Insulin Signaling and Action in Cultured Skeletal Muscle Cells From Lean Healthy Humans With High and Low Insulin Sensitivity

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The aim of these studies was to investigate whether insulin resistance is primary to skeletal muscle. Myoblasts were isolated from muscle biopsies of 8 lean insulin-resistant and 8 carefully matched insulin-sensitive subjects (metabolic clearance rates as determined by euglycemic-hyperinsulinemic clamp: 5.8 ± 0.5 vs. 12.3 ± 1.7 ml · kg^{-1} · min^{-1}, respectively; $P \leq 0.05$) and differentiated to myotubes. In these cells, insulin stimulation of glucose uptake, glycogen synthesis, insulin receptor (IR) kinase activity, and insulin receptor substrate 1–associated phosphatidylinositol 3-kinase (PI 3-kinase) activity were measured. Furthermore, insulin activation of protein kinase B (PKB) was compared with immunoblotting of serine residues at position 473. Basal glucose uptake (1.05 ± 0.07 vs. 0.95 ± 0.07 relative units, respectively; $P = 0.49$) and basal glycogen synthesis (1.02 ± 0.11 vs. 0.98 ± 0.11 relative units, respectively; $P = 0.89$) were not different in myotubes from insulin-resistant and insulin-sensitive subjects. Maximal insulin responsiveness of glucose uptake (1.35 ± 0.03–fold vs. 1.41 ± 0.05–fold over basal for insulin-resistant and insulin-sensitive subjects, respectively; $P = 0.43$) and glycogen synthesis (2.00 ± 0.13–fold vs. 2.10 ± 0.16–fold over basal for insulin-resistant and insulin-sensitive subjects, respectively; $P = 0.66$) were also not different. Insulin stimulation (1 nmol/l) of IR kinase and PI 3-kinase were maximal within 5 min (~8- and 5-fold over basal, respectively), and insulin activation of PKB was maximal within 15 min (~3.5-fold over basal). These time kinetics were not significantly different between groups. In summary, our data show that insulin action and signaling in cultured skeletal muscle cells from normoglycemic lean insulin-resistant subjects is not different from that in cells from insulin-sensitive subjects. This suggests an important role of environmental factors in the development of insulin resistance in skeletal muscle. *Diabetes* 49:992–998, 2000

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Received for publication 13 October 1999 and accepted in revised form 24 January 2000.

AAS, antibiotic antimycotic solution; α-MEM, α-minimum essential medium; ANOVA, analysis of variance; BSA, bovine serum albumin; CEE, chicken embryo extract; DOG, 2-deoxy-D-glucose; FBS, fetal bovine serum; FDR, first-degree relative of a patient with type 2 diabetes; IR, insulin receptor; IRS, insulin receptor substrate; MCR, metabolic clearance rate; OGTT, oral glucose tolerance test; PBS, phosphate-buffered saline; PI 3-kinase, phosphatidylinositol 3-kinase; PKB, protein kinase B; TLC, thin-layer chromatography.

Insulin resistance of skeletal muscle resulting in impaired glucose transport (1) and glycogen synthesis (2,3) is an important factor in the pathogenesis of type 2 diabetes (4). Whether insulin resistance in muscle is caused by defects in the initial insulin-signaling events in type 2 diabetes and where these defects are located remain controversial. Impaired (5,6) or normal (7) insulin-induced insulin receptor (IR) kinase activation, reduced insulin receptor substrate (IRS)-1– and IRS-2–associated phosphatidylinositol 3-kinase (PI 3-kinase) activity (8,9), and decreased (10) or normal (9) protein kinase B (PKB) activation have been described. Moreover, whether these potential defects and/or insulin resistance are primary to muscle tissue (i.e., genetically determined) or secondary to environmental factors such as hyperinsulinemia, hyperlipidacidemia, or hyperglycemia, all of which are frequently present in type 2 diabetes (11,12), is unclear.

Therefore, ideal populations for studying insulin resistance include normoglycemic first-degree relatives of patients with type 2 diabetes (FDRs) who have an increased risk of developing the disease (13). Insulin resistance has been demonstrated to be more prevalent in this group compared with subjects without a family history of type 2 diabetes (14). Moreover, impaired insulin activation of glycogen synthase (15) and IR kinase (16) have been described in muscle biopsies from FDRs. Because these defects occurred in the absence of hyperinsulinemia and hyperglycemia, they were likely genetically determined in this population.

As part of an ongoing study addressing the pathogenesis of type 2 diabetes, we determined the insulin sensitivity of glucose disposal (metabolic clearance rate [MCR] of glucose) in more than 200 healthy FDRs with euglycemic-hyperinsulinemic clamps. Preliminary data suggested that, in our Caucasian population, insulin sensitivity of skeletal muscle is evenly distributed from very sensitive to very resistant (17). Primary defects in muscle, if they exist, should be maximally enriched in lean subjects at the resistant end of the sensitivity spectrum.

Human skeletal muscle cells in primary culture display numerous features of mature skeletal muscle (18–20). This system has been used in the past for studies of muscle metabolism in normoglycemic Pima Indians and in patients with type 2 diabetes in vitro (19,21–25). Muscle cells from different individuals can be grown and studied under controlled experimental conditions, minimizing the influence of environmental factors. Therefore, these cells are a useful tool to

search for primary defects in skeletal muscle. In this study, we compared insulin sensitivity in primary muscle cell cultures from 8 healthy lean insulin-resistant and 8 well-matched insulin-sensitive Caucasians in the population described above. We measured insulin stimulation of glucose uptake and glycogen synthesis and key elements of the insulin-signaling cascade (IR kinase, IRS-1–associated PI 3-kinase, and PKB) in the muscle cells from both groups.

RESEARCH DESIGN AND METHODS

Subjects. A total of 8 normoglycemic nonobese insulin-resistant and 8 insulin-sensitive Caucasian subjects were recruited from a study population to date of 200 FDRs. Insulin sensitivity was determined with the euglycemic-hyperinsulinemic glucose clamp and was expressed as MCR (ml · kg^{-1} · min^{-1} [17]). MCR values ≤7.5 and ≥8.5 ml · kg^{-1} · min^{-1} were cutoff points for insulin-resistant and insulin-sensitive subjects, respectively. Both groups were carefully matched for sex, age, and BMI. The study protocol was approved by the local ethics committee and was carried out in accordance with the Helsinki guidelines. All probands gave their informed written consent before they participated. Glucose tolerance was determined by an oral glucose tolerance test (OGTT) with 40 g glucose/m^2 (17). The clinical data are summarized in Table 1.

Materials. Collagenase type I, α-minimum essential medium (α-MEM), Ham's F-12 medium, fetal bovine serum (FBS), chicken embryo extract (CEE), and a mixture of antibiotic antimycotic solution (AAS; 250 µg/ml amphotericin B, 10,000 U/ml penicillin, 10,000 µg/ml streptomycin) were obtained from Gibco (Eggenstein, Germany). 2-[G-^{3}H]deoxy-D-glucose (5–10 Ci/mmol/l), D-[U-^{14}C]glucose (250–360 mCi/mmol/l), and [γ-^{32}P]ATP (3,000 Ci/mmol/l) were from Du Pont-NEN (Boston, MA). Human insulin (Huminsulin) was from Lilly (Giessen, Germany), rat recombinant IRS-1 was from Upstate Biotechnology (Lake Placid, NY), phosphatidylinositol and wortmannin were from Sigma (Munich, Germany), silica gel thin-layer chromatography (TLC) plates were from Merck (Darmstadt, Germany), polyclonal antibodies against Ser^{473} in PKB and PKB protein were from New England Biolabs (Schwalbach, Germany), peroxidase-labeled antibodies against rabbit IgG were from Bio-Rad (Munich, Germany), and the enhanced chemiluminescence kit was from Amersham (Braunschweig, Germany).

Cell culture. Primary cultures were grown from satellite cells obtained from percutaneous biopsies performed on the lateral portion of the quadriceps femoris (vastus lateralis) muscle using side-cutting needles 5 mm in diameter (Popper & Sons, New Hyde Park, NY). The tissue (~80 mg) was immediately placed in 15 ml Ham's F-12 medium at 4°C. After discarding excess fat and connective tissue, the samples were digested in buffer A (100 mmol/l HEPES, 120 mmol/l NaCl, 48 mmol/l KCl, 1 mmol/l $CaCl_2$, 5.5 mmol/l glucose, 0.1% collagenase, 1.5% bovine serum albumin [BSA], pH 7.4) for 15 min at 37°C with agitation. After a spin at 80*g*, the supernatants were collected in Ham's F-12 medium and 10% FBS (final concentration) to stop enzymatic digestion, and the pellet was subjected to 2 further rounds of digestion by resuspension and centrifugation. The supernatants and the remaining cell debris were pooled and decanted, and the cells were sedimented at 300*g* for 5 min. The cell pellet was then resuspended in a 1:1 mixture of α-MEM and Ham's F-12 supplemented with 20% FBS, 1% CEE, and 0.2% AAS according to Sarabia et al. (20) and plated in 60-cm^2 dishes. All experiments were performed on subcultured cells (first pass) that were plated either at ~5 × 10^4 cells in 60-cm^2 dishes or at 2.5 × 10^3 cells/well in 6-well plates. When myoblasts reached 80–90% confluence (after 4–7 population doublings [21]), the cells were fused for 4 days in α-MEM with 2% FBS and 0.2% AAS according to Henry et al. (19).

2-Deoxy-D-glucose uptake and glycogen synthesis. Assays for glucose uptake and glycogen synthesis were modified from those described by Berti et al. (26) for C_2C_{12} myotubes. Fused cells in 6-well plates were washed 3 times with HEPES-buffered saline solution (20 mmol/l HEPES, 140 mmol/l NaCl, 5 mmol/l KCl, 2.5 mmol/l $MgSO_4$, 1 mmol/l $CaCl_2$, 0.1% BSA, pH 7.4) and then incubated in the same solution with insulin (0–1,000 nmol/l) for 1 h at 37°C with 5% CO_2. To measure glucose uptake, 2-deoxy-D-glucose (DOG)/2-[^{3}H]DOG (10 µmol/l final concentration, 0.08 µCi/well) was added for a further 10 min. Supernatants were then aspirated rapidly, and the cells were washed 3 times with ice-cold phosphate-buffered saline (PBS) and lysed in 0.2 N NaOH. After neutralization with 1 N HCl, radioactivity was determined by liquid scintillation counting. For the determination of nonspecific uptake, parallel incubations were performed in the presence of 10 µmol/l cytochalasin B instead of insulin, and the results were subtracted from the respective incubations in the absence of cytochalasin B.

For the measurement of glycogen synthesis, D-glucose/D-[^{14}C]glucose (5 mmol/l final concentration, 0.3 µCi/well) was added to the wells instead of DOG. After 1 h at 37°C, the supernatants were aspirated rapidly, and the cells were washed 3 times with ice-cold PBS and lysed in 30% (vol/vol) KOH. Aliquots (75 µl/well) were removed for protein analysis with the Bradford dye method (27) using the Bio-Rad reagent and BSA as a standard. The extracts were heated for 30 min at 95°C and were cooled on ice. Glycogen was precipitated with 95% ethanol and was centrifuged for 5 min at 9,000*g*. The resulting pellet was washed once with resuspension and precipitation. The final glycogen pellet was resuspended in 500 µl distilled water, and radioactivity was determined by liquid scintillation counting.

The insulin effect on glucose uptake and glycogen synthesis was expressed as counts per minute obtained in the presence of insulin divided by cpm obtained in the absence of insulin. Basal glucose uptake and glycogen synthesis were expressed as cpm per well per milligram of protein. To verify assay reproducibility, the cells of 6 subjects were plated, grown, and assayed on 2 different occasions. The interassay coefficients of variation for the effect of 100 nmol/l insulin were means ± SE 5 ± 1% (range 2–9) for glucose uptake and 8 ± 3% (1–18) for glycogen synthesis.

IR kinase and IRS-1–associated PI 3-kinase. Myotubes in 60-cm^2 dishes were serum-starved in α-MEM containing 0.5% FBS 16 h before the end of the 4-day fusion period. Myotubes were then stimulated for 0–64 min with insulin (0–100 nmol/l), washed 3 times with ice-cold PBS, and solubilized in buffer B (50 mmol/l HEPES, 1% Triton X-100, 20 mmol/l EDTA, 400 mmol/l sodium fluoride, 50 mmol/l sodium pyrophosphate, 5 mmol/l sodium orthovanadate, 8 mmol/l phenylmethylsulfonyl fluoride, 3 mg/ml benzamidine, 2 µg/ml aprotinin, 2.5 µg/ml leupeptin, 2.5 µg/ml pepstatin A, pH 7.4). After being on ice for 30 min, lysates were centrifuged for 20 min at 12,000*g*, and the supernatants were stored at –80°C until further use. Protein contents were determined as described above. For the IR kinase assays, 40 µl lysates (3.5 mg/ml) were added to microwells coated with anti-IR antibody (28) at 4°C. After overnight incubation, the wells were washed, and the kinase activity of immobilized IR was measured in the presence of 0.3 µmol/l ATP containing 3 µCi/well [γ-^{32}P]ATP and 2.4 µg/ml recombinant IRS-1 as previously described (29). Wells were then washed again, and insulin binding activity (defined as the amount of insulin specifically bound at 8.7 nmol/l) was analyzed as described earlier (28). IR kinase activity was expressed as attomoles phosphate incorporated into IRS-1 per minute per femtomole of insulin binding.

For the measurement of IRS-1–associated PI 3-kinase, a newly developed microwell-based assay system was used. A total of 40 µl of lysates (3.5 mg/ml) was added to microwells coated with anti–IRS-1 antibody (30) at 4°C. After overnight incubation, the wells with the immobilized IRS-1 were washed 5 times with buffer C (0.05% Triton X-100, 100 mmol/l NaCl, 2.5 mmol/l KCl, 1 mmol/l $CaCl_2$, 20 mmol/l HEPES, 0.5% BSA, 10% glycerol, pH 7.4) followed by 5 washes with buffer D (20 mmol/l HEPES, 10 mmol/l $MgCl_2$, 0.4 mmol/l EGTA, 0.4 mmol/l NaH_2PO_4, pH 7.5). Phosphorylation reactions were then initiated by adding 35 µl of buffer D (5 µg phosphatidylinositol, 50 µmol/l ATP, 12 µCi [γ-^{32}P]ATP) to each well. Parallel incubations in the presence of 100 nmol/l wortmannin served as negative controls. After 2 h at 30°C, the reactions were ended by adding 20 µl 4 N HCl to the wells. Samples were then transferred to reaction cups on ice, and lipids were extracted with 95 µl chloroform/methanol (1:1 vol/vol). After centrifugation for 5 min at 9,000*g*, the lower organic phase was applied to silica gel TLC plates. Plates were developed in $CHCl_3$:CH_3OH:H_2O:NH_4 (60:53:15:2), dried, and visualized by autoradiography. Radioactivity incorporated into phosphatidylinositol was quantified by a phosphoimager (Fujix BAS 2000 BioImage System; Fuji, Japan).

TABLE 1
Clinical characteristics of subjects

	Subjects		
	Insulin-sensitive	Insulin-resistant	*P*
n (F/M)	5/3	5/3	—
Age (years)	29 ± 2	26 ± 1	0.150
BMI (kg/m^2)	22 ± 1	24 ± 1	0.176
Fasting glucose (mg%)	85 ± 3	84 ± 3	0.713
2-h Glucose (mg%)*	87 ± 7	103 ± 5	0.101
Fasting insulin (pmol/l)	33 ± 5	46 ± 8	0.191
2-h Insulin (pmol/l)*	110 ± 15	259 ± 53	0.017
MCR (ml · kg^{-1} · min^{-1})†	12.3 ± 1.7	5.8 ± 0.5	0.003

Data are means ± SE. Significance was determined by Student's *t* test for unpaired data. *By OGTT; †by euglycemic-hyperinsulinemic clamp.

Immunoblotting. The activation of PKB that occurs by its phosphorylation at threonine 308 and Ser^{473} (31) was determined with immunoblotting of the Ser^{473}. Cell lysates used for kinase assays were adjusted for the same protein content by dilution in Laemmli sample-buffer (32) that contained 10% (vol/vol) β-mercaptoethanol. Samples were boiled, subjected to 7.5% SDS-PAGE (45 µg total protein/lane for detection of Ser^{473} in PKB, 15 µg/lane for detection of PKB protein), and the proteins were transferred to nitrocellulose membranes. Ser^{473} in PKB and PKB protein were detected with specific primary antibodies, peroxidase-labeled secondary antibodies, and enhanced chemiluminescence (33). Bands were evaluated by scanning densitometry.

Statistics. Myotubes were grown and analyzed in pairs containing cells from 1 insulin-resistant and 1 insulin-sensitive subject. In every paired experiment, results are expressed relative to the mean absolute value. This permitted us to compare experiments performed on separate days. Statistical significance was evaluated by 2-tailed Student's *t* test for paired or unpaired data or by analysis of variance (ANOVA) for time courses and dose-response curves as indicated. *P* values ≤0.05 were considered to be statistically significant.

RESULTS

Effect of different concentrations of insulin on glucose uptake, glycogen synthesis, and insulin signaling in myotubes. Figure 1*A* and *B* show insulin dose-response curves for 2-deoxyglucose uptake and glycogen synthesis in myotubes. Dose responses were not different in insulin-sensitive and insulin-resistant subjects. Maximal insulin effects were achieved at 100 nmol/l insulin, at which glucose uptake was increased ~1.4-fold and glycogen synthesis was increased ~2-fold over basal levels. Insulin effect (100 nmol/l) on glucose uptake was completely inhibitable by 0.1 µmol/l wortmannin (data not shown). Cytochalasin B decreased basal glucose uptake by >90% (data not shown), which confirmed that glucose uptake resulted from specific carrier-mediated transport. Figure 1*C*–*E* demonstrates insulin dose-response curves for IR kinase, IRS-1–associated PI 3-kinase, and phosphorylation of Ser^{473} in PKB, which were not significantly different between myotubes from insulin-sensitive and insulin-resistant subjects (NS by ANOVA). Insulin at a concentration that elicited maximal glucose uptake and glycogen synthesis (100 nmol/l) activated IR kinase ~15-fold and IRS-1–associated PI 3-kinase ~11-fold and increased Ser^{473} phosphorylation in PKB ~60-fold over their respective basal values.

Insulin action in myotubes from insulin-resistant subjects compared with control subjects. As shown in Fig. 2, maximal insulin responsiveness of glucose uptake (1.35 ± 0.03–fold vs. 1.41 ± 0.05–fold over basal, respectively; $P = 0.43$) and glycogen synthesis (2.00 ± 0.13–fold vs. 2.10 ± 0.16–fold over basal, respectively; $P = 0.66$) were not significantly different between myotubes from 8 insulin-resistant and 8 insulin-sensitive subjects. Furthermore, basal glucose uptake (1.05 ± 0.07 vs. 0.95 ± 0.07 relative units, respectively; $P = 0.49$ [$n = 7$]) and basal glycogen synthesis (1.02 ± 0.11 vs. 0.98 ± 0.11 relative units, respectively; $P = 0.89$ [$n = 7$]) were not significantly different in insulin-resistant and insulin-sensitive subjects.

Time kinetics of insulin signaling in myotubes. Figure 3 shows that insulin at a physiological concentration (1 nmol/l) led to a rapid increase in the activity of IR kinase and IRS-1–associated PI 3-kinase to maximal levels within 5 min (~8- and 5-fold over basal, respectively). In addition, insulin elicited maximal levels of PKB activation within 15 min (~3.5-fold over basal). These time kinetics were not different in myotubes from insulin-resistant versus insulin-sensitive subjects (NS by ANOVA). Furthermore, the amount of PKB protein as detected by immunoblot did not differ from basal levels during insulin incubation for as long as 64 min (data not shown; NS) and was not different between groups at any time point (NS by ANOVA).

DISCUSSION

The present study demonstrates that neither insulin-stimulated glucose uptake, glycogen synthesis, nor insulin signaling in cultured skeletal muscle cells were different in insulin-resistant and insulin-sensitive subjects. This in vitro lack of differences in insulin action and signaling is remarkable because glucose disposal measured in vivo was clearly different in the 2 groups. This finding indicates that the clinical insulin resistance in our subjects largely depended on factors present only in the in vivo environment. Deprivation of these factors during controlled culture conditions clearly resulted in the loss of the differences in insulin sensitivity, which suggests that, in our population, insulin resistance was not caused by primary defects in skeletal muscle but rather by environmental factors.

This conclusion contrasts with the general perception that lean insulin-resistant subjects are prone to have a primary defect in skeletal muscle. However, this interpretation of the data would not preclude heredity to be important in the development of insulin resistance. For example, genetic factors may well control the susceptibility of muscle to exogenous factors that result in impaired insulin action, including mild hyperinsulinemia and/or glycemic excursions (e.g., postprandial) that have been shown to decrease glucose disposal in skeletal muscle in vivo (34). Recent observations showing that myotubes from patients with type 2 diabetes are more sensitive to the effect of hyperglycemia to downregulate glucose transport activity than cells from normal control subjects support this concept (25). Our findings are also consistent with a recent study that demonstrated that isolated adipocytes from insulin-resistant FDRs have no defect in insulin action on glucose uptake and lipolysis compared with control subjects (35). However, the in vivo insulin sensitivity was not as clearly separated in that study as in our study.

Our finding no difference in insulin action could also be explained by a loss of the individual muscle phenotype as a result of the growth and differentiation conditions in vitro. For example, whether the muscle-specific glucose transporter GLUT4, which is expressed in cultured muscle cells (19,20), contributes to insulin-stimulated glucose uptake in this cell model to the same extent as in mature skeletal muscle is not known. We cannot therefore exclude the possibility that GLUT1, which is also expressed in cultured muscle cells (19,20), confounded our results. The insulin responsiveness of glucose uptake in our study, however, was comparable with that in previous studies that used the same cell model (20,36). Insulin-stimulated glycogen synthesis, the other effector system in cultured muscle cells, was also of a similar magnitude as demonstrated in previous reports (21,37). Furthermore, we demonstrated for the first time that insulin signaling (IR kinase, PI 3-kinase, PKB) in differentiated muscle cells is highly responsive to insulin. Insulin dose-response characteristics of IR kinase were in a similar range as previously observed for IR kinase from mature skeletal muscle stimulated in vivo (38) or in vitro (16). These findings demonstrate that insulin effects in myotubes and mature muscle are at least in part comparable. Therefore, we consider

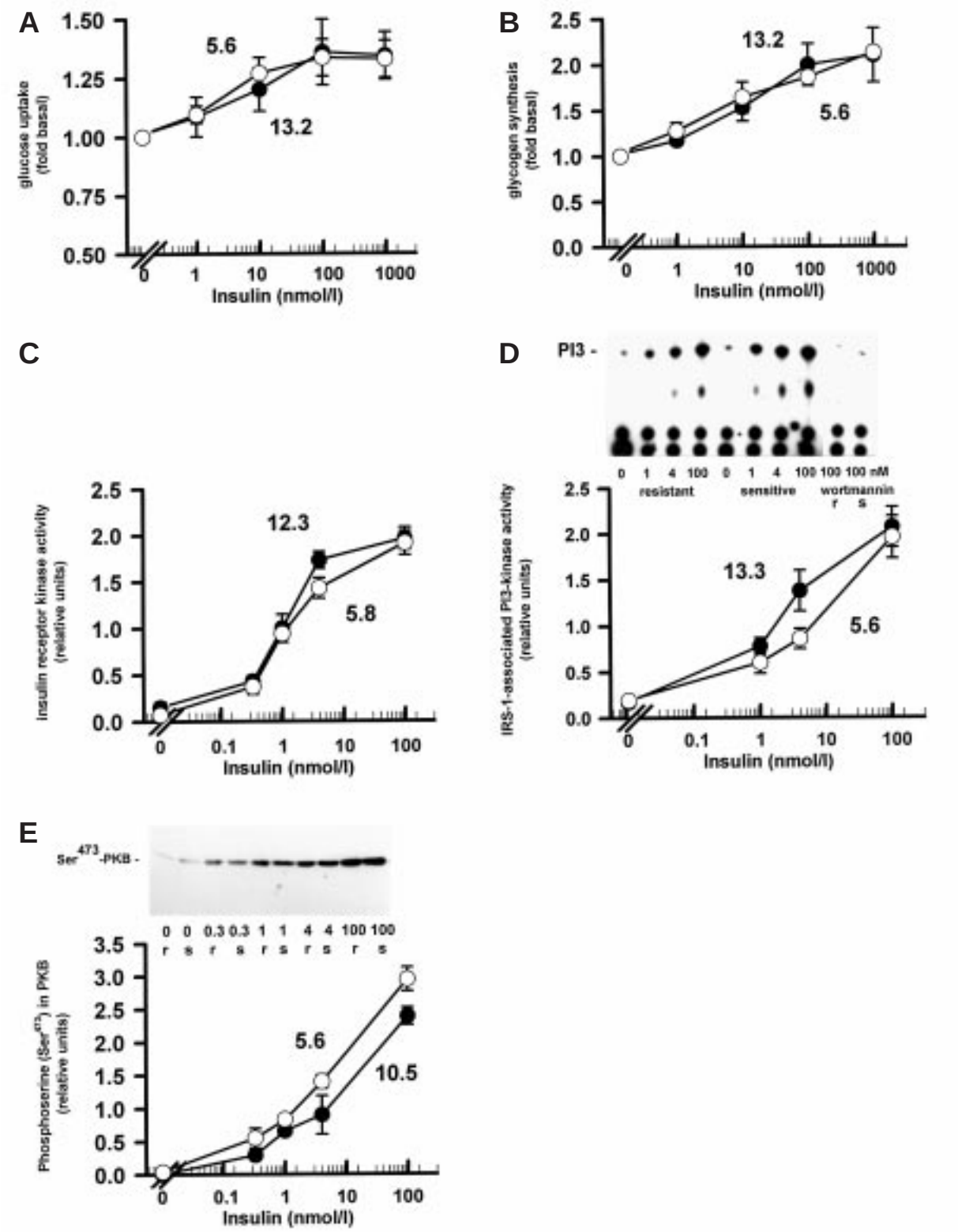

FIG. 1. Effect of increasing concentrations of insulin on glucose uptake (*A*), glycogen synthesis (*B*), IR kinase (*C*), IRS-1–associated PI 3-kinase (*D*), and Ser^{473} in PKB (*E*) in myotubes. Myotubes from insulin-sensitive (●) and insulin-resistant subjects (○) were stimulated with the indicated concentrations of insulin for 1 h (*A* and *B*), 4 min (*C* and *D*), or 15 min (*E*). *A* and *B*: Glucose uptake and glycogen synthesis of myotubes from 3 insulin-sensitive and 3 insulin-resistant subjects (MCR 13.2 ± 3.1 vs. 5.6 ± 1.1 mg · kg^{-1} · min^{-1}, respectively). *C*: IR kinase activities measured after immobilization of IR from the myotubes of 8 insulin-sensitive and 8 insulin-resistant subjects (MCR 12.3 ± 1.7 vs. 5.8 ± 0.5 mg · kg^{-1} · min^{-1}, respectively) to anti–IR microwells. *D*: IRS-1–associated PI 3-kinase activities measured after immobilization of IRS-1 from the myotubes of 3 insulin-sensitive and 3 insulin-resistant subjects (MCR 13.3 ± 3.7 vs. 5.6 ± 1.1 mg · kg^{-1} · min^{-1}, respectively) to anti–IRS-1 microwells. A representative autoradiograph is shown. *E*: Ser^{473} phosphorylation in PKB measured by immunoblot in myotubes from 2 insulin-sensitive and 2 insulin-resistant subjects (MCR 10.5 ± 1.4 vs. 5.6 ± 1.0 mg · kg^{-1} · min^{-1}, respectively). A representative immunoblot is shown. Data are means ± SE. The numbers next to the lines indicate the mean MCR of the subjects used for the respective panel.

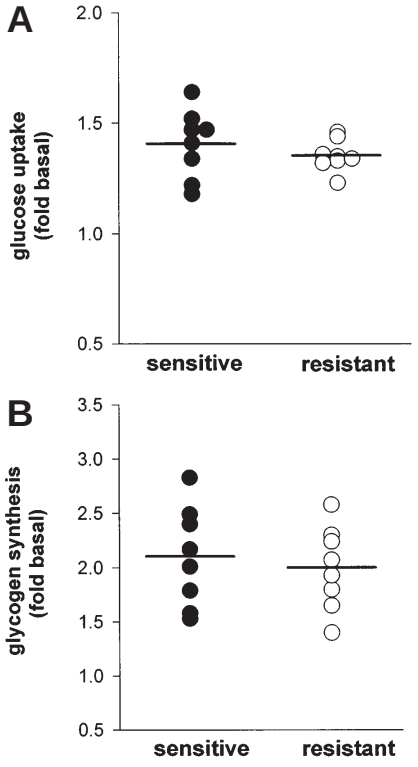

FIG. 2. Maximal insulin-stimulated glucose uptake (*A*) and glycogen synthesis (*B*) in myotubes from 8 insulin-sensitive (●) and 8 insulin-resistant subjects (○) stimulated with or without 100 nmol/l insulin for 60 min.

our cell culture system to be a valid model for excluding primary defects of major relevance in skeletal muscle.

In contrast with our data, previous studies have shown insulin resistance to be retained in cultured muscle cells. Myotubes from patients with type 2 diabetes showed no alterations in insulin responsiveness of glucose transport, whereas basal glucose transport activity was reduced (19). Furthermore, basal and insulin-stimulated glycogen synthase fractional velocity was impaired in myotubes from subjects with type 2 diabetes as shown by the same authors (24). In cultured myoblasts from nondiabetic Pima Indians, in vivo insulin resistance was also retained (21–23). Several possible explanations exist for these apparent discrepancies. Differences in age and body weight between control and diabetic subjects may have influenced the results (19,24). Moreover, insulin resistance in the cultured muscle cells from diabetic subjects may reflect irreversible acquired defects as a consequence of chronic hyperinsulinemia and/or hyperglycemia before their isolation. In addition, differences in genetic background may play a role, which would especially be the case for Pima Indians. Finally, we cannot exclude that, despite their hereditary status, the insulin-resistant subjects in our study will not develop diabetes. Thus, the primary muscle insulin resistance that definitely results in overt type 2 diabetes may have a different in vitro appearance.

One could argue that the relatively small maximal insulin effect on glucose uptake (~40% over basal) and on glycogen synthesis (~100% over basal), albeit in the range of previous studies, does not provide sufficient sensitivity to compare the groups at physiological insulin levels (dose-response curves

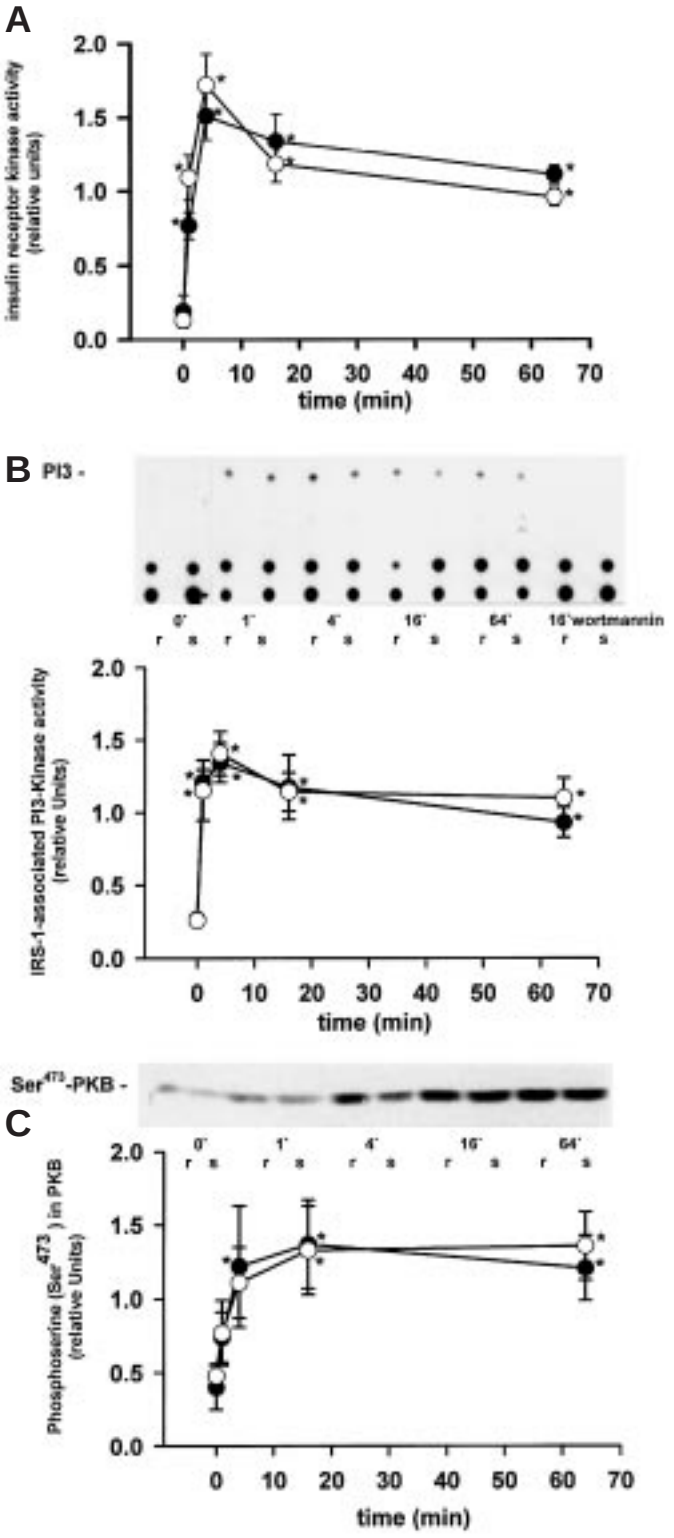

FIG. 3. Time dependence of the effect of 1 nmol/l insulin on IR kinase (*A*), IRS-1–associated PI 3-kinase (*B*), and Ser^{473} in PKB (*C*) in myotubes from insulin-sensitive (●) and insulin-resistant (○) subjects. *A*: IR kinase activities in myotubes from 8 insulin-sensitive and 8 insulin-resistant subjects. *B*: IRS-1–associated PI 3-kinase activities in myotubes from 7 insulin-sensitive and 7 insulin-resistant subjects. A representative autoradiograph is shown. *C*: Ser^{473} phosphorylation in PKB in myotubes from 7 insulin-sensitive and 7 insulin-resistant subjects. A representative immunoblot is shown. Data are means ± SE. *Significant difference from the corresponding basal value ($P \leq 0.05$ by Student's *t* test for paired data).

demonstrated for cell lines from 3 individuals in each group [Fig. 1]). Therefore, to produce a reasonable insulin effect, we had to use supraphysiogical insulin concentrations (100 nmol/l [Fig. 2]). This does not match the in vivo stimulation in which insulin concentrations of ~0.5 nmol/l were used. Consequently, we extended our comparative investigations to the insulin-signaling cascade. In this study, the high insulin responsiveness of IR kinase, PI 3-kinase, and PKB allowed us to investigate time kinetics at a physiological insulin concentration (1 nmol/l). However, also at this concentration, no differences between myotubes from insulin-resistant and insulin-sensitive subjects were detectable.

One may consider the fact that the insulin-sensitive subjects also had a family history of type 2 diabetes to be a disadvantage of this study. However, we regarded the highly insulin-sensitive subjects to be unaffected by potentially primary factors causing in vivo insulin resistance regardless of family history and therefore to be suitable for comparison with the insulin-resistant subjects.

In summary, our data show that insulin signaling and action in cultured skeletal muscle cells from normoglycemic lean insulin-resistant subjects are not impaired. This finding suggests an important role of environmental factors in the development of insulin resistance in skeletal muscle.

ACKNOWLEDGMENTS

This work was supported in part by grants from Deutsche Forschungsgemeinschaft (Stu-192/2-1 to M.S. and KL-503/7-3 to H.H.K.), the European Community (QLRT-1999-00674), and the Deutsche Diabetes-Gesellschaft (to M.S.).

We thank the volunteers for their participation. We gratefully acknowledge the superb technical assistance of E. Maerker.

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