated at room temperature for 1 h. Protein phosphorylation was visualized by ECL, and phosphorylation was quantified by densitometry.

Protein expression. For protein expression of insulin receptor, IRS-1/2, p85 α -subunit of PI 3-kinase, glycogen synthase, or MAP kinase (ERK1/ERK2), an aliquot (40 μg) of supernatant was resuspended in sample buffer and heated at 95°C for 1 min. Proteins were separated by SDS-PAGE, transferred to PVDF membranes, and blocked as described above. Membranes were incubated with the appropriate primary antibodies as indicated in Fig. 8 and were washed and incubated with appropriate secondary antibodies as recommended by the supplier (Amersham). Proteins were visualized by ECL and were quantified by densitometry.

Materials and antibodies. PI was purchased from Avanti Polar Lipids (Alabaster, AL), and the aluminum-backed Silica Gel 60 thin-layer chromatography plates were purchased from EM Separations (Gibbstown, NJ). All other chemicals were purchased from Sigma or Merck (Rahway, NJ). The insulin receptor antibody (CT-3) was raised against the COOH-terminal portion of the receptor and was obtained from Dr. Ken Sidde (Cambridge University, Cambridge, U.K.). Anti-phosphotyrosine antibodies were from Signal Transduction Laboratories (Lexington, KY). IRS-1 and IRS-2 antibodies were obtained from Dr. Morris White (Joslin Diabetes Center, Boston, MA). The p85 α -antibody was obtained from Dr. Jonathan Backer (Albert Einstein College of Medicine, Bronx, NY). Glycogen synthase antibodies were from Drs. Sten Lund and Oluf Pedersen (Steno Diabetes Center, Copenhagen). MAP kinase expression and phosphorylation were determined

TABLE 1
Characteristics of the study subjects

	Type 2 diabetic subjects	Control subjects
n	13	18
Age (years)	57.5 ± 1.7	55.9 ± 1.3
BMI (kg/m ²)	27.3 ± 1.0	25.9 ± 0.5
Body fat (%)	23.4 ± 2.0	23.4 ± 1.3
VO _{2max}	29.3 ± 1.4	32.3 ± 1.3
Fasting glucose (mmol/l)	9.2 ± 0.7*	5.4 ± 0.1
Fasting insulin (pmol/l)	90 ± 15*	38 ± 4
FFA (μmol/l)	631 ± 65	597 ± 57
Serum triglycerides (mmol/l)	1.33 ± 0.13	1.25 ± 0.12
Serum cholesterol (mmol/l)	4.88 ± 0.23	5.01 ± 0.18
HDL cholesterol	1.21 ± 0.09	1.35 ± 0.06
LDL cholesterol	3.06 ± 0.22	3.12 ± 0.17
HbA _{1c} (%)	6.7 ± 0.4*	4.0 ± 0.2
Glucose utilization (μmol · kg ⁻¹ · min ⁻¹)	27.8 ± 4.0†	39.1 ± 3.0
Duration of diabetes (years)	5.7 ± 1.2	—

Data are means ± SE. *P < 0.001 vs. control subjects; †P < 0.05 vs. control subjects.

with pan-ERK and phospho-specific ERK antibodies, respectively (New England Biolabs, Beverly, MA).

Statistics. Data are presented as means ± SE. Muscle from one type 2 diabetic subject and one healthy control subject was processed in parallel for each analysis. Statistical difference between the clinical characteristics of the study participants was determined by an unpaired Student's t test. Differences for insulin signal transduction within and between the subjects were determined through analysis of variance (ANOVA). A least-significant-difference test was used for post hoc analysis.

RESULTS

Subject characteristics. Type 2 diabetic and control subjects were matched for age, BMI, and physical fitness (Table 1). Because insulin resistance is closely associated with obesity, we also measured percentage of body fat in the study participants, which was similar in type 2 diabetic and control subjects. Thus, any physiological difference between the type 2 diabetic and control subjects cannot be explained by either poor physical fitness or increased whole-body adiposity. Fasting plasma glucose and serum insulin levels were elevated in the type 2 diabetic subjects (P > 0.001 vs. control subjects). However, the blood lipid and cholesterol profiles were similar between the type 2 diabetic and control subjects. HbA_{1c} levels were only moderately elevated, which suggests that the type 2 diabetic subjects had good metabolic control. Time course for the activation of the insulin-signaling molecules. Isolated skeletal muscle was incubated in the absence or presence of 60 nmol/l insulin for 4–40 min and was then immediately frozen in liquid nitrogen. Muscles were homogenized as described in RESEARCH DESIGN AND METHODS. Tyrosine phosphorylation of the insulin receptor was determined by immunoprecipitation of muscle lysates with an anti-phosphotyrosine antibody followed by immunoblot analysis with an anti-insulin receptor antibody. Insulin exposure led to a profound increase in phosphorylation of a 95-kDa band. Peak phosphorylation of the insulin receptor was noted at 8 min (Fig. 1A). An 18- to 23-fold increase in phosphorylation was noted at all time points. Tyrosine phosphorylation of IRS-1 was determined by immunoprecipitation

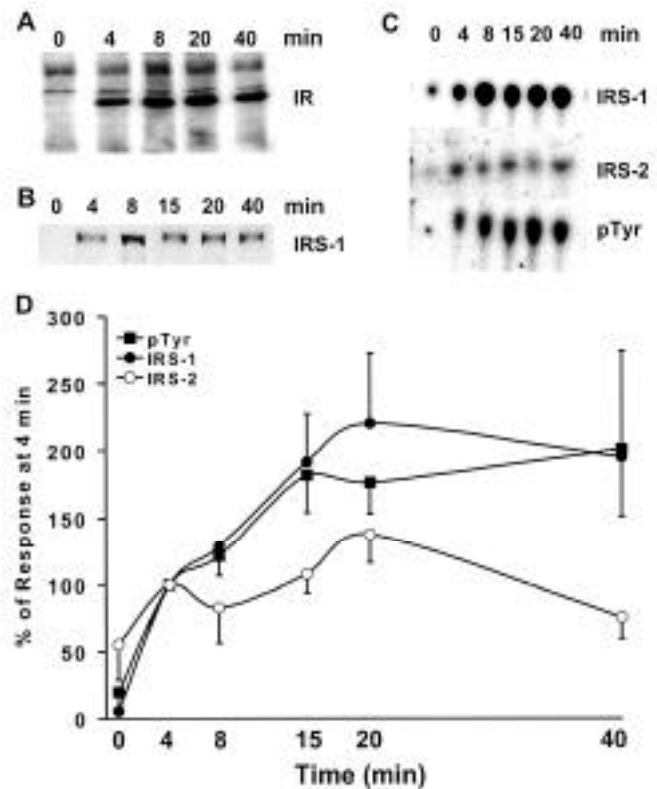


FIG. 1. Time course of activation of key insulin-signaling molecules. Isolated skeletal muscle was incubated in vitro in the absence or presence of 60 nmol/l insulin for 4–40 min. A: Insulin receptor autophosphorylation was assessed by anti-phosphotyrosine immunoprecipitation of muscle lysate followed by immunoblotting with an insulin-receptor antibody. Similar results were obtained for two subjects. B: Tyrosine phosphorylation of IRS-1 was determined in IRS-1 immunoprecipitates as described in RESEARCH DESIGN AND METHODS. Similar results were obtained in four subjects. C: Insulin-stimulated PI 3-kinase activity was assessed in anti-phosphotyrosine-, IRS-1-, or IRS-2 immunoprecipitates. The panel shows a representative autoradiogram after separation of reaction products by thin-layer chromatography. Similar results were obtained in five subjects. D: Quantitation of anti-phosphotyrosine-, IRS-1-, or IRS-2-associated PI 3-kinase activity in human skeletal muscle. PI 3-kinase activity was assessed in muscle samples as indicated in C. Data are expressed as percent of each respective (anti-phosphotyrosine-, IRS-1-, or IRS-2-associated) PI 3-kinase activity at 4 min. Data are means ± SE for five subjects.

of muscle lysates with an anti-IRS-1 antibody followed by immunoblot analysis with an anti-phosphotyrosine antibody. Insulin exposure led to an increase in phosphorylation of a 170-kDa protein (Fig. 1B). Peak IRS-1 tyrosine phosphorylation was noted at 8–15 min, and phosphorylation was sustained at all time points studied (11- to 23-fold increase in IRS-1 tyrosine phosphorylation). PI 3-kinase activity was determined in phosphotyrosine-, IRS-1-, or IRS-2 immunoprecipitates as described in RESEARCH DESIGN AND METHODS (Fig. 1C). Anti-phosphotyrosine- and IRS-1-associated PI 3-kinase activities were increased and sustained throughout the 40-min insulin exposure. The time course experiment revealed that both anti-phosphotyrosine- and IRS-1-associated PI 3-kinase activities occurred in parallel, which suggests that IRS-1 is the predominant tyrosine-phosphorylated molecule that transmits the insulin signal to PI 3-kinase in human skeletal muscle (Fig. 1D). IRS-2-associated PI 3-kinase activity reached its highest level at 20 min of insulin exposure; in

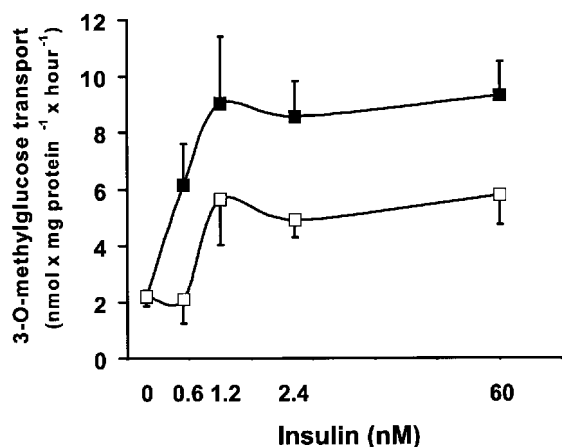


FIG. 2. Insulin-stimulated 3-O-methylglucose transport in isolated skeletal muscle from control (■) or type 2 diabetic subjects (□). Muscle strips were incubated at 35°C for 40 min in the absence or presence of 0.6, 1.2, 2.4, or 60 nmol/l insulin. 3-O-methylglucose transport was assessed as described in RESEARCH DESIGN AND METHODS. Data are means \pm SE (control subjects $n = 13$, type 2 diabetic subjects $n = 11$). The insulin response was compared using a two-way ANOVA. Curves are significantly different ($P < 0.001$).

contrast to anti-phosphotyrosine- and IRS-1-associated PI 3-kinase activity, IRS-2-associated PI 3-kinase activity decreased at 40 min. For subsequent analysis between type 2 diabetic and control subjects, anti-phosphotyrosine-associated PI 3-kinase activity was assessed. When performing these types of experiments in human subjects, there naturally are limits to the amount of samples that can be obtained. Thus, we have scaled down the signaling assays to measure several of the insulin-signaling intermediates in small muscle samples (20–30 mg). For all subsequent experiments, muscle specimens were exposed to insulin for a total of 40 min, because phosphorylation or activity of each of the signaling intermediates studied was increased at this time point. Furthermore, in human skeletal muscle, glucose transport activity is at steady state between 30 min and 4 h (32). Thus, we assessed insulin signal transduction and glucose transport for each insulin concentration in the same muscle specimen.

Skeletal muscle glucose transport. Isolated muscle was incubated in the absence or presence of insulin (0.6–60 nmol/l), and 3-O-methylglucose transport was determined (Fig. 2). In skeletal muscle from control subjects, insulin led to dose-dependent increases in glucose transport activity with a maximal $4.3 (\pm 0.9)$ -fold response observed in the presence of 60 nmol/l insulin ($P < 0.01$). In type 2 diabetic subjects, insulin-stimulated glucose transport activity was markedly blunted across the entire range of insulin concentrations ($P < 0.001$ vs. control subjects). Thus, insulin sensitivity and responsiveness are reduced in muscle from people with type 2 diabetes. These findings are consistent with previous studies from our laboratory in different cohorts of type 2 diabetic subjects (4,5,13–15).

Insulin receptor tyrosine phosphorylation. The decrease in insulin-stimulated glucose transport may be a consequence of impaired insulin receptor tyrosine kinase phosphorylation. Isolated muscle was incubated for 40 min in the absence or presence of insulin (0.6–60 nmol/l), and tyrosine phosphorylation of the insulin receptor was determined in

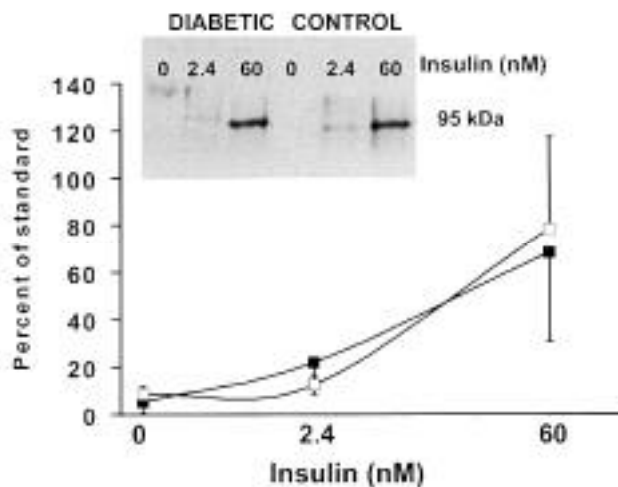


FIG. 3. Insulin-stimulated autophosphorylation of the insulin receptor was determined in isolated skeletal muscle incubated in vitro in the presence or absence of insulin at the indicated concentrations for 40 min. Muscles were snap frozen, homogenized, and analyzed as described in RESEARCH DESIGN AND METHODS. Data show means \pm SE for skeletal muscle from control (■) ($n = 7$) or type 2 diabetic (□) ($n = 6$) subjects. Results were normalized by comparison with a standard sample (insulin-stimulated rat liver standard). Inset shows representative immunoblot analysis.

lysates of skeletal muscle from type 2 diabetic and control subjects (Fig. 3). Preliminary experiments revealed a relatively low level of insulin receptor tyrosine phosphorylation in human skeletal muscle after stimulation with 0.6 nmol/l insulin compared with the background signal as assessed by immunoblot analysis. Thus, insulin receptor tyrosine phosphorylation was assessed only in muscles exposed to 2.4 or 60 nmol/l insulin. Insulin-stimulated receptor phosphorylation was similar between the two groups. This finding is consistent with some (36,37) but not all previous studies (38–40). Because we observed a profound reduction in insulin-stimulated glucose transport, despite normal insulin receptor tyrosine kinase activity, in skeletal muscle from type 2 diabetic subjects, postreceptor signal transduction defects are likely to account for impaired insulin action.

Insulin-stimulated IRS-1 tyrosine-phosphorylation. We established an insulin dose-response curve for tyrosine phosphorylation of IRS-1 (Fig. 4). Muscle was processed as described above for the time course analysis. Insulin led to a concentration-dependent increase in IRS-1 tyrosine phosphorylation in muscle from type 2 diabetic and control subjects. In response to 0.6, 1.2, or 2.4 nmol/l insulin, IRS-1 tyrosine phosphorylation was similar between type 2 diabetic and control subjects. At 2.4 nmol/l insulin, there was a tendency toward reduced IRS-1 phosphorylation in skeletal muscle from type 2 diabetic subjects, although this reduction did not reach statistical significance ($P = 0.14$) because of individual variations. However, in contrast to our results for insulin receptor phosphorylation, at the highest level of insulin studied (60 nmol/l), IRS-1 tyrosine phosphorylation was 60% lower in skeletal muscle from diabetic subjects than in that from control subjects ($P < 0.01$). Thus, diabetes-associated defects in insulin-stimulated tyrosine phosphorylation of IRS-1 appear to be more pronounced at very high insulin concentrations.

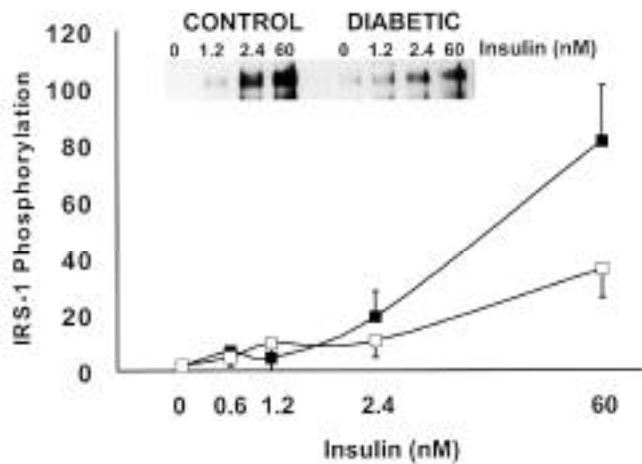


FIG. 4. Insulin-stimulated IRS-1 tyrosine phosphorylation. Muscles were incubated as described for Fig. 3. IRS-1 phosphorylation was assessed as described in RESEARCH DESIGN AND METHODS. Data are means \pm SE for skeletal muscle from control (■) (n = 8) or type 2 diabetic (□) (n = 8) subjects. Results were normalized as described in Fig. 3. Inset shows representative immunoblot analysis. Curves are significantly different (P < 0.01).

Phosphotyrosine-associated PI 3-kinase activity. We next determined the insulin concentration dependence on phosphotyrosine-associated PI 3-kinase activity in skeletal muscle (Fig. 5). In parallel with IRS-1 tyrosine phosphorylation, insulin led to a concentration-dependent increase in phosphotyrosine-associated PI 3-kinase activity in muscle from type 2 diabetic and control subjects. Consistent with our results for IRS-1 phosphorylation, insulin-stimulated (60 nmol/l) anti-phosphotyrosine-associated PI 3-kinase was 45% lower in skeletal muscle from type 2 diabetic subjects compared with that from control subjects (P < 0.001). However, in contrast to our finding for insulin-stimulated IRS-1 tyrosine phosphorylation, submaximal (2.4 nmol/l) insulin-

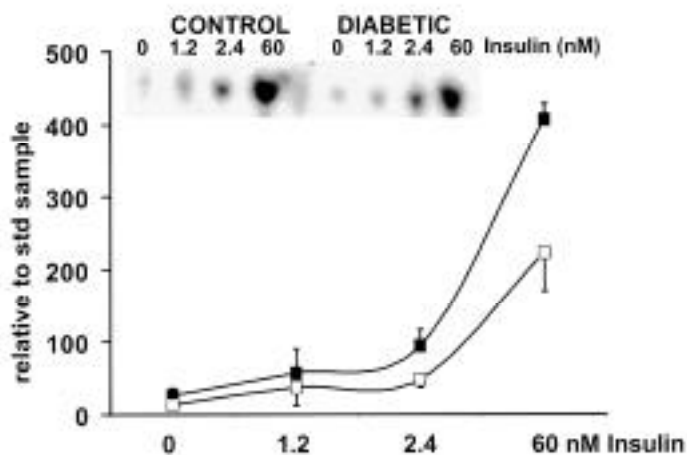


FIG. 5. Phosphotyrosine-associated PI 3-kinase activity. Muscles were incubated as described for Fig. 3. PI 3-kinase activity was assessed in anti-phosphotyrosine immunocomplexes as described in RESEARCH DESIGN AND METHODS. Muscles were incubated for 40 min in the absence or presence of insulin as indicated. Data are means \pm SE for skeletal muscle from control (■) (n = 11) and type 2 diabetic (□) (n = 9) subjects. Results were normalized as described in Fig. 3. Inset shows representative autoradiogram of thin-layer chromatographic separation of reaction products. Curves are significantly different (P < 0.001).

stimulated anti-phosphotyrosine-associated PI 3-kinase was also reduced (51% of control activity, P = 0.07). Consistent with IRS-1, impairments of insulin-stimulated tyrosine-associated PI 3-kinase are more pronounced at higher concentrations of insulin.

In a subset of control subjects (n = 5) and type 2 diabetic patients (n = 4), sufficient material was available to assess IRS-1- and IRS-2-associated PI 3-kinase. In control and type 2 diabetic subjects, there was a close correlation between IRS-1- and IRS-2-associated PI 3-kinase activity, so that if IRS-1 activity was reduced, IRS-2 activity was reduced in parallel (data not shown). Thus, IRS-2 does not appear to compensate for reduced anti-phosphotyrosine-associated PI 3-kinase activity in skeletal muscle from type 2 diabetic subjects.

Insulin-stimulated glycogen synthase activity. Insulin is believed to partly mediate glycogen synthase activity via a PI 3-kinase-dependent mechanism (17). We assessed insulin signal transduction to glycogen synthase in skeletal muscle from type 2 diabetic and control subjects (Fig. 6). Insulin led to a significant dose-dependent increase of the proportion of glycogen synthase present in the active form in muscle from type 2 diabetic and control subjects. However, in contrast to our results for glucose transport, insulin-stimulated glycogen synthase activity was not altered between type 2 diabetic and control subjects. Thus, full activation of PI 3-kinase does not appear to be necessary to achieve an insulin-stimulated increase in glycogen synthase activity. Furthermore, the divergence in the effect of insulin on glucose transport versus that on glycogen synthase suggests that downstream pathways from PI 3-kinase along glucose transport rather than those along glycogen synthase are selectively impaired in muscle of type 2 diabetic patients.

Insulin-stimulated MAP kinase phosphorylation. MAP kinase phosphorylation was determined in skeletal muscle through use of an antibody that recognizes MAP (ERK) kinase when phosphorylated on Thr 202 and Tyr 204 (Fig. 7). Insulin led to a 1.5-fold increase in MAP kinase phosphorylation in skeletal muscle from control subjects (P < 0.05 vs. non-insulin-stimulated conditions). Basal (non-insulin-stimulated) MAP kinase phosphorylation was similar between type 2 diabetic and control subjects (data not shown).

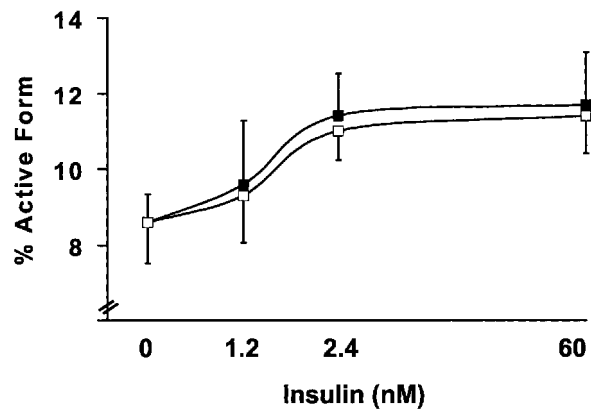


FIG. 6. Insulin-stimulated glycogen synthase activity. Muscles were incubated as described for Fig. 3. Glycogen synthase was assessed as described in RESEARCH DESIGN AND METHODS. Data are expressed as percentage enzyme in active form (means \pm SE) from control (■) (n = 7) or type 2 diabetic (□) (n = 5) subjects.

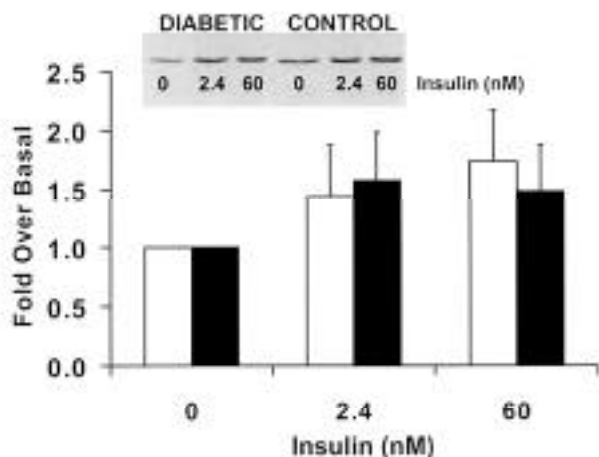


FIG. 7. Insulin-stimulated MAP-kinase phosphorylation. Muscles were incubated as described for Fig. 3. Phosphorylation of p42/44 MAP kinase on Thr 202 and Tyr 204 was assessed in total lysates by immunoblot analysis by use of a phospho-specific antibody. Data are expressed as means \pm SE for skeletal muscle from control (■; n = 8) or type 2 diabetic (□; n = 8) subjects. Inset shows representative immunoblot analysis.

Insulin-stimulated MAP kinase phosphorylation was not impaired in skeletal muscle from type 2 diabetic subjects.

Expression of key signaling proteins in skeletal muscle. We next determined whether altered protein expression of the signaling intermediates account for the functional changes reported above (Fig. 8). The expression of a number of key signaling proteins was determined by immunoblot analysis. Expression of IRS-1, IRS-2, the p85 α -subunit of PI 3-kinase, glycogen synthase, and MAP kinase was similar between type 2 diabetic and control subjects. Thus, aberrant expression of key proteins involved in insulin-signal transduction cannot account for the impaired insulin action in skeletal muscle from moderately overweight people with type 2 diabetes.

DISCUSSION

Here we provide evidence for differential defects in insulin signal transduction between metabolic and mitogenic pathways in skeletal muscle from people with type 2 diabetes. Post-receptor signal transduction via IRS-1/PI 3-kinase is markedly impaired, whereas phosphorylation of the insulin receptor and MAP kinase is intact. Furthermore, reduced glucose transport rather than impaired glycogen synthase activity in skeletal muscle accompanies the defects in whole-body insulin-mediated glucose uptake from these moderately overweight type 2 diabetic patients. Importantly, increased total body fat or a poor level of physical fitness cannot account for these defects in insulin action.

We have previously reported that *in vivo* insulin stimulation, as achieved by means of the euglycemic-hyperinsulinemic clamp, leads to a fivefold increase in IRS-1 tyrosine phosphorylation and a twofold increase in anti-phosphotyrosine-associated PI 3-kinase activity in skeletal muscle from healthy subjects (13). Both of these steps in insulin signal transduction are severely impaired in muscle from type 2 diabetic subjects (13). We now provide a full characterization of the nature of the diabetes-associated insulin-signaling defects in skeletal muscle. These findings could not have been predicted from animal studies, because limited knowledge is available regarding

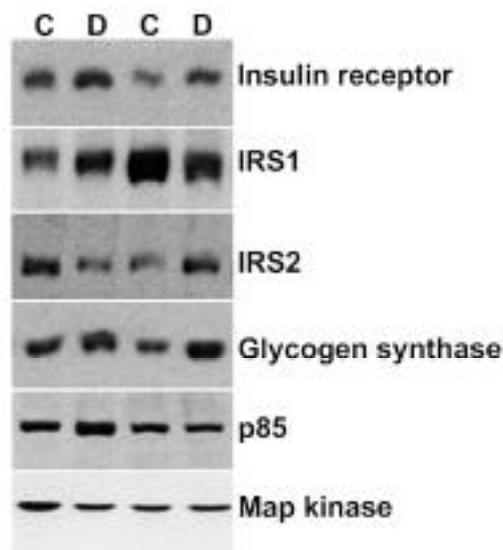


FIG. 8. Expression of key signaling proteins. Protein expression in skeletal muscle from control (n = 11) or type 2 diabetic (n = 11) subjects. Muscle samples were prepared as described in RESEARCH DESIGN AND METHODS. Representative immunoblots are shown for control (C) and type 2 diabetic (D) subjects.

the full insulin dose-response characterization of these signaling cascades in skeletal muscle. Furthermore, many of the animal models studied have severe forms of insulin resistance that are accompanied by extreme obesity.

Poor physical fitness, advanced age, and increased percentage of body fat are associated with insulin resistance (41). Thus, the type 2 diabetic and control subjects were carefully matched for these criteria to determine whether defects in signal transduction are independent of obesity or poor physical fitness. In morbidly obese (BMI \sim 53 kg/m²) insulin-resistant subjects (42), reduced insulin signal transduction can be explained in part by decreased expression of several key proteins in the insulin-signaling cascade. However, this is not the case in moderately overweight (BMI \sim 27 kg/m²) type 2 diabetic patients, because protein expression of signal transduction intermediates was similar to non-diabetic subjects. Our results clearly show that functional defects rather than aberrant protein expression of signal transduction intermediates account for insulin-signaling defects in moderately overweight type 2 diabetic patients.

In a previous study (13), the impairment in insulin-stimulated PI 3-kinase activity was noted at lower (0.6 nmol/l) insulin levels. In the present study, however, when insulin action was studied *in vitro*, impaired signal transduction was noted at very high, rather than physiological, insulin concentrations. Differences in the insulin effect between these studies may be because of the *in vivo* versus *in vitro* experimental conditions. For example, there may be differences in the degree of insulin-mediated vasodilatation between type 2 diabetic and control subjects. Under *in vivo* conditions, the actual insulin concentration at the muscle level may have been greater in control subjects, and thus a higher insulin level may have been achieved in the muscle extracellular space, which would lead to increased signal transduction. Although blood flow is similar between diabetic and control subjects (43), there could be differences at the capillary level. Alternatively,

differences in the *in vivo* milieu between the diabetic and control subjects may exacerbate the insulin resistance of the muscle, which would result in signaling defects at more physiological insulin levels. For example, hyperglycemia (44,45), FFA (46), and tumor necrosis factor- α (TNF- α) (45,47) inhibit insulin signal transduction at the level of IRS-1. Taken together, our results suggest there is an intrinsic defect in insulin signal transduction in skeletal muscle from type 2 diabetic subjects. However, this defect is likely to be exacerbated by a deleterious *in vivo* environment and external risk factors, such as obesity and inactivity.

Reduced insulin receptor tyrosine kinase activity has been associated with type 2 diabetes and obesity (38–40). However, this finding is inconsistent with some studies that provide evidence that insulin receptor function is normal in skeletal muscle from type 2 diabetic patients (36,37). Insulin-signaling defects in skeletal muscle from moderately overweight type 2 diabetic subjects appeared to be downstream of the insulin receptor; we observed a similar level of insulin stimulation of receptor tyrosine kinase activity between diabetic and control subjects. Yet, despite normal receptor auto-phosphorylation, the ability of insulin to mediate tyrosine phosphorylation of IRS-1 was markedly reduced. In contrast to our results for IRS-1 phosphorylation, insulin signaling to MAP kinase phosphorylation was not impaired, which further provides indirect evidence that kinase activity and signaling capacity of the insulin receptor is intact. Thus, divergent defects in the insulin-signaling cascade are present in muscle from people with type 2 diabetes. Importantly, IRS-1 appears to be the site of the first postreceptor defect in the insulin-signaling cascade in muscle from people with type 2 diabetes.

Because we observed selective resistance at the level of IRS-1 and downstream signal transduction, factors that inhibit insulin action at the level of IRS-1 must be considered. Intense interest has now focused on determining whether increased serine phosphorylation of IRS-1 leads to impaired insulin signal transduction and insulin resistance associated with diabetes (48–50). In tissue culture systems, serine phosphorylation of IRS-1 occurs in response to elevations in the level of glucose (44,45), insulin (50), or TNF- α (45,47) and reduces subsequent downstream signal transduction. Numerous proteins, including adaptors, transforming proteins, structural proteins, and enzymes (17), have now been shown to modulate both the interaction between IRS-1 and the insulin receptor and the interaction between IRS-1 and the downstream signaling machinery. In addition, activation of the protein kinase C–signaling pathways have been shown to induce serine phosphorylation of IRS-1, which provides a potential mechanism for insulin resistance in some models of insulin resistance (49). Whether serine phosphorylation of IRS-1 is increased in skeletal muscle from type 2 diabetic subjects remains to be determined.

Insulin-stimulated glycogen synthase activity was not impaired in skeletal muscle from type 2 diabetic subjects. This finding is consistent with some (51–53) but not all (54–57) studies of skeletal muscle in type 2 diabetic subjects. Although we and others (51) show that glycogen synthase activity is not impaired in skeletal muscle from lean type 2 diabetic subjects, obesity appears to be negatively correlated with insulin-stimulated glycogen synthase activity (51). Thus, reduced insulin-mediated glycogen synthase activity may be a direct consequence of obesity rather than diabetes.

Although signaling defects in skeletal muscle from type 2 diabetic subjects in the present study correspond with impaired insulin-mediated glucose transport, the signaling defects were apparent only at high insulin concentrations (2.4–60 nmol/l). In contrast, defects in glucose transport were noted at the lowest concentration of insulin tested (0.6 nmol/l). Therefore, there appears to be a complex relationship between insulin signal transduction and the biological response at the level of glucose transport activity. Because IRS-1/2 and PI 3-kinase are targeted to intracellular locations, such as the actin cytoskeleton, signals for glucose transport may be compartmentalized within the cell (58,59). Thus, the possibility remains that signal transduction may be impaired to a greater extent in critical compartments that are important for activation of glucose transport. Because of the small sample size (20-mg muscle pieces), detailed studies of intracellular location of the insulin signal will be a challenge to perform.

We have performed the first detailed study that includes time course and insulin concentration-dependent characterization of key components of the insulin signal transduction pathways in isolated skeletal muscle from moderately overweight type 2 diabetic patients. We show that there are intrinsic defects in insulin signal transduction in skeletal muscle at the level of the IRS-1 that appear to result in reduced phosphotyrosine-associated PI 3-kinase activity. The reduced PI 3-kinase activity may partly account for reduced insulin-stimulated glucose transport, but it appears that additional defects contribute to impaired insulin action on glucose transport. In contrast, insulin receptor tyrosine phosphorylation, glycogen synthase activity, and signal transduction via the MAP kinase pathway are intact. Thus, type 2 diabetes is associated with selective defects in insulin signal transduction. Whether these defects are a cause or a consequence of type 2 diabetes remains to be determined. Future studies performed in skeletal muscle biopsies from first-degree relatives of patients with type 2 diabetes will allow us to address this important issue.

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