Decreased Insulin Responsiveness of Glucose Uptake in Cultured Human Skeletal Muscle Cells From Insulin-Resistant Nondiabetic Relatives of Type 2 Diabetic Families

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To investigate the contribution of inherited biochemical defects to the peripheral insulin resistance of type 2 diabetes, we studied cultured skeletal muscle from 10 insulin-resistant nondiabetic first-degree relatives of type 2 diabetic families and 6 control subjects. Insulin stimulation of glucose uptake and glycogen synthesis was maximal in myoblasts. Insulin-stimulated glucose uptake (fold-stimulation over basal uptake) was decreased in relative compared with control myoblasts at 0.001 µmol/l (0.93 ± 0.05 [mean ± SE] vs. 1.15 ± 0.06, *P* < 0.05) and 0.1 µmol/l (1.38 ± 0.10 vs. 1.69 ± 0.08, *P* = 0.025) insulin. Insulin responsiveness was markedly impaired in 5 of the relative myoblast cultures, and in 4 of these, there was an associated increase in basal glucose uptake (76.7 ± 7.0 vs. 47.4 ± 5.5 pmol · min^{-1} · mg^{-1} protein, relative vs. control; *P* < 0.02). Expression of insulin receptor substrate 1, phosphatidylinositol 3-kinase, protein kinase B, and glycogen synthase was normal in the relative cultures with impaired insulin responsiveness. Glycogen synthesis was also normal in the relative cultures. We conclude that the persistence of impaired insulin responsiveness in some of the relative cultures supports the role of inherited factors in the insulin resistance of type 2 diabetes and that the association with increased basal glucose uptake suggests that the 2 abnormalities may be linked. *Diabetes* 49:1169–1177, 2000

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FBG, fasting blood glucose; GS, glycogen synthesis; IRS, insulin receptor substrate; ITT, insulin tolerance test; K_{ITT}, insulin resistance index; MEM, minimum essential medium; OGTT, oral glucose tolerance test; PBS, phosphate-buffered saline; PED/PEA-15, phospho-protein enriched in diabetes; PI, phosphatidylinositol; PKB, protein kinase B; S_G, glucose effectiveness; WHR, waist-to-hip ratio.

Type 2 diabetes is a heterogeneous condition characterized by insulin resistance, pancreatic β-cell dysfunction, dyslipidemia, and a high prevalence of obesity. The relative importance of these characteristics in the pathogenesis of the disease is unclear because once type 2 diabetes is fully developed, it is impossible to determine the initial event or events that precipitated its onset (1,2). There is strong evidence, however, for the role of genetic factors in the development of type 2 diabetes. First, there is a high rate of concordance of type 2 diabetes in monozygotic twins (70–80%) compared with dizygotic twins (10–20%) (3,4), and second, there is clear clustering within families. The lifetime risk of nondiabetic sibs and offspring of type 2 diabetic patients developing diabetes has been estimated at ~40% (5).

Insulin resistance is one of the earliest recognizable metabolic events in nondiabetic first-degree relatives of type 2 diabetic patients, even in the presence of normal glucose tolerance (6–10). Importantly, prospective studies in families and populations at risk of developing type 2 diabetes have shown that insulin resistance is a major predictor for the subsequent development of diabetes (11,12). Skeletal muscle has been shown to be the principal peripheral site of insulin resistance in type 2 diabetes (13,14), although the underlying mechanisms remain to be clearly defined. In healthy humans, skeletal muscle accounts for ~70–80% of the insulin-stimulated glucose uptake in vivo (15), and most of the metabolized glucose is stored nonoxidatively (15) as glycogen (16). Both glucose transport, the rate-limiting step in glucose utilization by muscle (17,18), and glycogen synthesis are stimulated by insulin, and defects in each of these processes have been implicated in the development of peripheral insulin resistance in type 2 diabetes (16,19–23).

There are several lines of evidence that implicate inherited factors in the development of peripheral insulin resistance. First, not only is impaired peripheral insulin action an early metabolic feature of nondiabetic relatives of type 2 diabetic patients, but 2 studies reported clear familial clustering of impaired peripheral insulin action in type 2 diabetic families (24,25). Second, defects of insulin action are retained in cultured fibroblasts (26,27) and skeletal muscle cells (28,29) from type 2 diabetic patients.

Human skeletal muscle culture provides a powerful tool for the investigation of the biochemical and genetic basis of peripheral insulin resistance in type 2 diabetes. Importantly, cultured human muscle cells maintain morphological, biochemical, and metabolic properties of skeletal muscle (28–34), and in particular, glucose uptake and glycogen synthesis remain responsive to insulin in cultured muscle cells from control subjects (28–33). One of the difficulties in investigating patients with established type 2 diabetes is that secondary metabolic changes such as chronic hyperglycemia and lipid abnormalities contribute to peripheral insulin resistance (35,36). Therefore, to limit the influence of these secondary changes and focus on subjects that are likely to harbor inherited defects of insulin action, we have identified insulin-resistant nondiabetic relatives of type 2 diabetic families and established muscle cultures. In these cultures, we have studied the effect of insulin on glucose uptake and glycogen synthesis—the 2 putative sites of insulin resistance in type 2 diabetes.

RESEARCH DESIGN AND METHODS

Subjects. Nondiabetic first-degree relatives of type 2 diabetic patients from families of northern European extraction with at least 2 living type 2 diabetic relatives were recruited (9). There were 93 relatives who underwent a 75-g oral glucose tolerance test (OGTT) and an insulin tolerance test (ITT) (0.05 U insulin/kg body wt) to assess whole-body insulin sensitivity (37). Insulin resistance has been previously defined as an insulin resistance index (K_{ITT}) <2.5% min using the ITT (38), and this result corresponds exactly with the cutoff for the lower tertile of the relatives' K_{ITT} distribution. The difference in K_{ITT} values between the 31 insulin-resistant relatives from the lower tertile and the other 62 relatives was independent of BMI as a covariate. Of the insulin-resistant relatives, 10 had impaired glucose tolerance, and the rest had normal glucose tolerance. To try to limit the potential confounding effect of hyperglycemia on insulin sensitivity, we primarily recruited for muscle biopsy relatives with normal glucose tolerance, and as shown in Table 1, only 1 of the 10 relatives was classified as having impaired glucose tolerance.

There were 94 nondiabetic control subjects with no family history of diabetes recruited from the local background population (9) who underwent an OGTT and ITT. The 6 subjects with normal glucose tolerance classified as insulin sensitive based on a K_{ITT} value above the cutoff (>3.7% min) for the upper tertile of their K_{ITT} distribution were recruited for muscle biopsy. Their characteristics are shown in Table 1.

All subjects gave written informed consent, and the study was approved by the Newcastle and North Tyneside Joint Ethics Committee.

Materials. Cell culture materials were supplied by Life Technologies (Paisley, Scotland, U.K.), except for minimum essential medium (MEM)-α modification, which was supplied by Sigma (Poole, U.K.). All cell culture plastic ware was purchased from Nunc (Paisley, Scotland, U.K.), except for 6-well tissue culture plates, which were supplied by Costar (Littel Chalfont, Bucks, U.K.). 2-Deoxy-D-glucose-^{3}H (G), cytochalasin B (from Helminotosporium dematiodeum), and glycogen type II were obtained from Sigma. D-[U-^{14}C]glucose was purchased from Amersham Life Sciences (Little Chalfont, Bucks, U.K.). Human actrapid insulin was supplied by Novo Nordisk (West Sussex, U.K.). 2-Deoxy-D-glucose was obtained from ICN Biomedicals (Thame, U.K.). Mouse anti-human desmin and rabbit anti-mouse immunoglobulins were purchased from Dako (High Wycombe, U.K.). Mouse anti-human titin was supplied by NovoCastra (Newcastle upon Tyne, U.K.). Goat polyclonal anti-GLUT1 IgG (C-20) and rabbit antibody to insulin receptor substrate (IRS)-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Upstate Biotechnology (Lake Placid, NY), respectively. Rabbit antibodies to GLUT4, phosphatidylinositol (PI) 3-kinase, protein kinase B (PKB), and glycogen synthase were gifts from Gus Leinard (Dartmouth Medical School, Hanover, NH), Peter Shepherd (University College London, London), Brian Hemmings (Basel, Switzerland), and Leif Groop (Malmo, Sweden), respectively. Donkey anti-rabbit IgG fluorescein-conjugated secondary antibody and donkey anti-goat IgG rhodamine-conjugated secondary antibody, both absorbed for dual labeling, were obtained from Chemicon International (Harrow, U.K.). The secondary goat anti-rabbit antibody linked to horseradish peroxidase for Western blotting was obtained from Sigma.

Human primary muscle cultures. Aneural muscle cultures were established according to the methods of Blau and Webster (34) and Yasin et al. (39) and from ~50 mg muscle obtained from a needle biopsy of each of the subjects described above. Cultures were maintained in Nutrient Mixture Ham's F10, supplemented with 20% fetal bovine serum, 1% chick embryo extract, 100 U/ml penicillin, and 100 μg/ml streptomycin in an atmosphere of 5% CO_2 in air. Cell monolayers were harvested by trypsinization when 80% confluence was attained and subcultured using a split ratio of 1 to 4.

Once the cell monolayer attained ~90% confluence, myoblast fusion was initiated by changing the growth media to MEM-α, supplemented with 2% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.1 μmol/l insulin. The medium was changed every 3 days. Cell fusion and differentiation into multinucleated myotubes was monitored visually by phase contrast microscopy.

TABLE 1
Characteristics of insulin-resistant relatives and insulin-sensitive control subjects

	Family number	Sex	Age (years)	BMI (kg/m^2)	WHR	FBG mmol/l	2-h Blood glucose (mmol/l)	K_{ITT} (% min)
Relatives								
1	4	M	48	30.8	0.96	5.1	5.4	1.4
2	10	M	49	24.4	0.89	5.1	5.5	2.3
3	14	F	42	37.9	0.90	4.4	7.0	2.1
4	14	M	29	27.5	0.90	4.4	2.9	1.6
5	18	M	38	34.0	1.00	6.0	9.8	1.9
6	23	F	35	27.3	0.83	5.5	6.9	2.2
7	23	M	58	28.0	0.88	4.9	4.5	2.0
8	26	F	52	28.4	0.82	4.8	5.7	2.2
9	37	F	36	31.3	0.84	4.6	6.0	2.0
10	37	F	25	26.8	0.83	4.2	4.7	2.1
Means ± SE	—	—	41 ± 3	29.6 ± 1.3	0.88 ± 0.02	4.9 ± 0.2	5.8 ± 0.6	2.0 ± 0.1
Control subjects								
1	—	F	64	24.3	0.80	4.2	4.2	5.1
2	—	F	30	24.6	0.85	4.6	4.5	5.5
3	—	M	35	22.3	0.77	4.4	5.0	4.2
4	—	M	48	24.0	0.82	4.8	3.5	4.7
5	—	F	43	23.3	0.74	4.1	4.3	4.3
6	—	F	24	23.1	0.75	4.4	4.6	4.1
Means ± SE	—	—	41 ± 6	23.6 ± 0.4	0.79 ± 0.02	4.4 ± 0.1	4.4 ± 0.2	4.7 ± 0.2

Muscle cell origin was confirmed in each culture immunohistochemically using antibodies to the muscle-specific proteins desmin and titin (40,41). Greater than 95% of cells in each culture were of muscle origin. Before assay, all cultures were confirmed free of mycoplasma contamination using a polymerase chain reaction enzyme-linked immunosorbent assay–based kit (Boehringer Mannheim, Lewes, U.K.).

Measurement of 2-deoxy-D-[3H]glucose uptake. Cells were grown in 6-well cluster plates. Before assay, the medium was removed from the cell monolayer and replaced with 2 ml serum-free MEM-α. The cells were then incubated at 37°C for 15 min, after which the medium was removed and the procedure was repeated once more. The cell monolayers were then incubated in 2 ml serum-free MEM-α for 16 h, after which time the media were replaced with 1 ml serum-free MEM-α, including insulin when stated, and the cells incubated at 37°C for 1 h. 2-Deoxy-D-[^{3}H]glucose uptake was then determined using a modification of the method of Klip et al. (42). The plates were washed 3 times with 2 ml uptake buffer: 118 mmol/l NaCl, 5 mmol/l KCl, 1.3 mmol/l $CaCl_2$, 1.2 mmol/l $MgSO_4$, and 1.2 mmol/l KH_2PO_4, pH 7.4, at 37°C. Then, 1 ml of this buffer was added to each well, and uptake was initiated by the addition of 10 μmol/l 2-deoxy-D-[^{3}H]glucose (0.7 μCi/ml final concentration). After a 10-min incubation at 37°C, uptake was stopped by pouring off the incubation medium and rapidly rinsing the monolayer 5 times with ice-cold Dulbecco's phosphate-buffered saline (PBS). The cells were then solubilized in 0.5 ml 0.05% SDS for 30 min. The wells were then aspirated and washed twice with 200 μl distilled water, and cell-associated radioactivity in each well was determined in 750 μl of the combined aspirate/washings by scintillation counting. Under these conditions, transport across the cell membrane and not phosphorylation is the rate-limiting step in the uptake of 2-deoxyglucose (30,43). Non–carrier-mediated uptake was determined in parallel incubations in which the cells were exposed to 10 μmol/l cytochalasin B, a potent inhibitor of transport (31,44), for 15 min before initiation of uptake. The value obtained was subtracted from all experimental uptake values and was typically <10% of the basal value determined in the absence of the inhibitor.

Glycogen synthesis. Total glycogen synthesis was determined by measuring the incorporation of D-[U-^{14}C]glucose into glycogen in cells cultured in parallel to those used in the glucose uptake studies.

Before assay, cells cultured in 6-well plates were washed in serum-free MEM-α and exposed to this medium for 16 h exactly as described above. After this period of serum starvation, the medium was replaced with 1 ml serum-free MEM-α, and the cells were incubated in the presence or absence of insulin for 1 h. D-[U-^{14}C]glucose was then added (1.25 μCi/ml final concentration), and the cells were incubated for a further 1 h at 37°C. After this time, incorporation was stopped by pouring off the medium and rapidly rinsing the monolayer 5 times with ice-cold Dulbecco's PBS. The cells were then solubilized by adding 250 μl of 20% (wt/vol) KOH to each well. When the cell monolayer was completely solubilized, 250 μl of 1 mol/l HCl was added to each well. The wells were then aspirated and washed twice with 200 μl water, and glycogen was extracted from 650 μl of the combined aspirate/washings by ethanol precipitation after the addition of 12 mg/ml (final concentration) carrier glycogen. Glycogen-associated radioactivity was determined by scintillation counting.

Protein determination. Protein content of the cell extracts was determined by the method of Bradford (45) using Pierce Coomassie Protein Reagent (Pierce, Rockford, IL).

Immunofluorescent detection of GLUT1 and GLUT4. Myoblasts and myotubes were grown on chamber slides to ~80% confluence, at which time the growth medium was replaced with serum-free MEM-α. After 16 h of serum deprivation, the medium was replaced, and the cells were incubated for a further 1 h in the presence or absence of insulin. The monolayer was rinsed 3 times with PBS and fixed for 15 min at room temperature with 3.7% formalin in PBS. The cells were further rinsed in PBS, blocked in 10% normal goat serum in PBS for 30 min, and then exposed to the anti-GLUT1 and anti-GLUT4 antibodies (Santa Cruz Biotechnology) diluted in 1.5% bovine serum albumin in PBS (5 μg/ml) for 1 h. After 3 washes with PBS, the slides were incubated with fluorescein-conjugated donkey anti-rabbit serum and rhodamine-conjugated donkey anti-goat serum (10 and 20 μg/ml in 1.5% bovine serum albumin in PBS, respectively) for 1 h, washed 4 times in PBS, and mounted in glycerol/PBS mounting medium (Citifluor, Canterbury, U.K.). In some instances, the cells were labeled for GLUT4 only, in which case the cells were counter-stained with probidium iodide (5 μg/ml in PBS) for 10 min, which stains both nuclear DNA and cytoplasmic RNA. Confocal microscopy was performed at the Biomedical Electron Microscopy Unit, University of Newcastle upon Tyne.

Western blotting. Myoblasts were harvested from 6-well plates in 200 μl of extraction buffer (50 mmol/l Tris-HCl, pH 7.4; 1 mmol/l sodium vanadate; 1 mmol/l EGTA; 1 mmol/l sodium fluoride; 150 mmol/l sodium chloride; 1 mmol/l sodium pyrophosphate; 1% NP-40; 0.25% sodium deoxycholate; 1 μg/ml each of pepstatin, antipain, and leupeptin; and 1 mmol/l phenylmethylsulfonyl fluoride). Samples were snap-frozen and stored at –80°C until needed. The samples were defrosted, and the protein concentration was determined. Aliquots of 10 μg were boiled at 100°C in Laemmli sample buffer containing 6.25% 2-mercaptoethanol for 5 min before being loaded onto 10% polyacrylamide gels. The gels were run at 20 mAmps for ~45 min before transfer to nitrocellulose membranes. The membranes were briefly washed with PBS-0.1% Tween before being blocked in 10% nonfat dry milk and 0.1% sodium azide (PBS-MILK) for 1 h at room temperature with constant agitation. The membranes were then incubated for 1 h at room temperature with rabbit antibodies against IRS-1, p85 subunit of PI 3-kinase, PKB, or glycogen synthase diluted in PBS-0.1% Tween. After repeat washes, membranes were incubated for 1 h at room temperature with constant agitation with the secondary antibody horseradish peroxidase–linked goat anti-rabbit IgG (1:1,000) diluted in PBS-0.1% Tween. After further washes, the proteins were detected using enhanced chemiluminescence (Amersham Life Sciences).

Statistical analyses. Data are presented as mean ± SE and as mean and individual data points in Figs. 2 and 4. Comparisons between groups were determined by a *t* test and analysis of covariance and within groups by a paired *t* test.

RESULTS

Subject characteristics. Table 1 summarizes the metabolic and anthropometric characteristics of the relatives and control subjects from whom muscle cultures were established. There were significant differences between the relatives and control subjects for BMI ($P < 0.001$), waist-to-hip ratio (WHR) ($P < 0.01$), fasting blood glucose (FBG) ($P = 0.03$), 2-h blood glucose ($P = 0.03$), and K_{ITT} ($P = 0.0001$). Importantly, the difference between the groups for K_{ITT} remained significant ($P = 0.0001$) after correcting for BMI, WHR, FBG, and 2-h blood glucose as covariates. Therefore, the relatives remained significantly more insulin resistant compared with the control subjects after correction for potential metabolic and anthropometric confounders.

Glucose uptake. To select the most appropriate stage at which to study glucose transport in cultured skeletal muscle cells, 2-deoxy-D-glucose uptake was measured in myoblasts (day 0) and in myotubes fused for 4, 8, and 11 days from 5 control subjects. As shown in Fig. 1, the basal rate (i.e., the rate in the absence of acute insulin stimulation) of 2-deoxy-D-glu-

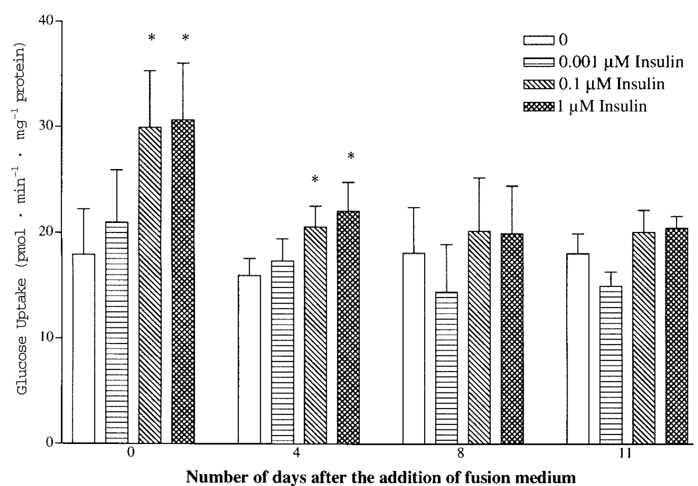

FIG. 1. Effect of insulin exposure on 2-deoxy-D-glucose uptake in cultured myoblasts and myotubes. Myoblasts from 5 control subjects were cultured in normal growth medium until ~90% confluence and then allowed to fuse in α-MEM for the number of days indicated. Glucose uptake was determined in triplicate after 16 h of serum depletion and after exposure to 0 (basal), 0.001, 0.1, and 1 μmol/l insulin as indicated. Data are presented as mean ± SE. *$P < 0.05$ compared with the corresponding basal values.

TABLE 2
2-Deoxy-D-glucose uptake in cultured myoblasts from control subjects and insulin-resistant relatives

	Insulin concentration			
	0	0.001 μmol/l	0.1 μmol/l	1 μmol/l
Control subjects: first batch of substrate (n = 5)	17.9 ± 4.3	21.0 ± 4.9	29.9 ± 5.4	30.7 ± 5.4
Lower 95% CI	5.9	7.2	15.0	15.8
Upper 95% CI	29.9	34.7	44.8	45.5
Relatives: first batch of substrate				
Subject				
3	24.3 ± 1.4	25.2 ± 1.4	36.2 ± 3.4	41.7 ± 2.2
4	9.2 ± 1.1	7.8 ± 0.8	13.1 ± 1.6	15.3 ± 1.3
5	20.8 ± 0.9	19.3 ± 3.5	26.5 ± 3.2	26.2 ± 2.0
8	12.4 ± 0.7	14.7 ± 0.9	23.5 ± 1.5	28.2 ± 2.2
9	49.4 ± 4.1	41.1 ± 3.7	40.8 ± 3.7	43.9 ± 2.6
10	5.9 ± 0.2	5.6 ± 0.3	11.5 ± 1.5	12.3 ± 1.6
Control subjects: second batch of substrate (n = 6)	47.4 ± 5.5	—	70.2 ± 5.5	73.6 ± 5.5
Lower 95% CI	33.2	—	56.1	59.4
Upper 95% CI	61.5	—	84.0	87.7
Relatives: second batch of substrate				
Subject				
1	66.6 ± 3.3	—	73.8 ± 1.7	83.1 ± 2.8
2	70.3 ± 9.8	—	90.6 ± 14.1	89.1 ± 16.9
6	97.6 ± 12.9	—	109.7 ± 15.3	106.5 ± 16.8
7	27.4 ± 0.3	—	37.1 ± 3.8	41.0 ± 1.4
9	72.1 ± 4.3	—	77.2 ± 0.5	71.1 ± 3.6

Data are means ± SE for triplicate measurements repeated on at least 2 separate occasions unless otherwise indicated. The values given are rates of glucose uptake expressed as picomoles per minute per milligram protein.

cose uptake, expressed in relation to cellular protein content, remained unchanged on fusion of myoblasts to myotubes. The effect of acute (1-h) exposure to insulin on glucose uptake was studied using a range of insulin concentrations (0.001, 0.1, and 1 μmol/l). The greatest stimulatory effect of insulin was seen in myoblasts with 1 μmol/l insulin (Fig. 1). This effect produced a significant 1.81 ± 0.20–fold increase above basal (30.7 ± 5.4 vs. 17.9 ± 4.3 pmol · min^{-1} · mg^{-1} protein, 1 μmol/l insulin vs. basal, $P < 0.005$). A similar increase was seen in myoblasts after exposure to 0.1 μmol/l insulin (1.75 ± 0.1–fold over basal, $P < 0.005$), whereas exposure to 0.001 μmol/l insulin produced only a small and nonsignificant increase in glucose uptake. The effect of insulin on glucose uptake was diminished after myoblast fusion (Fig. 1). By day 4, the fold-increase in glucose uptake after exposure to 0.1 or 1 μmol/l insulin, while still significant ($P < 0.05$), had fallen to 1.3 ± 0.1– and 1.4 ± 0.1–fold, respectively. This increase is similar to that found by other workers in comparable day 4 human myotubes (28,30,31). Insulin had no significant effect on the rate of glucose uptake in myotubes fused for 8 or 11 days.

In light of these findings, myoblasts were selected for the comparison of insulin action on glucose transport between the relative and control cultures. Initial studies were performed using myoblasts from the 5 control subjects and 6 of the relatives (Table 2). A different batch of 2-deoxy-[^{3}H]glucose was then used to study the remaining relative cultures because the initial supply of this substrate was exhausted. The basal rates of glucose uptake were found to be higher with the new batch of substrate; therefore, myoblasts from the same control individuals plus relative #9 were restudied along with the remaining relatives and a further control (Table 2). Although the basal rates of 2-deoxy-D-glucose uptake determined with the 2 different batches of substrate differed, the magnitude of the response to insulin was unaffected by the batch of substrate used. This result allowed direct comparison of the insulin responses (fold-stimulation) between the relative and control cultures.

The same pattern of insulin stimulation was seen in the relative (Fig. 2*A*, Table 2) and control myoblast cultures (Fig. 1). Comparison between relative and control cultures showed that fold-stimulation of glucose uptake was significantly decreased in the relative cultures at 0.001 μmol/l (0.93 ± 0.05 vs. 1.15 ± 0.06, $P < 0.05$) and 0.1 μmol/l (1.38 ± 0.10 vs. 1.69 ± 0.08, $P = 0.025$) insulin concentrations. The difference at 1 μmol/l insulin, however, failed to reach statistical significance (1.49 ± 0.14 and 1.73 ± 0.12). Interestingly, 5 of the 10 relative myoblast cultures (subject numbers 1, 2, 5, 6, and 9, Table 2) exhibited a negligible increase in glucose uptake in that the rates of fold-stimulation were well below the control ranges at both 0.1 and 1 μmol/l insulin. To determine whether these 5 relatives exhibited any particular in vivo characteristics, the anthropometric and metabolic features listed in Table 1 were compared between these relatives and the remaining 5 relatives (subject numbers 3, 4, 7, 8, and 10). Only the FBG was different between the groups, being higher in the relatives with the negligible insulin response in muscle culture (5.3 ± 0.23 vs. 4.5 ± 0.13 mmol/l, $P = 0.04$).

Basal rates of glucose uptake were compared between relative and control cultures studied with the same batch of substrate. In 4 (subject numbers 1, 2, 6, and 9) of the 5 relative cultures with the negligible response to insulin, basal glucose uptake was markedly increased compared with that

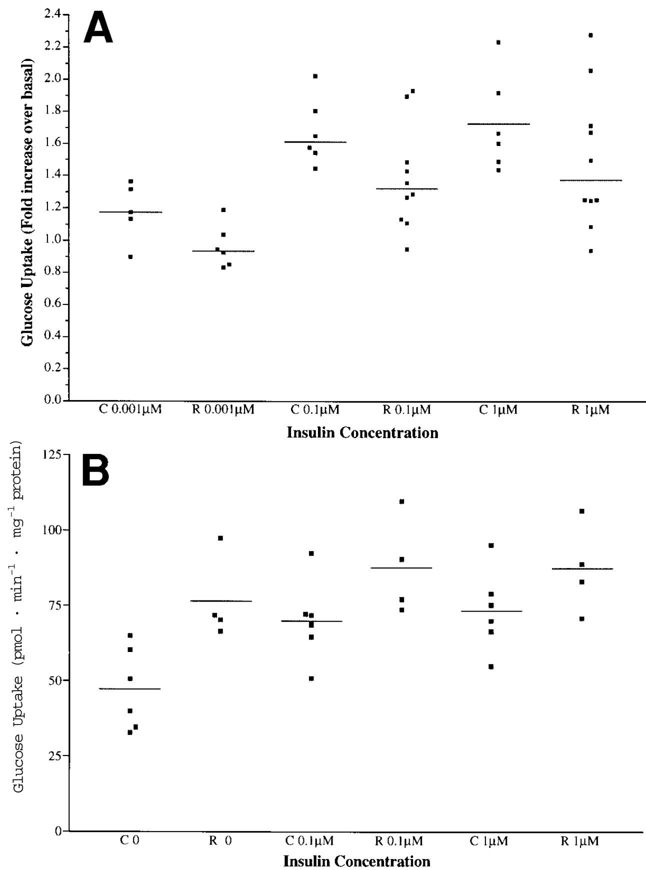

FIG. 2. Glucose uptake in myoblasts from insulin-resistant nondiabetic relatives and control subjects. Myoblasts from 6 control subjects (C) and 10 insulin-resistant relatives (R) were cultured to ~90% confluence. The monolayers were exposed to serum-free α-MEM for 16 h and then incubated in the absence or presence of the indicated concentration of insulin for 1 h at 37°C. Glucose uptake was determined in triplicate as described in RESEARCH DESIGN AND METHODS. *A*: The results are expressed as fold-increase over the corresponding basal value. Glucose uptake after exposure to 0.001 μmol/l was determined only in 5 and 6 of the control and relative cultures, respectively. *B*: Comparison of the absolute rates of basal and insulin-stimulated glucose uptake in 4 of the 5 relative cultures (R) with impaired insulin responsiveness (subjects 1, 2 , 6, and 9) and the 6 control cultures (C). The horizontal bar represents the mean for each condition.

measured in the control subjects (Table 2 and Fig. 2*B*; 76.7 ± 7.0 vs. 47.4 ± 5.5 pmol · min^{-1} · mg^{-1} protein, $P < 0.02$). Thus, whereas the response to insulin was impaired in the 4 relative cultures, because of the high basal uptake rates, the absolute rates of insulin-stimulated glucose uptake were comparable to the control values (Fig. 2*B*).

Glycogen synthesis. Total glycogen synthesis was studied in myoblasts (day 0) and in myotubes from control subjects 4, 8, and 11 days after the addition of fusion medium. There was no change in basal glycogen synthesis after cell fusion (Fig. 3). As with glucose uptake, the greatest response to insulin occurred in myoblasts, with 0.1 and 1 μmol/l insulin for 1 h resulting in a 3.5 ± 1.1– and 4.3 ± 1.3–fold stimulation of glycogen synthesis (both $P < 0.05$ vs. basal), respectively. The stimulatory effect of insulin on glycogen synthesis was also apparent in myotubes (Fig. 3) and persisted to 11 days after fusion (2.2 ± 0.3– and 2.4 ± 0.3–fold at 0.1 and 1 μmol/l insulin, both $P < 0.05$ vs. basal).

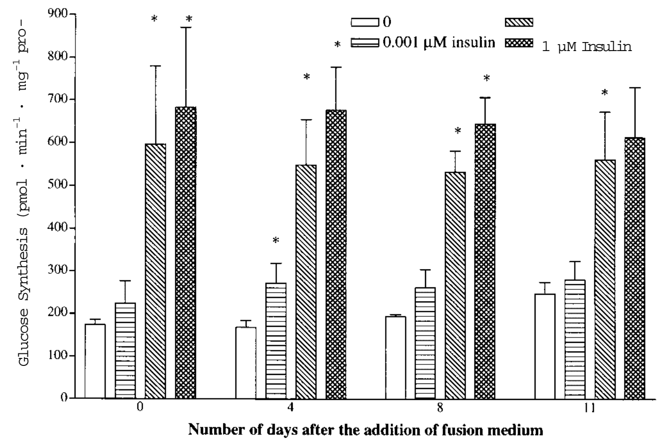

FIG. 3. Effect of insulin exposure on glycogen synthesis in cultured myoblasts and myotubes. Myoblasts from 5 control subjects were cultured in normal growth medium until ~90% confluence and then allowed to fuse in α-MEM for the number of days indicated. Glycogen synthesis was determined in triplicate after 16 h of serum depletion and after exposure to the concentrations of insulin indicated for 1 h at 37°C. Data are presented as means ± SE and are expressed as picomoles glucose incorporated per minute per milligram protein. *$P < 0.05$ compared with the corresponding basal values.

In view of the magnitude of the response to insulin, myoblast cultures were used for the subsequent comparisons of glycogen synthesis rates in relative and control muscle cultures. However, there were no significant differences between the groups for glycogen synthesis in the basal and insulin-stimulated states (Fig. 4).

Expression of GLUT1 and GLUT4 glucose transporter proteins. GLUT1 is believed to mediate glucose uptake in the basal state, whereas GLUT4 is responsible for insulin-stimulated glucose transport (46). The changes in insulin respon-

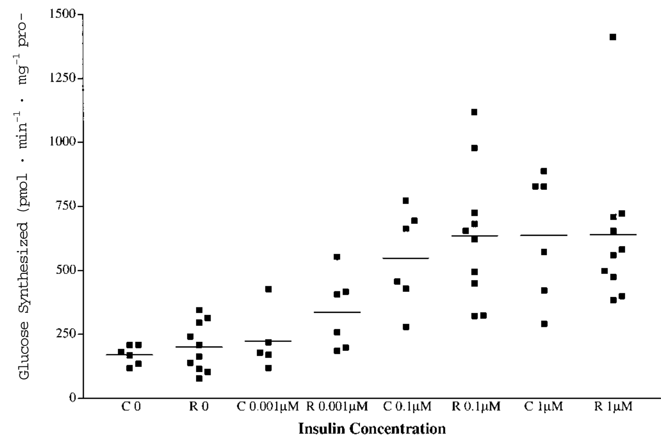

FIG. 4. Glycogen synthesis in cultured myoblasts from insulin-resistant nondiabetic relatives and control subjects. Myoblasts from 6 control subjects (C) and 10 insulin-resistant relatives (R) were grown in normal growth medium until ~90% confluence. The monolayers were exposed to serum-free α-MEM for 16 h and then incubated with the indicated concentration of insulin for 1 h at 37°C. The rate of glycogen synthesis was then determined in triplicate as described in RESEARCH DESIGN AND METHODS. The effect of 0.001 μmol/l insulin was determined in only 5 and 6 of the control and relative cultures, respectively. Data are expressed as picomoles glucose incorporated per minute per milligram protein. The horizontal bar represents the mean for each condition.

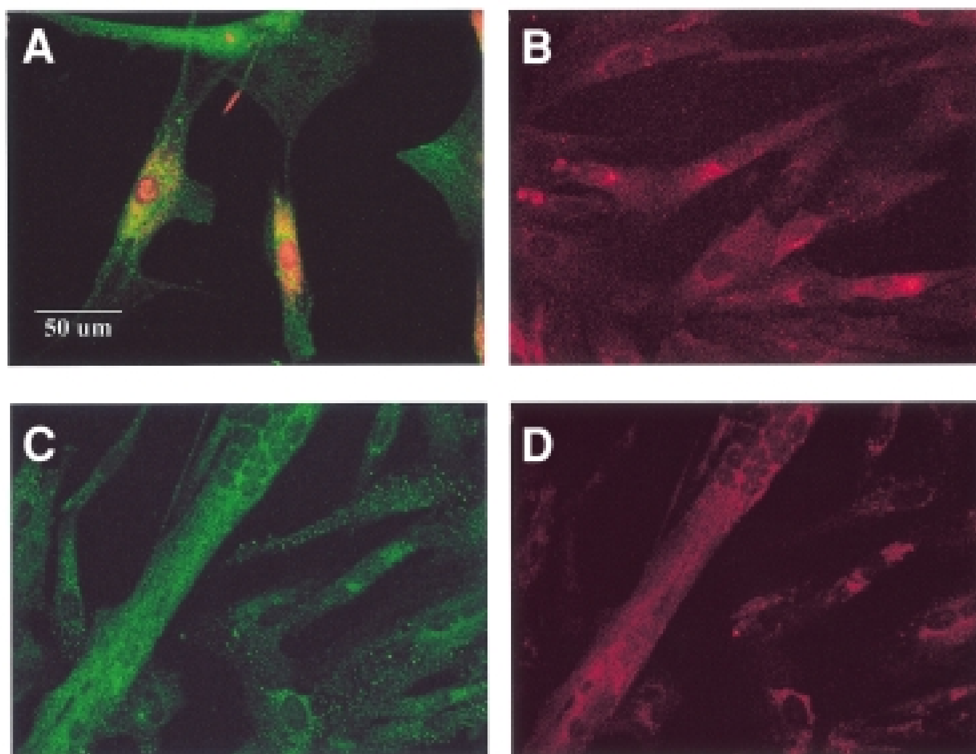

FIG. 5. Immunofluorescent detection of GLUT1 and GLUT4 in cultured myoblasts and myotubes. Myoblasts (*A* and *B*) and 4-day fused myotubes (*C* and *D*) were deprived of serum for 16 h. GLUT4 (green fluorescence, *A* and *C*) and GLUT1 (red fluorescence, *B* and *D*) were detected by indirect immunofluorescence. Myoblasts in *A* were counterstained with propidium iodide for 10 min to visualize the nucleus (red fluorescence). Under the culture conditions used for the fluorescence studies, a number of myoblasts remain unfused in *C* and *D*.

siveness and basal glucose uptake identified in some of the relative cultures lead us to investigate the expression of GLUT1 and GLUT4 in our culture system. As other workers have reported a lower level of expression of GLUT4 in myoblasts compared with myotubes (31), we studied GLUT4 expression in myoblasts and day 4 myotubes. A strong signal for GLUT4 was detected in both myoblasts and myotubes (Fig. 5), whereas GLUT1 was also expressed by both cell types. Both transporters were detected as punctate fluorescence visible throughout the cells (Fig. 5), the pattern of which was not appreciably different after insulin administration using this system of visualization (data not shown).

Expression levels of proteins involved in insulin signaling. Current evidence indicates that the control of glucose uptake involves IRS-1, PI 3-kinase, and PKB. Western blotting detected all of these in extracts of the cultured myoblasts. The levels of each protein varied between cells from different subjects, but there was no significant difference in the levels of any of these proteins between myoblasts from control subjects and the relatives, either as the whole group or as the subset that showed impaired glucose uptake in response to insulin (Fig. 6). Furthermore, the levels of glucose synthesis (GS) did not differ between the different groups.

DISCUSSION

Despite the increasing use of human muscle cultures in the investigation of type 2 diabetes, to the best of our knowledge, insulin-stimulated glucose utilization in this system has not been comprehensively characterized at all stages of cell differentiation. Because the magnitude of insulin stimulation of glucose uptake is low in vitro (typically 1.5-fold in muscle strips [47]), it is important to use cells at a stage when insulin stimulation is maximal to facilitate the detection of differences between muscle cultures from different groups under study. Under the culture conditions we used, insulin stimulation of glucose uptake and glycogen synthesis was maximal in myoblasts.

The novel and important finding of our study is the impaired insulin-stimulated glucose uptake in muscle cultures derived from 5 of the insulin-resistant relatives. Under the assay conditions used for the measurement of glucose uptake in this study, glucose transport is the rate-limiting step and indicates that glucose transport is the major site of impaired insulin action in these insulin-resistant relatives. This finding therefore reinforces and extends the findings of in vivo nuclear magnetic resonance studies in comparable insulin-resistant nondiabetic relatives, which found that decreased glucose uptake and/or phosphorylation represented the principle defect in the prediabetic state (20). Decreased insulin-stimulated glucose uptake has been described in skeletal muscle strips (22) and cultured muscle cells (28) from type 2 diabetic patients. However, an acknowledged potential drawback of tissue derived from diabetic patients is that chronic hyperglycemia in vivo may induce acquired but nonreversible changes in insulin action that persist in vitro (28). Clearly, this result is not an issue in cultures derived from nondiabetic relatives and therefore provides evidence that the observed defect in insulin-stimulated glucose uptake is likely to have an inherited basis.

Mean fasting blood glucose concentrations were higher in the relatives with the impaired insulin response in cul-

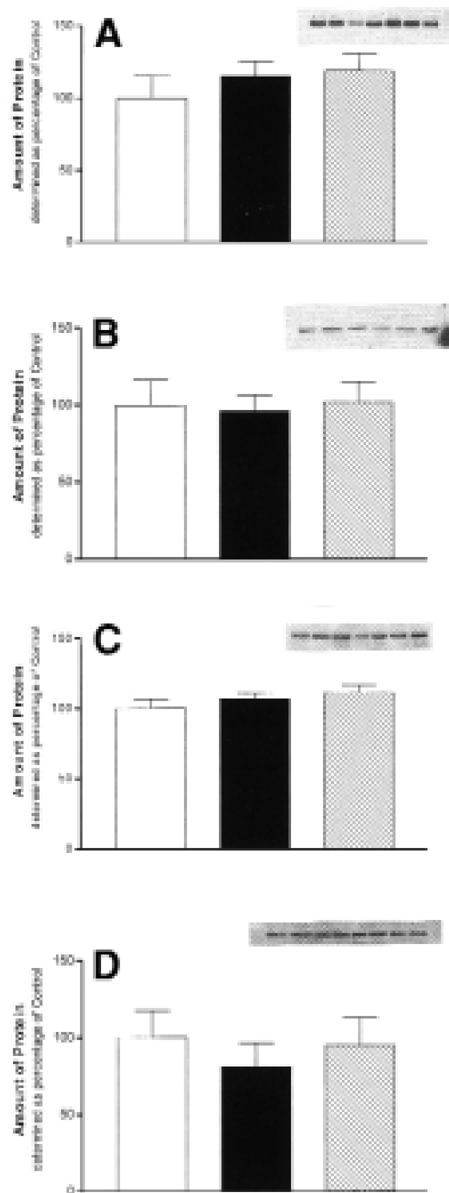

FIG. 6. Expression levels of IRS-1 (*A*), PKB (*B*), p85 subunit of PI-3 kinase (*C*), and (*D*) glycogen synthase in myoblasts from control subjects and insulin-resistant nondiabetic relatives. Myoblasts were cultured to ~90% confluence. The monolayers were exposed to serum-free Ham's F10 for 2 h before being harvested as described in RESEARCH DESIGN AND METHODS. Each panel shows the amount of protein, as determined by density analysis, from the 6 control cultures (□), the 10 insulin-resistant relatives (■), and the subgroup of relatives with impaired insulin responsiveness for glucose uptake (▨). Data are presented as mean ± SE. The insert shows a representative example of the Western blot for a selection of the control subjects and relatives.

tured muscle cells compared with the relatives with a normal response, although there was no difference in the degree of insulin resistance (K_{ITT}). However, a difference in muscle-specific insulin sensitivity may have gone undetected because K_{ITT} provides an index of whole-body insulin sensitivity, and the subject numbers are small. Nonetheless, it may be that the difference in fasting blood glucose levels reflects the differences in insulin action at the cellular level.

The mechanisms underlying the impaired insulin responsiveness in the cultured muscle cells remains to be determined. However, we have shown that the insulin-sensitive GLUT4 glucose transporter is expressed in cultured human myoblasts and that the expression of IRS-1, PI 3-kinase, and PKB as key components of the insulin-signaling pathway involved with glucose uptake (46) was normal. Further work is therefore required to examine the functional aspects of the insulin responsive glucose transport system in our cultures. Previous work has reported normal expression of GLUT4 in type 2 diabetic skeletal muscle (48,49) and cultured human muscle cells (28), but there is evidence of impaired GLUT4 translocation in response to insulin (21,22). Recently, impaired IRS-1 phosphorylation, PI 3-kinase activity, and glucose transport activity have been reported in skeletal muscle strips from type 2 diabetic patients (50), although the expression of IRS-1 and the p85 subunit of PI 3-kinase was normal, which is consistent with our own findings.

Another key finding of the present study is that basal glucose uptake was significantly increased in 4 of the 5 relative cultures with impaired insulin-stimulated glucose transport. Others have reported increased (27), unchanged (22), and decreased (28) basal glucose uptake rates in tissue preparations from type 2 diabetic patients, and this apparent conflict may reflect methodological differences and the heterogeneity of type 2 diabetes. Nonetheless, the increased basal glucose uptake we have observed could represent a compensatory response to try to maintain glucose uptake in the face of the impaired response to insulin, or alternatively, it might represent a primary defect that secondarily leads to decreased insulin-stimulated glucose uptake. A similar finding of increased basal glucose uptake associated with decreased insulin-stimulated glucose uptake was recently reported in cultured fibroblasts from type 2 diabetic subjects with familial diabetes (27). Total cellular GLUT1 content was normal in these cells, but the proportion of GLUT1 was increased at the plasma membrane. Subsequent work using the same fibroblast cultures identified a novel protein (phospho-protein enriched in diabetes [PED/PEA-15]), the tissue expression of which was increased specifically in type 2 diabetes (51). Furthermore, transfection of PED/PEA-15 into L6 skeletal muscle cells increased plasma membrane GLUT1 content and decreased insulin-stimulated glucose transport due to an impairment of GLUT4 transporter translocation. A link between increased non–insulin-mediated glucose uptake and decreased insulin-stimulated glucose transport has been reported in other studies. Overexpression of GLUT1 in a transgenic mouse model lead to a 2- to 8-fold increase in basal glucose uptake in isolated skeletal muscle, and this result in turn was associated with a marked decrease in insulin-stimulated glucose uptake (52). Similarly, increased non–insulin-mediated glucose flux into muscle strips from type 2 diabetic patients resulted in the subsequent downregulation of insulin-stimulated glucose uptake (53). As recently hypothesized, increased activity of the hexosamine pathway could provide the link between increased GLUT1-mediated glucose flux into the cell and a decrease in insulin-stimulated GLUT4 transporter translocation (54).

The association between perturbed basal and insulin-stimulated glucose uptake may explain an in vivo observation. When normal glucose-tolerant first-degree relatives were studied using the frequently sampled intravenous glucose tolerance test with minimal model assessment, decreased insulin sensitivity but increased glucose effectiveness (S_G) was found (55).

Whereas there has been some debate about the physiological relevance of S_G, recent studies have shown that it primarily represents non–insulin-mediated glucose uptake into skeletal muscle (56,57). It was presumed that the increase in S_G in relatives might represent a compensatory response to maintain normal glucose tolerance in the face of decreased insulin sensitivity. However, as considered above, the increase in non–insulin-mediated glucose uptake might actually represent a primary defect rather than a secondary adaptation.

Under the conditions used in our study, we found no evidence of impaired insulin stimulation of glycogen synthesis in myoblast cultures from the relatives—even in those cultures in which the insulin-stimulated glucose uptake was impaired. It might be expected that the impaired response to insulin in the latter cells might lead to a reduction in the rate of glycogen synthesis secondary to the reduction in substrate supply. However, it is important to note that because of the increased non–insulin-mediated glucose flux into these cells, glucose uptake after insulin exposure was not significantly different to that in the control myoblasts (Fig. 2*B*). Rothman et al. (20) concluded that the impaired insulin-stimulated glycogen synthesis observed in the nondiabetic first-degree relatives they studied was secondary to a decrease in insulin-stimulated glucose transport/phosphorylation. However, when these relatives were exercised and glucose uptake was normalized, an additional and independent defect of glycogen synthesis became apparent (58). Certainly, decreased activity of glycogen synthase has been reported in native skeletal muscle from nondiabetic relatives (10) and in cultured myotubes from type 2 diabetic patients (29). We also studied glycogen synthesis in myotubes from 6 of the relatives, although no defect in insulin-stimulated glycogen synthesis was apparent (data not shown). The expression of GS was also normal in the relative myoblasts and is consistent with a recent report of normal GS expression in muscle strips from type 2 diabetic patients (50). Further work is therefore required with our relative cultures to examine glycogen synthesis and glycogen synthase activity under different culture conditions.

It is important to recognize that whereas insulin response for glucose transport was impaired in 5 of the relative cultures, it was normal in the remaining 5 cultures (Fig. 2*A*). This finding suggests that other factors contribute to the in vivo insulin resistance in those relatives with normal insulin response in culture. These factors might include lifestyle influences such as physical activity and dietary composition (59), together with other determinants such as vascular function (60) and muscle fiber type (61), which may be under genetic control but could not be assessed in our muscle culture system.

In conclusion, we have demonstrated that abnormalities of insulin-stimulated glucose uptake persist in cultured myoblasts from some insulin-resistant nondiabetic relatives of type 2 diabetic patients, in keeping with a primary inherited defect. Moreover, we found an association between the impaired response to insulin and increased basal glucose uptake, suggesting that the 2 abnormalities may be linked.

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