Insulin Signal Transduction in Skeletal Muscle From Glucose-Intolerant Relatives With Type 2 Diabetes

Heidi Storgaard,[1] Xiao Mei Song,[2] Christine B. Jensen,[1] Sten Madsbad,[1] Marie Björnholm,[2] Allan Vaag,[1,3] and Juleen R. Zierath[2]

To determine whether defects in the insulin signal transduction cascade are present in skeletal muscle from prediabetic individuals, we excised biopsies from eight glucose-intolerant male first-degree relatives of patients with type 2 diabetes (IGT relatives) and nine matched control subjects before and during a euglycemic-hyperinsulinemic clamp. IGT relatives were insulin-resistant in oxidative and nonoxidative pathways for glucose metabolism. In vivo insulin infusion increased skeletal muscle insulin receptor substrate-1 (IRS-1) tyrosine phosphorylation ($P = 0.01$) and phosphatidylinositide 3-kinase (PI 3-kinsase) activity (phosphotyrosine and IRS-1 associated) in control subjects ($P < 0.02$) but not in IGT relatives (NS). The incremental increase in insulin action on IRS-1 tyrosine phosphorylation was lower in IGT relatives versus control subjects ($P < 0.05$). The incremental defects in signal transduction noted for IRS-1 and PI 3-kinase may be attributed to elevated basal phosphorylation/activity of these parameters, because absolute phosphorylation/activity under insulin-stimulated conditions was similar between IGT relatives and control subjects. Insulin increased Akt serine phosphorylation in control subjects and IGT relatives, with a tendency for reduced phosphorylation in IGT relatives ($P = 0.12$). In conclusion, aberrant phosphorylation/activity of IRS-1, PI 3-kinase, and Akt is observed in skeletal muscle from relatives of patients with type 2 diabetes with IGT. However, the elevated basal activity of these signaling intermediates and the lack of a strong correlation between these parameters to glucose metabolism suggests that other defects of insulin signal transduction and/or downstream components of glucose metabolism may play a greater role in the development of insulin resistance in skeletal muscle from relatives of patients with type 2 diabetes. *Diabetes* 50:2770–2778, 2001

From the [1]Department of Endocrinology, Hvidovre Hospital and Clinical Trial Unit, University of Copenhagen, Copenhagen, Denmark; the [2]Department of Clinical Physiology, Karolinska Hospital, Karolinska Institutet, Stockholm, Sweden; and the [3]Steno Diabetes Center, Gentofte, Denmark.

Address correspondence and reprint requests to Heidi Storgaard, Department of Endocrinology, Hvidovre Hospital, DK-2650 Hvidovre, University of Copenhagen, Copenhagen, Denmark. E-mail: hstorgaard@dadlnet.dk. Or Juleen R. Zierath, Department of Clinical Physiology, and Department of Physiology, Karolinska Institute, von Eulers väg 4, II, SE-171 77 Stockholm, Sweden. E-mail: juleen.zierath@fyfa.ki.se.

Received for publication 2 November 2000 and accepted in revised form 5 September 2001.

H.S. and X.M.S. contributed equally to this study.

AUC, area under the curve; DTT, dithiothreitol; ECL, enhanced chemiluminescence; FFA, free fatty acid; FFM, fat-free body mass; IGT, impaired glucose tolerance; IGT relatives, glucose-intolerant first-degree relatives of type 2 diabetic patients; IRS, insulin receptor substrate; IRTK, insulin receptor tyrosine kinase; IVGTT, intravenous glucose tolerance test; HGP, hepatic glucose production; OGTT, oral glucose tolerance test; PI 3-kinase, phosphoinositide 3-kinase; PVDF, polyvinylidene difluoride; SA, specific activity; Rd, glucose disposal rate.

Insulin resistance in skeletal muscle is a hallmark feature of type 2 (non–insulin-dependent) diabetes (1–3). In normal glucose-tolerant relatives of patients with type 2 diabetes, insulin resistance in skeletal muscle has been observed several years before the development of overt diabetes (4). Importantly, skeletal muscle insulin resistance predicts the development of type 2 diabetes in different populations (5,6). One current hypothesis is that skeletal muscle insulin resistance may occur in part from a genetically determined defect(s) that orchestrates the complicated pathophysiological events that lead to overt type 2 diabetes.

In patients with type 2 diabetes, defects in insulin-stimulated glucose metabolism in skeletal muscle have been attributed to impaired glucose transport (7,8), glycogen synthesis (9), and glycogen synthase activation (10). These defects may result from impaired insulin signal transduction (11). The molecular signaling mechanisms by which insulin regulates glucose uptake and storage is initiated by the binding of insulin to its specific cell-surface receptor, which results in receptor autophosphorylation and activation of insulin receptor tyrosine kinase (IRTK). IRTK phosphorylates tyrosine residues on insulin receptor substrates (IRS-1–4). IRS-1 seems to be the major postreceptor component of the signaling machinery involved in mediating GLUT4 translocation, glucose transport, and glucose metabolism in human skeletal muscle (12). Furthermore, in insulin-resistant rodent skeletal muscle, the decrease in total phosphotyrosine-associated phosphoinositide 3-kinase (PI 3-kinase) activity closely parallels the reduction in IRS-1–associated PI 3-kinase activity (13). Binding of IRS-1 to the regulatory subunit of PI 3-kinase results in activation of the kinase and initiation of a phosphorylation cascade involving phosphoinositide-dependent protein kinase-1 and the downstream kinase Akt (also known as protein kinase B) (14,15). Among other insulin-mediated effects, Akt phosphorylates and inactivates glycogen synthase kinase-3, resulting in a greater degree of dephosphorylation and thereby activation of glycogen synthase (16). Impaired insulin action on glycogen synthase in skeletal muscle represents a major defect that correlates with insulin resistance in the pathway of nonoxidative glucose metabolism in patients with type 2 diabetes (12,17) and their nondiabetic first-degree relatives (4,18). Thus, impaired insulin action on Akt in skeletal muscle from prediabetic and diabetic individuals may contribute to the pathophysiology of type 2 diabetes.

TABLE 1
Clinical characteristics of the study participants

	IGT relatives	Control subjects	*P*
n	8	9	
Age (years)	55 ± 2	53 ± 2	NS
Weight (kg)	102 ± 5	106 ± 5	NS
Height (cm)	178 ± 3	181 ± 1	NS
BMI (kg/m^2)	31.9 ± 1.0	32.3 ± 1.3	NS
Waist/hip ratio	1.00 ± 0.01	1.00 ± 0.02	NS
FFM (kg)	67.1 ± 2.0	69.0 ± 3.2	NS
Fasting plasma glucose (mmol/l)	6.6 ± 0.2	5.9 ± 0.1	0.02
2-h plasma glucose (mmol/l)	9.3 ± 0.4	5.5 ± 0.3	<0.0001
Plasma FFA (mmol/l)	0.40 ± 0.06	0.44 ± 0.04	NS
Plasma triglyceride (mmol/l)	2.2 ± 0.5	1.3 ± 0.2	NS
Plasma cholesterol (mmol/l)	5.5 ± 0.2	5.7 ± 0.4	NS
HbA_{1c} (%)	6.0 ± 0.3	6.0 ± 0.1	NS
Plasma C-peptide (pmol/l)			
Basal	1,202 ± 195	619 ± 86	0.0037
IS	1,066 ± 174	648 ± 71	0.046
Plasma insulin (pmol/l)			
Basal	96.1 ± 27.7	39.1 ± 4.8	NS
IS	487.5 ± 54.2	442.7 ± 24.9	NS
Hepatic glucose production (mg · kg FFM^{-1} · min^{-1})			
Basal	2.81 ± 0.24	2.38 ± 0.20	NS
ISu	1.52 ± 0.41	2.09 ± 0.38	NS
$AUC_{10\ min}$ insulin (pmol · l^{-1} · min^{-1})	2,530 ± 1032	1,698 ± 454	NS

Data are means ± SE. IS, insulin-stimulated (during euglycemic clamp); ISu, insulin suppressed (during euglycemic clamp); NS, not significant.

Insulin receptor binding and IRTK activity have been reported to be either normal (19) or decreased (17) in muscle biopsies from patients with type 2 diabetes. Despite the inconsistent findings for immediate insulin signaling events at the level of the receptor, insulin action on PI 3-kinase in skeletal muscle from patients with overt type 2 diabetes is impaired (11,12,20,21). Moreover, we recently reported decreased Akt activity in isolated skeletal muscle from patients with type 2 diabetes in response to supraphysiological insulin concentrations (22). In contrast, Akt activity is normal in muscle biopsies obtained from patients with type 2 diabetes during a euglycemic-hyperinsulinemic clamp, whereby insulin levels were maintained at a high physiological level (12,21). Therefore, the role of Akt in the development of insulin resistance in type 2 diabetes is unclear.

One explanation for the conflicting results on IRTK activity may be the presence of hyperglycemia and its dual effects on glucose metabolism in patients with overt type 2 diabetes. Hyperglycemia (or "glucose toxicity") may be a major cause of insulin resistance and defective signal transduction in skeletal muscle from patients with type 2 diabetes (23). Hyperglycemia also has a compensatory effect on insulin action on signal transduction and downstream metabolic responses in skeletal muscle, allowing for normal insulin-stimulated glucose uptake and muscle glycogen synthase activation when patients with type 2 diabetes are studied during their ambient hyperglycemic state (24,25). One way to avoid the influence of hyperglycemia on glucose uptake and metabolism is to study nondiabetic first-degree relatives of patients with type 2 diabetes with a known high risk for the development of overt type 2 diabetes (4,18,26,27). Importantly, this approach may also reveal a potential primary defect(s) at the level of signal transduction in people who are at high risk for the development of type 2 diabetes.

The aim of the present study was to determine whether impaired insulin signal transduction at the level of IRS-1, PI 3-kinase, and/or Akt is associated with defects in glucose uptake and metabolism (oxidative and/or nonoxidative) in prediabetic individuals. To ensure an a priori high risk for the later progression to overt type 2 diabetes, we studied first-degree relatives of patients with type 2 diabetes with impaired glucose tolerance but normal HbA_{1c} levels.

RESEARCH DESIGN AND METHODS

Participants. Seventy-one men (>40 years) with or without family history of type 2 diabetes were recruited through local newspaper advertisements. All participants underwent an oral glucose (75 g) tolerance test (OGTT) to classify the population according to glucose tolerance and family history of type 2 diabetes. On the basis of the results from this initial screening program, we included eight glucose-intolerant first-degree relatives of patients with type 2 diabetes (IGT relatives) and nine age- and body mass index (BMI)-matched control subjects with normal glucose tolerance and no family history of diabetes (Table 1). Five of the IGT relatives had more than one relative with type 2 diabetes. Glucose tolerance was diagnosed according to the World Health Organization criteria (IGT: 2 h plasma glucose during OGTT >7.8 and <11.1 mmol/l). According to the definition of the two study groups, the 2-h plasma glucose concentration after the OGTT was higher in the IGT relatives (Table 1). The participants were not participating in strenuous exercise on a regular basis, and they were instructed to avoid excessive physical exercise and alcohol intake for at least 2 and 1 days, respectively, before participating in the experimental testing procedure. The participants were also instructed not to alter body weight or lifestyle habits (eating, drinking, smoking, and exercise) during their participation in the study. All participants agreed to participate in the experimental protocols after receiving oral and written information regarding the details of the study. The Copenhagen County Ethical Committee approved the study, and the study was conducted according to the principles of the Helsinki Declaration.

Body fat determinations. A dual-energy X-ray absorptiometry scan was performed using a Norland XR-36 scanner to measure total body fat (28).

Protocol for in vivo studies. All studies commenced at 0800 h after a 10-h overnight fast. A polyethylene catheter was inserted into an antecubital vein for infusion of test substances. Another polyethylene catheter was inserted into a contralateral wrist vein for blood sampling. This hand was kept in a heated plexiglas box throughout the test to obtain arterialized venous blood (29). Basal samples for insulin, C-peptide, free fatty acid (FFA), and glucose determination were obtained. Each study was initiated with a basal period (−160 min), at which time the tracer bolus was given. Indirect calorimetry was performed during the predefined basal steady state period (−70 to −40 min) for basal glucose oxidation measurements. Thereafter, a basal (non–insulin-stimulated) muscle biopsy was obtained (−40 to −30 min). From −30 to 0 min, an intravenous glucose tolerance test (IVGTT) was performed to characterize the first-phase insulin response.

Euglycemic-hyperinsulinemic clamp. Squared priming was performed (0–9 min) with a stepwise decline in the insulin (Actrapid; Novo Nordisk, Bagsvaerd, Denmark) infusion rate every third minute, thereby reducing the insulin infusion rate from 100–80 to 60–40 mU · m^{-2} · min^{-1}. Thereafter, the insulin infusion rate was fixed at 40 mU · m^{-2} · min^{-1} from 9 to 120 min. Plasma glucose concentration was maintained constant at euglycemia, using a variable glucose infusion (180 g/l) (30). Plasma glucose concentration was monitored every 5 min using an automated glucose oxidase method (Glucose Analyzer 2; Beckman Instruments, Fullerton, CA). Indirect calorimetry was performed during the insulin-stimulated steady-state period (90–120 min) to measure insulin-stimulated glucose oxidation.

Tritiated glucose. At 160 min before the start of insulin (−160 min), an adjusted primed (11 μCi · [fasting plasma glucose concentration/5 mmol/l]) continuous (0.11 μCi/min) infusion of 3-[^{3}H]glucose (New England Nuclear, Boston, MA) was initiated to ensure isotope equilibrium (31). The tracer infusion was continued throughout the 120-min period of insulin infusion. To obtain constant specific activity (SA) during insulin infusion, we added tritiated glucose to the infused glucose solution (54 μCi/500 ml in an 18% glucose solution) (32). Blood samples were drawn in fluoride-treated tubes at the beginning and end of the 30-min basal steady-state period and every 10 min thereafter during the 30-min insulin-stimulated steady-state period for determination of 3-[^{3}H]glucose activity. Plasma 3-[^{3}H]glucose was measured every 30 min during the rest of the study. Plasma 3-[^{3}H]glucose concentration was determined from radioactivity in the evaporated (to avoid 3-[^{3}H]water) 0.5-ml plasma samples (31).

Indirect calorimetry. A ventilated canopy was placed over the participant's head (Deltatrac; Datex, Helsinki, Finland), and continuous gas exchange was determined. Inspired and expired air was analyzed for oxygen content using a paramagnetic differential oxygen sensor and for carbon dioxide tension using an infrared carbon dioxide sensor. Oxygen consumption and carbon dioxide production were recorded and calculated each minute. After an equilibrium period of 10 min, the average gas exchange during the two 30-min steady-state periods (basal and insulin-stimulated) (−70 to −40 min and 90–120 min) were used to calculate rates of glucose oxidation as previously described (33,34).

Muscle biopsy. Muscle biopsies were obtained under local anesthesia, from m. vastus lateralis using a modified Bergström's needle (including suction), at −40 (basal) and 120 min (insulin-stimulated). Biopsies were frozen in liquid nitrogen and stored at −80°C for later analysis for signaling transduction studies. Previous in vitro and in vivo studies showed that insulin-mediated signal transduction in human muscle is sustained between 15 min and 3 h (12,21).

IVGTT. An IVGTT was performed from −30 min to 0 min. A bolus of 300 mg glucose/kg body mass (18% glucose) was infused over 1 min. Blood samples were collected at −10, −5, 0, 2, 4, 6, 8, 10, 15, and 30 min. All samples were analyzed for plasma glucose, insulin, and C-peptide.

Blood chemistry. Plasma glucose was determined using an automated glucose oxidase method (Glucose Analyzer 2; Beckman Instruments). Tritiated glucose was measured as described by Hother-Nielsen and Beck-Nielsen (31). Plasma insulin and C-peptide concentration were determined using the 1234 AutoDELFIA immunoassay system (Wallac Oy, Turku, Finland). Plasma FFA were quantified using an enzymatic colorimetric method (Wako Chemicals, Neuss, Germany).

Materials and antibodies. PI was from Avanti Polar Lipids (Alabaster, AL). The aluminum-backed Silica Gel 60 thin-layer chromatography plates were from EM Separations (Gibbstown, NJ). Protein A-Sepharose was purchased from Pharmacia (Uppsala, Sweden). All other chemicals were purchased from Sigma (St. Louis, MO) or Merck (Rahway, NJ). Antiphosphotyrosine and p85α antibodies were from Transduction Laboratories (Lexington, KY). IRS-1 polyclonal antibody was from Dr. Ton Maassen (Leiden University, Leiden, the Netherlands). Polyclonal antibodies to detect Akt phosphorylation or Akt protein were from New England Biolabs (Beverly, MA). These antibodies were produced by immunizing rabbits with a KLH-coupled synthetic phosphopeptide (Akt phosphorylation) or synthetic peptide (Akt protein expression) corresponding to residues 466–479 of mouse Akt.

Tissue processing. Muscle biopsies 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 Na_3VO_4, 10 mmol/l NaF, 30 mmol/l $Na_4P_2O_7$, 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) using a glass-on-glass rotating homogenizer and subjected to centrifugation (12,000 *g* for 10 min at 4°C). Protein was determined in the supernatant using a commercial kit (Bio-Rad, Richmond, CA).

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

PI 3-kinase activity. Muscle specimens were homogenized as described above, and an aliquot of the supernatant (800 μg) was immunoprecipitated overnight (4°C) with antiphosphotyrosine or anti–IRS-1 antibody coupled to protein A-Sepharose. PI 3-kinase activity was assessed directly on the protein A-Sepharose beads as previously reported (35). Reaction products were resolved by thin-layer chromatography and quantified using a PhosphorImager (Bio-Rad). Results are presented as arbitrary phosphoimager units.

Akt phosphorylation. Aliquots of muscle homogenate (40 μg) were solubilized in Laemmli sample buffer with 100 mmol/l DTT and heated (95°C) for 6 min. Proteins were separated by SDS-PAGE, transferred to PVDF membranes, and immunoblotted as described above, using a phospho-specific antibody that recognizes Akt when phosphorylated at Ser 473 (New England Biolabs). Phosphorylated Akt was visualized by ECL and quantified by densitometric scanning.

Protein expression. For protein expression of IRS-1, p85 α subunit of PI 3-kinase, and Akt, an aliquot (40 μg) of supernatant was resuspended in sample buffer and heated (95°C) for 6 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 above, washed, and incubated with appropriate secondary antibodies as recommended by the supplier (Amersham). Proteins were visualized by ECL and quantified by densitometry.

Calculations. Hepatic glucose production (HGP) and glucose disposal rate (Rd) were calculated during the steady-state periods using Steele's non–steady-state equation (36). During the insulin-stimulated steady-state period, Rd and HGP were calculated at 10-min intervals. In the calculations, the distribution volume of glucose was taken as 200 ml/kg body wt and the pool fraction of 0.65 (36). HGP during the basal steady-state period was equal to the rate of appearance of 3-[^{3}H]glucose (Ra), whereas HGP during the clamp steady-state period was calculated as the difference between Ra and the glucose infusion rate. Nonoxidative glucose metabolism was calculated by subtracting the glucose oxidation rate (determined by indirect calorimetry) from Rd. Glucose metabolism data were expressed as mg · kg FFM^{-1} · min^{-1}. The acute insulin response to intravenous glucose was calculated as the incremental area under the plasma insulin concentration curve (AUC) from 0 to 10 min ($AUC_{10\ min}$ insulin).

Statistical analysis. The results are represented as means ± SE. Differences between groups were compared by Mann-Whitney rank-sum test. Paired comparisons were performed using Wilcoxon matched-pairs signed rank test. Correlation analyses were performed using Spearman correlation analysis. Significance was accepted at $P < 0.05$.

RESULTS

Clinical characteristics. IGT relatives and control subjects were matched for sex, age, and BMI (Table 1). Fasting C-peptide and 2-h plasma glucose concentration after the OGTT were higher in IGT relatives (Table 1). Fasting plasma glucose concentration during the basal steady-state period was higher in IGT relatives (6.6 ± 0.2 vs. 5.9 ± 0.2 mmol/l, for IGT versus control subjects, respectively; $P = 0.02$). However, during the insulin-stimulated steady-state period (clamp), plasma glucose concentration was similar between the IGT relatives

(5.0 ± 0.2 mmol/l; variation coefficient 11.0%) and control subjects (5.0 ± 0.1 mmol/l; variation coefficient 7.2%). Fasting plasma insulin levels tended to be greater in IGT relatives (Table 1). Although insulin infusion rates were the same between IGT relatives and control subjects, the insulin concentration in the IGT relatives tended to be greater (Table 1). This indicates a lower insulin clearance in the IGT relatives during the insulin-stimulated state. The magnitude of the first-phase insulin response was measured as $AUC_{10\ min}$. Insulin was higher in the IGT relatives, but this increase was not significantly different from the control subjects (Table 1).

Glucose metabolism. SA for tritiated glucose was similar between the IGT relatives and control subjects under basal (210 ± 14 vs. 205 ± 8 cpm, respectively) and insulin-stimulated (246 ± 12 vs. 229 ± 10 cpm, respectively) steady-state periods. Basal and insulin suppressed rates of HGP were similar in the IGT relatives and control subjects (Table 1).

Insulin increased the Rd in control subjects 3.8-fold (basal versus insulin-stimulated, 2.30 ± 0.24 vs. 8.79 ± 0.67 mg · kg FFM^{-1} · min^{-1}; $P = 0.004$) and 1.7-fold in IGT relatives (3.02 ± 0.21 vs. 5.03 ± 0.42; $P = 0.008$). The mean basal Rd were similar between the IGT relatives and control subjects, whereas the insulin-stimulated Rd was lower in IGT relatives compared with control subjects ($P = 0.002$; Fig. 1). The insulin-stimulated increase in Rd over basal levels was significantly lower in IGT relatives (2.01 ± 0.45 vs. 6.39 ± 0.84; $P = 0.002$). Basal glucose metabolism was similar between IGT relatives and control subjects, with similar rates of basal oxidative (IGT relatives versus control subjects, 1.88 ± 0.20 and 1.61 ± 0.16, respectively) and nonoxidative (IGT relatives versus control subjects, 1.14 ± 0.27 and 0.69 ± 0.24, respectively) components of glucose metabolism. During insulin stimulation, oxidative (2.76 ± 0.22 vs. 3.48 ± 0.13, respectively; $P = 0.02$) and nonoxidative (2.27 ± 0.55 vs. 5.31 ± 0.68, respectively; $P = 0.01$) glucose metabolism was lower in IGT relatives versus control subjects (Fig. 1). Furthermore, the insulin-stimulated increase over basal glucose metabolism was lower in IGT relatives versus control subjects for oxidative (0.89 ± 0.17 vs. 1.85 ± 0.11, respectively; $P < 0.0001$) and nonoxidative (1.13 ± 0.40 vs. 4.53 ± 0.80, respectively; $P = 0.006$) components of glucose metabolism.

Lipid metabolism. Basal and insulin-stimulated rates of lipid oxidation were similar between IGT relatives and control subjects (1.11 ± 0.10 vs. 1.09 ± 0.05 and 0.69 ± 0.10 vs. 0.76 ± 0.03, respectively, both comparisons NS).

IRS-1 tyrosine phosphorylation. Physiological hyperinsulinemia increased IRS-1 tyrosine phosphorylation in skeletal muscle from control subjects 3.9-fold (basal versus insulin-stimulated, 80.2 ± 18.4 vs. 314.8 ± 57.6 arbitrary units; $P = 0.008$; Fig. 2*A*). Conversely, insulin infusion did not significantly alter IRS-1 tyrosine phosphorylation in skeletal muscle from IGT relatives (201.0 ± 108.1 vs. 190.9 ± 35.1 arbitrary units; NS). Basal IRS-1 tyrosine phosphorylation tended to be higher in IGT relatives, although this difference was not significant ($P = 0.82$). Insulin-stimulated IRS-1 tyrosine phosphorylation was slightly lower in IGT relatives compared with control subjects, although this difference did not reach statistical significance. The insulin-stimulated increase in IRS-1 tyrosine phosphorylation over basal was greater in control subjects versus IGT relatives (234.6 ± 59.4 vs. −10.0 ± 81.4 arbitrary units, respectively; $P = 0.046$). IRS-1 protein expression was similar between IGT relatives and control subjects (1.1 ± 0.3 vs. 1.2 ± 0.3 arbitrary units; NS).

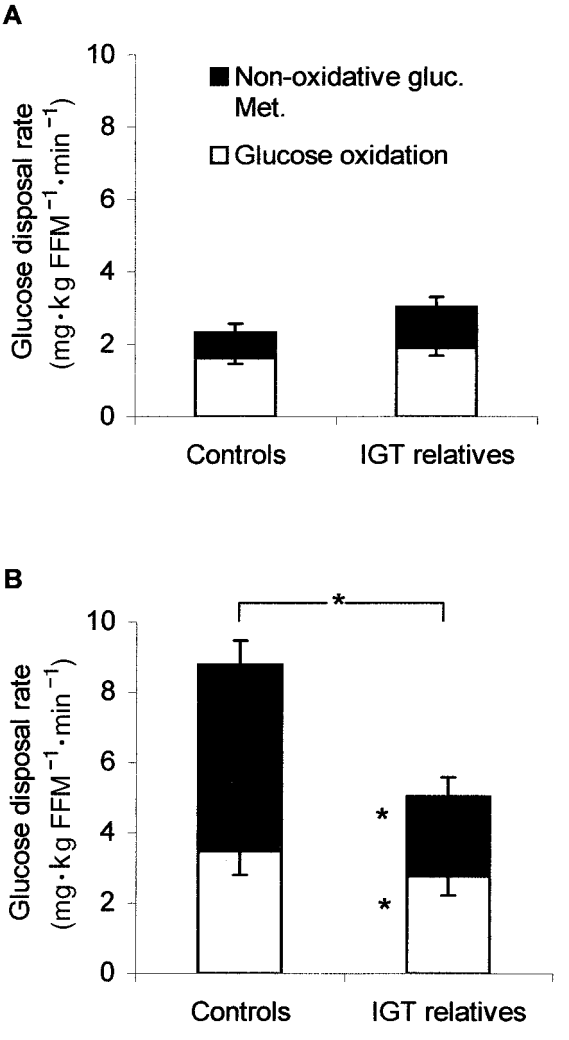

FIG. 1. Glucose disposal in IGT first-degree relatives of patients with type 2 diabetes and control subjects. Rds are presented as oxidative (□) and nonoxidative (■) glucose metabolism during basal (*A*) and insulin-stimulated conditions (*B*). Data are the means ± SE for eight IGT first-degree relatives of patients with type 2 diabetes and nine normal glucose-tolerant control subjects. *$P < 0.02$.

Phosphotyrosine-associated PI 3-kinase activity. PI 3-kinase activity was assessed in antiphosphotyrosine immunoprecipitates of basal and in vivo insulin-stimulated muscle from IGT relatives and control subjects (Fig. 2*B*). Physiological hyperinsulinemia increased tyrosine-associated PI 3-kinase activity 1.7-fold in control subjects (basal versus insulin-stimulated, 155.1 ± 22.7 vs. 267.6 ± 33.5 arbitrary units; $P = 0.02$). In contrast, basal and insulin-stimulated PI 3-kinase activity was similar in IGT relatives (221.33 ± 49.7 and 253.3 ± 43.7 arbitrary units, respectively; NS). IGT relatives tended to have a higher basal PI 3-kinase activity compared with control subjects (NS, $P = 0.37$). The insulin-stimulated increase in PI 3-kinase activity over basal was greater in control subjects compared with IGT relatives (112.5 ± 31.7 vs. 31.9 ± 57.0 arbitrary

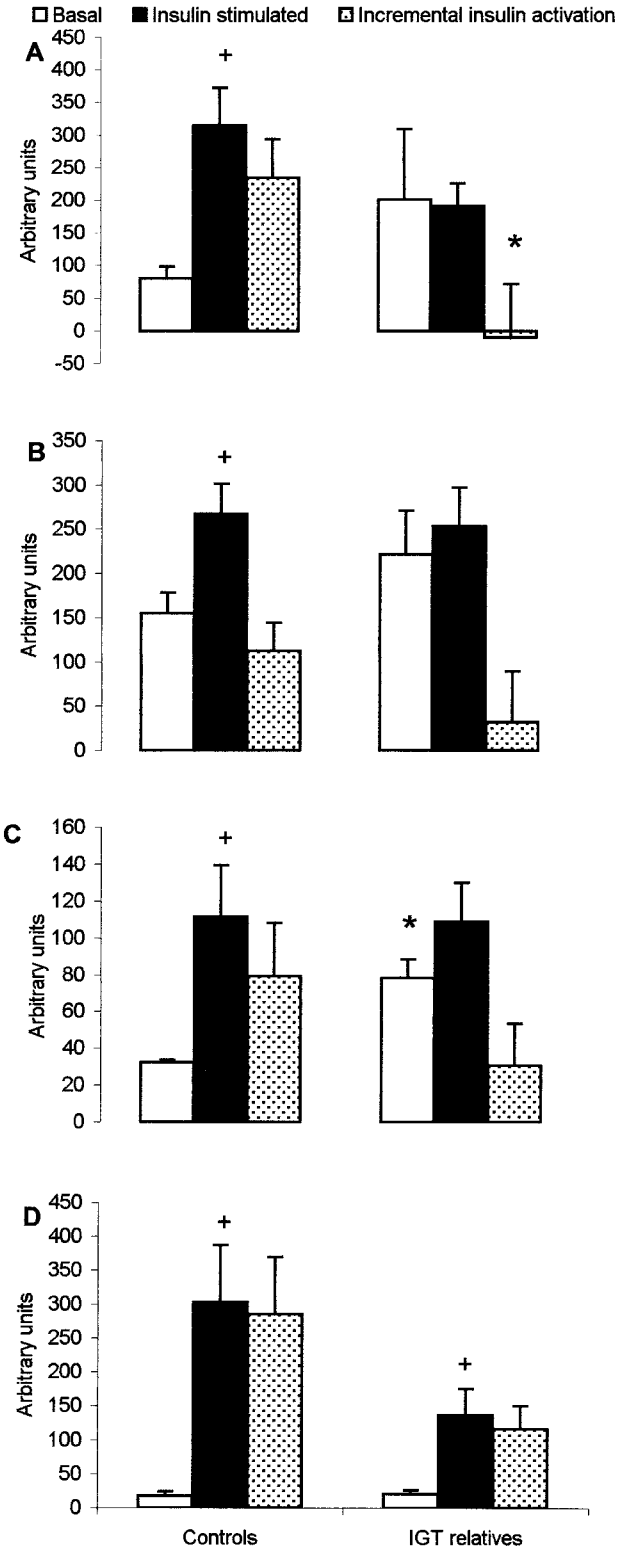

FIG. 2. Insulin action on IRS-1 tyrosine phosphorylation (*A*), Phosphotyrosine-associated PI 3-kinase activity (*B*), IRS-1–associated PI 3-kinase activity (*C*), and Akt serine phosphorylation (*D*) in skeletal muscle from IGT first-degree relatives of patients with type 2 diabetes and control subjects. Muscle biopsies were obtained under basal (□) and insulin-stimulated (■) conditions as described in RESEARCH DESIGN AND METHODS. Results are also presented as incremental insulin activation (insulin-stimulated effect minus basal effect) (▨). IRS-1 tyrosine phosphorylation was determined in skeletal muscle from six IGT first-degree relatives of patients with type 2 diabetes and seven control subjects. IRS-1–associated PI 3-kinase was determined in skeletal muscle from six IGT first-degree relatives of patients with type 2-diabetes and six control subjects. Phosphotyrosine-associated PI 3-kinase activity and Akt serine phosphorylation were determined in skeletal muscle from eight IGT first-degree relatives of patients with type 2 diabetes and nine control subjects. Data are means ± SE. *P < 0.05 versus control subjects. +P < 0.05 versus corresponding basal values.

units, respectively), although because of the large degree of variability within the groups, this difference was not significant (P = 0.24). Protein expression of the p85α regulatory subunit of PI 3-kinase was similar between IGT relatives and control subjects (1.5 ± 0.2 vs. 1.4 ± 0.2 arbitrary units, respectively).

IRS-1–associated PI 3-kinase activity. In a subset of IGT first-degree relatives of patients with type 2 diabetes (n = 6) and control subjects (n = 6), sufficient material was available to assess IRS-1–associated PI 3-kinase in basal and in vivo insulin-stimulated muscle (Fig. 2*C*). Physiological hyperinsulinemia increased IRS-1–associated PI 3-kinase activity 3.5-fold in control subjects (basal versus insulin-stimulated, 32.2 ± 1.6 vs. 111.3 ± 27.9 arbitrary units; P = 0.03). In contrast, insulin did not increase IRS-1–associated PI 3-kinase activity in skeletal muscle from IGT relatives (78.1 ± 10.3 vs. 108.7 ± 21.2 arbitrary units, for basal and insulin-stimulated conditions, respectively; NS), a finding consistent with data for phosphotyrosine-associated PI 3-kinase activity. When comparing responses for IRS-1–associated PI 3-kinase activity between IGT relatives and control subjects, basal activity was greater in IGT relatives (P = 0.002), and insulin-stimulated activity was similar. Consistent with data for phosphotyrosine-associated PI 3-kinase activity, the insulin-stimulated increase in IRS-1–associated PI 3-kinase activity over basal was greater in control subjects compared with IGT relatives (79.1 ± 28.7 vs. 30. 6 ± 22.7 arbitrary units, respectively), although because of the large degree of variability within the groups, this difference was not significant (P = 0.43).

Akt phosphorylation. The serine/threonine kinase Akt (PKB/Rac) is a downstream target of PI 3-kinase (37–39). In vivo basal and insulin-stimulated Akt phosphorylation was assessed in skeletal muscle from IGT relatives and control subjects (Fig. 2*D*). Insulin increased serine phosphorylation of Akt 16.8-fold in control subjects (basal versus insulin-stimulated, 18 ± 6 vs. 303 ± 85 arbitrary units; P = 0.004) and 6.7-fold in IGT relatives (20 ± 5 vs. 137 ± 38 arbitrary units; P = 0.008). In contrast to our results for IRS-1 and PI 3-kinase, basal serine phosphorylation of Akt was similar between IGT relatives and control subjects, and insulin-stimulated Akt serine phosphorylation tended to be lower in IGT relatives (P = 0.11). The insulin-stimulated increase in Akt serine phosphorylation over basal was lower in the IGT relatives versus control subjects (116 ± 34 vs. 285 ± 85 arbitrary units; P = 0.11). Akt protein expression was similar between IGT relatives and control subjects (2.1 ± 0.9 vs. 2.9 ± 0.6 arbitrary units, respectively).

Correlation analysis. The incremental increase in IRS-1 tyrosine phosphorylation, PI 3-kinase activity, and Akt serine phosphorylation during in vivo insulin infusion was weakly correlated to the increase in glucose disposal, as measured in the total study population (n = 15–17; IRS-1 tyrosine phosphorylation: r = 0.52 and P = 0.04; PI 3-kinase activity: r = 0.42 and P = 0.09; and Akt serine

phosphorylation: $r = 0.43$ and $P = 0.08$). The incremental increase in IRS-1–associated PI 3-kinase activity did not correlate with the increase in glucose disposal ($r = -0.02$ and $P = 0.93$). When the two groups were considered separately, insulin-stimulated glucose disposal was not significantly correlated to phosphorylation/activity of any the components of the insulin signal transduction cascade measured, including IRS-1 tyrosine phosphorylation, PI 3-kinase activity, and Akt serine phosphorylation.

Because elevated basal IRS-1 tyrosine phosphorylation and PI 3-kinase activity was noted in the IGT relatives, correlation analysis was performed between these parameters and plasma insulin and glucose levels. Basal levels of phosphotyrosine-associated PI 3-kinase activity, IRS-1–associated PI 3-kinase activity, and Akt phosphorylation were independently correlated with basal insulin levels in all participants (phosphotyrosine-associated PI 3-kinase activity: $r = 0.59$ and $P = 0.012$; IRS-1–associated PI 3-kinase activity: $r = 0.72$ and $P = 0.008$; Akt phosphorylation: $r = 0.63$ and $P = 0.007$). When parameters were correlated for the individual study groups, basal insulin level was also correlated with PI 3-kinase activity and Akt serine phosphorylation (PI 3-kinase activity: $r = 0.83$ and $P = 0.008$; Akt serine phosphorylation: $r = 0.81$ and $P = 0.01$) in control subjects but not in IGT relatives (Fig. 3*B* and *D*). IRS-1 tyrosine phosphorylation and insulin level were not correlated under basal conditions (Fig. 3*A*). Furthermore, when comparisons were made for all participants or for the individual study groups, basal phosphorylation/activation of the insulin signaling intermediates was not correlated with basal plasma glucose level (data not shown).

DISCUSSION

This study provides the first evidence that in vivo insulin-mediated IRS-1 tyrosine phosphorylation, PI 3-kinase activity, and Akt serine phosphorylation are impaired in insulin-resistant first-degree relatives of patients with type 2 diabetes when expressed as the relative insulin-stimulated increments over the basal values. Because of known family history of type 2 diabetes, coupled with impaired glucose tolerance, these IGT relatives are likely to be at very high risk for later progression to overt type 2 diabetes. Our findings may have important clinical relevance as they suggest that defects proximal in the insulin signal transduction system in skeletal muscle are present in individuals with a genetically determined high risk for the development of type 2 diabetes.

We observed impairments in both oxidative and nonoxidative components of insulin-stimulated glucose metabolism in the IGT relatives. Thus, our findings are consistent with the hypothesis that defects in both glucose transport and glycogen synthesis contribute to whole-body insulin resistance in skeletal muscle from IGT relatives. Peripheral insulin resistance in first-degree relatives of patients with type 2 diabetes and in people with IGT is primarily due to a quantitatively major defect in the pathway of nonoxidative glucose metabolism (40), which is tightly correlated with a defect in insulin activation of muscle glycogen synthesis and muscle glycogen synthase enzyme activity (4,18). The extent to which defective muscle glycogen synthesis in IGT relatives is explained by decreased glucose transport (41) or defective glycogen synthase enzyme activity is a topic of current debate (4,18), and the relative importance of each pathway may depend on the study design, the analytical methods used (42,43), and the individuals investigated.

The extent to which muscle insulin resistance in first-degree relatives represents a primary underlying genetic defect responsible for the risk of developing type 2 diabetes is unknown. One hypothesis is that a primary β-cell abnormality, associated with early insulin hypersecretion, causes insulin resistance as a result of hyperinsulinemia (44). Alternatively other endocrine or paracrine substances, such as FFA and TNF-α (45), may induce insulin resistance. We have reported normal insulin-stimulated glucose uptake and metabolism in isolated muscle strips from patients with type 2 diabetes during in vitro incubation under euglycemic conditions, in the absence of endocrine and paracrine substances (9). This finding supports the hypothesis that in some patients with type 2 diabetes, muscle insulin resistance is secondary to an altered metabolic milieu. Similarly, a recent report provided evidence that proximal insulin-signaling parameters elicit a normal response in cultured myotubes prepared from muscle biopsies from insulin-resistant nondiabetic individuals (42).

Insulin increased IRS-1 tyrosine phosphorylation and PI 3-kinase activity in skeletal muscle from control subjects, consistent with our earlier report (11). Conversely, in skeletal muscle from IGT relatives, insulin infusion did not alter either IRS-1 tyrosine phosphorylation or PI 3-kinase activity, a finding similar to our earlier report in patients with insulin-resistant type 2 diabetes (11). Protein expression of IRS-1, the p85α subunit of PI 3-kinase, and Akt was similar between IGT relatives and control subjects. Given the degree of whole-body insulin resistance in the IGT participants, impaired insulin action at these early components of the insulin signaling machinery in skeletal muscle may precede the development of overt type 2 diabetes. In contrast to our findings for IRS-1 and PI 3-kinase, insulin increased serine phosphorylation of Akt in both control subjects and IGT relatives. Discordance between in vivo insulin action on PI 3-kinase and Akt has previously been noted in skeletal muscle from patients with type 2 diabetes (21). However, in the former study, the absolute level of insulin-stimulated PI 3-kinase activity was depressed in skeletal muscle from patients with diabetes, whereas the absolute level of insulin-stimulated Akt activity was normal, potentially denoting an important difference between these studies.

In the present study, insulin-stimulated phosphorylation/activity of IRS-1, PI 3-kinase, and Akt was not significantly decreased in an absolute sense in skeletal muscle from IGT relatives compared with control subjects. Rather, the elevated basal phosphorylation/activity of these signal transduction parameters may possibly mask or inhibit any further increase in signal transduction in response to insulin in skeletal muscle from the IGT relatives. However, the precise molecular mechanism that accounts for the lack of insulin response is unknown. It is interesting that the finding of elevated basal signal transduction at the level of these proximal parameters in skeletal muscle from insulin-resistant IGT relatives is

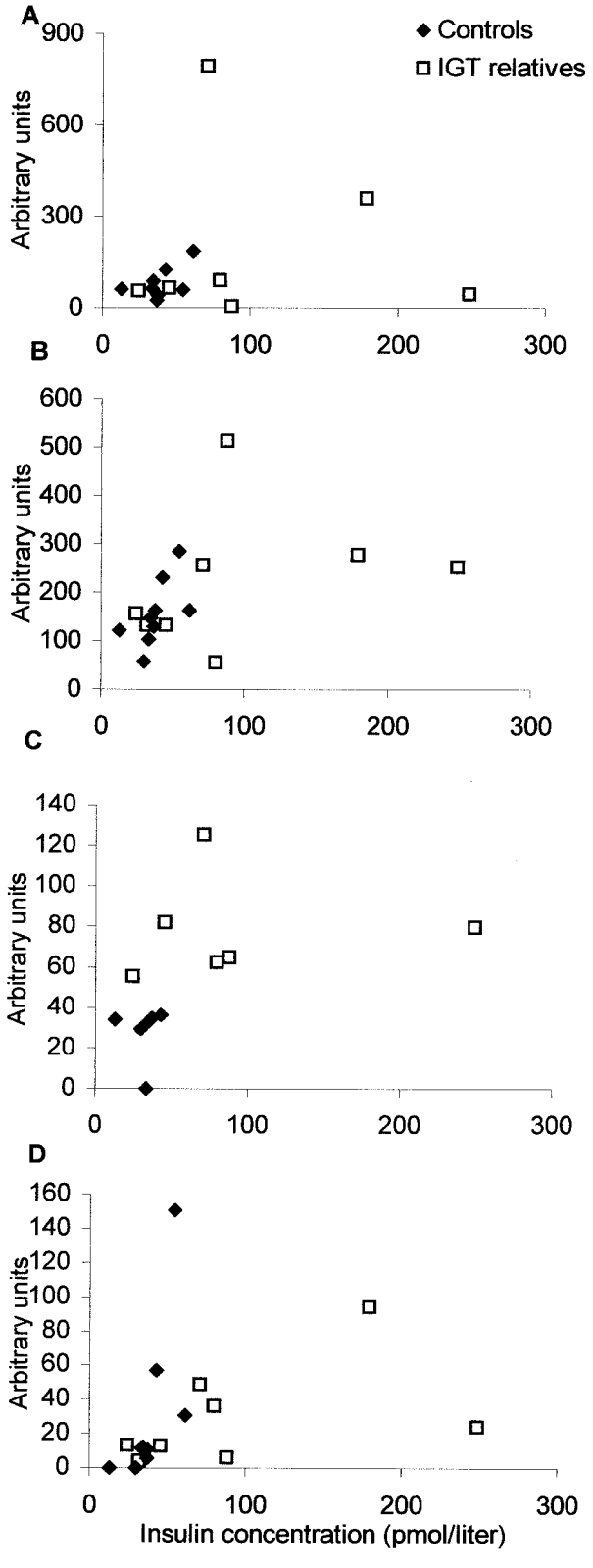

FIG. 3. Correlation between basal insulin concentration and basal IRS-1 tyrosine phosphorylation (*A*), phosphotyrosine-associated PI 3-kinase activity (*B*), IRS-1–associated PI 3-kinase activity (*C*), or Akt serine phosphorylation (*D*). Results for IRS-1 tyrosine phosphorylation (*A*) are for seven IGT first-degree relatives of patients with type 2 diabetes (□) and eight control subjects (◆). Spearman rank-sum correlation analysis for all participants ($n = 15$) is $r = 0.18$ and $P = 0.50$. Results for phosphotyrosine-associated PI 3-kinase activity (*B*) are for eight IGT first-degree relatives of patients with type 2 diabetes (□) and nine control subjects (◆). Spearman rank-sum correlation analysis for all participants ($n = 17$) is $r = 0.59$ and $P = 0.01$. Results for IRS-1–associated PI 3-kinase activity (*C*) are for six IGT first-degree relatives of patients with type 2 diabetes (□) and six control subjects (◆). Spearman rank-sum correlation analysis for all participants ($n = 12$) is $r = 0.72$ and $P = 0.008$. *D*: Serine phosphorylation of Akt was determined for eight IGT first-degree relatives of patients with type 2 diabetes (□) and nine control subjects (◆). Note that although it appears that there are only eight control subjects, two have almost an identical insulin concentration and Akt phosphorylation. Spearman rank-sum correlation analysis for all participants ($n = 17$) is $r = 0.63$ and $P = 0.007$.

consistent with a recent report of a significantly greater basal IRS-1 tyrosine phosphorylation (1.7-fold) in skeletal muscle from insulin-resistant patients with type 2 diabetes, compared with lean control subjects (20). Furthermore, insulin did not elicit a further increase in IRS-1 phosphorylation in skeletal muscle from patients with type 2 diabetes (20), a finding comparable with that noted for IGT relatives. Elevated basal phosphorylation/activity of the signaling intermediates is also consistent with increased basal glucose transport in cultured muscle cells from insulin-resistant nondiabetic first-degree relatives of patients with type 2 diabetes (43).

The molecular mechanism for the observed increased basal tyrosine phosphorylation of IRS-1 in IGT relatives and patients with type 2 diabetes is elusive but may involve modulation at the level of the insulin receptor in response to high levels of insulin or glucose. Although the IGT relatives were not hyperglycemic, intermittent periods of postprandial hyperglycemia as a result of glucose intolerance may modulate insulin receptor and IRS-1 tyrosine phosphorylation in skeletal muscle. In various cultured cell systems, high extracellular glucose concentrations inhibit insulin action (46,47), presumably by inducing serine phosphorylation of the insulin receptor through a PKC-mediated mechanism (47). Glucose-induced serine phosphorylation of the insulin receptor is associated with decreased IRS-1 tyrosine-phosphorylation and attenuation of more distal signaling events, such as activation of PI 3-kinase (47) and Akt (47,48).

Hyperinsulinemia may be a likely candidate to account for increased basal signal transduction in skeletal muscle from the IGT relatives. The high basal IRS-1 tyrosine phosphorylation and PI 3-kinase activity in the IGT relatives may have prevented a further insulin-induced increase in signal transduction. Consistent with this, hyperinsulinemia may promote a constitutive activation of insulin signal transduction in skeletal muscle, which may induce a refractory state of the signaling intermediates when acutely stimulated by insulin during the in vivo experiments. This hypothetical phenomenon may underlie and be phenotypically recognized as insulin resistance in vivo. To test this possibility, we performed correlation analysis between basal insulin levels and basal IRS-1 tyrosine phosphorylation, PI 3-kinase activity, and Akt phosphorylation. The positive association noted between fasting plasma insulin and PI 3-kinase activity, as well as with Akt phosphorylation, for the entire study group, as well as in the control subjects, supports the hypothesis that hyperinsulinemia may partly account for the elevated signal transduction parameters in the IGT relatives in the "basal" state. However, the lack of a direct association between fasting plasma insulin levels and signal transduction parameters in the IGT relatives may to some extent

challenge this hypothesis. Alternatively, other proximal, distal, or even parallel defects of insulin signal transduction parameters may play a greater role for the development of in vivo insulin resistance.

Insulin-stimulated serine phosphorylation of Akt was 55% lower in IGT relatives compared with control subjects; however, this difference was not statistically significant ($P = 0.12$). Nevertheless, the tendency toward decreased insulin-induced serine phosphorylation of Akt (insulin-stimulated minus basal) was consistent with our finding of decreased IRS-1 tyrosine phosphorylation and PI 3-kinase activity in the IGT relatives. Therefore, we cannot exclude that decreased insulin action on Akt as well as on IRS-1 and PI 3-kinase are all likely to represent physiologically relevant defects of some importance for insulin resistance in the IGT relatives. Because these signal transducers constitute early components of the insulin signaling cascade, these defects are likely to contribute to impaired glucose transport and glycogen synthase activation. However, given the relative impairment of these signal transduction parameters, our data suggest the presence of a more proximal postreceptor defect. Although the extent to which muscle insulin resistance in patients with overt type 2 diabetes may be explained by defects of insulin receptor binding or tyrosine kinase activity is controversial (17,18), the only available data in nondiabetic first-degree relatives of patients with type 2 diabetes demonstrates defects in muscle insulin receptor binding and IRTK activity (49).

In conclusion, we have demonstrated that a high physiological insulin infusion does not increase IRS-1 tyrosine phosphorylation and PI 3-kinase activity in skeletal muscle from glucose-intolerant first-degree relatives of patients with type 2 diabetes. This impaired insulin response may partly be explained by elevated basal activation states of these molecules. Thus, when phosphorylation/activity of these signaling intermediates are expressed as increment over basal, defective insulin action on IRS-1, PI 3-kinase, and Akt was noted in IGT relatives. Therefore, insulin signaling defects may partly contribute to impaired insulin action on glucose transport and glycogen synthesis in skeletal muscle in glucose-intolerant relatives of patients with type 2 diabetes, and these signaling defects are present before the development of overt diabetes. However, because the absolute magnitude of insulin action on signal transduction events was not decreased in IGT relatives, other signaling parameters or downstream defects (impaired glucose transport) may play a greater role in the development of skeletal muscle insulin resistance.

ACKNOWLEDGMENTS

This study was supported by grants from the Swedish Society for Medical Research; the Novo-Nordisk Foundation; the Swedish Diabetes Association; the Marcus and Amalia Wallenbergs Foundation; a Junior Individual Grant and a Project Grant from the Foundation for Strategic Research; the Swedish Medical Research Council; the Danish Research Council; the Danish Diabetes Association; the Association of Danish Female Doctors; the Grosserer C. P. Frederiksens Foundation; the Beckett Foundation; the Direktør E. Danielsen and Wifes Foundation; Eli Lilly's Diabetes Research Foundation; the Handelsgartner Ove V. B. Olesens and Edith B. Olesens Foundation; the Ebba Celinders Foundation; the Christian the 3rd Foundation; and the H:S Research Foundation.

We thank all of the individuals who participated in the study for their cooperation and Susanne Reimer and Sussi Polmann, Hvidovre Hospital, for technical assistance.

REFERENCES

1. DeFronzo RA: Pathogenesis of type 2 diabetes: metabolic and molecular implications for identifying diabetes genes. *Diabetes Rev* 5:177–269, 1997
2. Vaag A: On the pathophysiology of late onset non-insulin dependent diabetes mellitus: current controversies and new insights. *Dan Med Bull* 46:197–234, 1999
3. Zierath JR, Krook A, Wallberg-Henriksson H: Insulin action and insulin resistance in human skeletal muscle. *Diabetologia* 43:821–835, 2000
4. Vaag A, Henriksen JE, Beck-Nielsen H: Decreased insulin activation of glycogen synthase in skeletal muscles in young nonobese Caucasian first-degree relatives of patients with non-insulin-dependent diabetes mellitus. *J Clin Invest* 89:782–788, 1992
5. Lillioja S, Mott DM, Spraul M, Ferraro R, Foley JE, Ravussin E, Knowler WC, Bennett PH, Bogardus C: Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus: prospective studies of Pima Indians. *N Engl J Med* 329:1988–1992, 1993
6. Warram JH, Martin BC, Krolewski AS, Soeldner JS, Kahn CR: Slow glucose removal rate and hyperinsulinemia precede the development of type II diabetes in the offspring of diabetic parents. *Ann Intern Med* 113:909–915, 1990
7. Dohm GL, Tapscott EB, Pories WJ, Dabbs DJ, Flickinger EG, Meelheim D, Fushiki T, Atkinson SM, Elton CW, Caro JF: An in vitro human muscle preparation suitable for metabolic studies: decreased insulin stimulation of glucose transport in muscle from morbidly obese and diabetic subjects. *J Clin Invest* 82:486–494, 1988
8. Ryder JW, Yang J, Galuska D, Rincon J, Björnholm M, Krook A, Lund S, Pedersen O, Wallberg-Henriksson H, Zierath JR, Holman GD: Use of a novel impermeable biotinylated photolabeling reagent to assess insulin- and hypoxia-stimulated cell surface GLUT 4 content in skeletal muscle from type 2 diabetic patients. *Diabetes* 49:647–654, 2000
9. Zierath JR, He L, Guma A, Odegoard-Wahlström E, Klip A, Wallberg-Henriksson H: Insulin action on glucose transport and plasma membrane GLUT4 content in skeletal muscle from patients with NIDDM. *Diabetologia* 39:1180–1189, 1996
10. Damsbo P, Vaag A, Hother-Nielsen O, Beck-Nielsen H: Reduced glycogen synthase activity in skeletal muscle from obese patients with and without type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 34:239–245, 1991
11. Björnholm 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
12. Krook A, Björnholm M, Galuska D, Jiang XJ, Fahlman R, Myers MG Jr, Wallberg-Henriksson H, Zierath JR: Characterization of signal transduction and glucose transport in skeletal muscle from type 2 diabetic patients. *Diabetes* 49:284–292, 2000
13. Kerouz NJ, Horsch D, Pons S, Kahn CR: Differential regulation of insulin receptor substrates-1 and −2 (IRS-1 and IRS-2) and phosphatidylinositol 3-kinase isoforms in liver and muscle of the obese diabetic (ob/ob) mouse. *J Clin Invest* 100:3164–3172, 1997
14. Shepherd PR, Kahn BB: Glucose transporters and insulin action: implications for insulin resistance and diabetes mellitus. *N Engl J Med* 341:248–257, 1999
15. Virkamaki A, Ueki K, Kahn CR: Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. *J Clin Invest* 103:931–943, 1999
16. Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA: Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 378:785–789, 1995
17. Klein HH, Vestergaard H, Kotzke G, Pedersen O: Elevation of serum insulin concentration during euglycemic hyperinsulinemic clamp studies leads to similar activation of insulin receptor kinase in skeletal muscle of subjects with and without NIDDM. *Diabetes* 44:1310–1317, 1995
18. Schalin-Jantti C, Harkonen M, Groop LC: Impaired activation of glycogen synthase in people at increased risk for developing NIDDM. *Diabetes* 41:598–604, 1992
19. 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
20. Cusi K, Maezono K, Osman A, Pendergrass M, Patti ME, Pratipanawatr T, DeFronzo RA, Kahn CR, Mandarino LJ: Insulin resistance differentially affects the PI 3-kinase- and MAP kinase-mediated signaling in human muscle. *J Clin Invest* 105:311–320, 2000
21. 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
22. Krook A, Roth RA, Jiang XJ, Zierath JR, Wallberg-Henriksson H: Insulin-stimulated Akt kinase activity is reduced in skeletal muscle from NIDDM subjects. *Diabetes* 47:1281–1286, 1998
23. Zierath JR, Galuska D, Nolte LA, Thörne A, Smedegaard-Kristensen J, Wallberg-Henriksson H: Effects of glycaemia on glucose transport in isolated skeletal muscle from patients with NIDDM: in vitro reversal of muscular insulin resistance. *Diabetologia* 37:270–277, 1994
24. Baron AD, Kolterman OG, Bell J, Mandarino LJ, Olefsky JM: Rates of noninsulin-mediated glucose uptake are elevated in type II diabetic subjects. *J Clin Invest* 76:1782–1788, 1985
25. Vaag A, Damsbo P, Hother-Nielsen O, Beck-Nielsen H: Hyperglycaemia compensates for the defects in insulin-mediated glucose metabolism and in the activation of glycogen synthase in the skeletal muscle of patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 35:80–88, 1992
26. Eriksson J, Franssila-Kallunki A, Ekstrand A, Saloranta C, Widen E, Schalin C, Groop L: Early metabolic defects in persons at increased risk for non-insulin-dependent diabetes mellitus. *N Engl J Med* 321:337–343, 1989
27. Grill V, Persson G, Carlsson S, Norman A, Alvarsson M, Östensson CG, Svanström L, Efendic S: Family history of diabetes in middle-aged Swedish men is a gender unrelated factor which associates with insulinopenia in newly diagnosed diabetic subjects. *Diabetologia* 42:15–23, 1999
28. Gotfredsen A, Baeksgaard L, Hilsted J: Body composition analysis by DEXA by using dynamically changing samarium filtration. *J Appl Physiol* 82:1200–1209, 1997
29. McGuire EA, Helderman JH, Tobin JD, Andres R, Berman M: Effects of arterial versus venous sampling on analysis of glucose kinetics in man. *J Appl Physiol* 41:565–573, 1976
30. DeFronzo RA, Tobin JD, Andres R: Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol (Endocrinol Metab)* 237:E214–E223, 1979
31. Hother-Nielsen O, Beck-Nielsen H: On the determination of basal glucose production rate in patients with type 2 (non-insulin-dependent) diabetes mellitus using primed-continuous 3-3H-glucose infusion. *Diabetologia* 33: 603–610, 1990
32. Molina JM, Baron AD, Edelman SV, Brechtel G, Wallace P, Olefsky JM: Use of a variable tracer infusion method to determine glucose turnover in humans. *Am J Physiol (Endocrinol Metab)* 258:E16–E23, 1990
33. Ferrannini E: The theoretical bases of indirect calorimetry: a review. *Metabolism* 37:287–301, 1988
34. Frayn KN: Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol* 55:628–634, 1983
35. Krook A, Whitehead JP, Dobson SP, Griffiths MR, Ouwens M, Baker C, Hayward AC, Sen SK, Maassen JA, Siddle K, Tavare JM, O'Rahilly S: Two naturally occurring insulin receptor tyrosine kinase domain mutants provide evidence that phosphoinositide 3-kinase activation alone is not sufficient for the mediation of insulin's metabolic and mitogenic effects. *J Biol Chem* 272:30208–30214, 1997
36. Steele R: Influence of glucose loading and of injected insulin on hepatic glucose output. *Ann N Y Acad Sci* 82:420–430, 1959
37. Burgering BM, Coffer PJ: Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* 376:599–602, 1995
38. Didichenko SA, Tilton B, Hemmings BA, Ballmer-Hofer K, Thelen M: Constitutive activation of protein kinase B and phosphorylation of p47phox by a membrane-targeted phosphoinositide 3-kinase. *Curr Biol* 6:1271–1278, 1996
39. Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, Tsichlis PN: The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* 81:727–736, 1995
40. Berrish TS, Hetherington CS, Alberti KG, Walker M: Peripheral and hepatic insulin sensitivity in subjects with impaired glucose tolerance. *Diabetologia* 38:699–704, 1995
41. Rothman DL, Shulman RG, Shulman GI: ^{31}P nuclear magnetic resonance measurements of muscle glucose-6-phosphate: evidence for reduced insulin-dependent muscle glucose transport or phosphorylation activity in non-insulin-dependent diabetes mellitus. *J Clin Invest* 89:1069–1075, 1992
42. 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
43. Jackson S, Bagstaff SM, Lynn S, Yeaman SJ, Turnbull DM, Walker M: Decreased insulin responsiveness of glucose uptake in cultured human skeletal muscle cells from insulin-resistant nondiabetic relatives of type 2 diabetic families. *Diabetes* 49:1169–1177, 2000
44. Del Prato S, Leonetti F, Simonson DC, Sheehan P, Matsuda M, DeFronzo RA: Effect of sustained physiologic hyperinsulinaemia and hyperglycaemia on insulin secretion and insulin sensitivity in man. *Diabetologia* 37:1025–1035, 1994
45. Hotamisligil GS: The role of TNFα and TNF receptors in obesity and insulin resistance. *J Intern Med* 245:621–625, 1999
46. Kroder G, Bossenmaier B, Kellerer M, Capp E, Stoyanov B, Muhlhofer A, Berti L, Horikoshi H, Ullrich A, Haring H: Tumor necrosis factor-a- and hyperglycemia-induced insulin resistance: evidence for different mechanisms and different effects on insulin signaling. *J Clin Invest* 97:1471–1477, 1996
47. Pillay TS, Xiao S, Olefsky JM: Glucose-induced phosphorylation of the insulin receptor. Functional effects and characterization of phosphorylation sites. *J Clin Invest* 97:613–620, 1996
48. Nakajima K, Yamauchi K, Shigematsu S, Ikeo S, Komatsu M, Aizawa T, Hashizume K: Selective attenuation of metabolic branch of insulin receptor down-signaling by high glucose in a hepatoma cell line, HepG2 cells. *J Biol Chem* 275:20880–20886, 2000
49. Handberg A, Vaag A, Vinten J, Beck-Nielsen H: Decreased tyrosine kinase activity in partially purified insulin receptors from muscle of young, non-obese first degree relatives of patients with type 2 (non-insulin-dependent) diabetes mellitus. *Diabetologia* 36:668–674, 1993