

The Diabetic Phenotype Is Conserved in Myotubes Established From Diabetic Subjects

Evidence for Primary Defects in Glucose Transport and Glycogen Synthase Activity

Michael Gaster, Ingrid Petersen, Kurt Højlund, Pernille Poulsen, and Henning Beck-Nielsen

The most well-described defect in the pathophysiology of type 2 diabetes is reduced insulin-mediated glycogen synthesis in skeletal muscles. It is unclear whether this defect is primary or acquired secondary to dyslipidemia, hyperinsulinemia, or hyperglycemia. We determined the glycogen synthase (GS) activity; the content of glucose-6-phosphate, glucose, and glycogen; and the glucose transport in satellite cell cultures established from diabetic and control subjects. Myotubes were precultured in increasing insulin concentrations for 4 days and subsequently stimulated acutely by insulin. The present study shows that the basal glucose uptake as well as insulin-stimulated GS activity is reduced in satellite cell cultures established from patients with type 2 diabetes. Moreover, increasing insulin concentrations could compensate for the reduced GS activity to a certain extent, whereas chronic supraphysiological insulin concentrations induced insulin resistance in GS and glucose transport activity. Our data suggest that insulin resistance in patients with type 2 diabetes comprises at least two important defects under physiological insulin concentrations: a reduced glucose transport under basal conditions and a reduced GS activity under acute insulin stimulation, implicating a reduced glucose uptake in the fasting state and a diminished insulin-mediated storage of glucose as glycogen after a meal. *Diabetes* 51: 921–927, 2002

Human satellite cell cultures display numerous features of mature skeletal muscles (1) and have been used for studies of muscle metabolism in cultures established from patients with type 2 diabetes and healthy control subjects (1–5,8,9,21,22). Several groups (3,8,9) have worked with this cell model to clarify whether insulin resistance is caused by a primary defect. Furthermore, the model has been used to study the molecular background for the

impaired glucose transport and glycogen synthesis associated with insulin resistance in skeletal muscles.

The most well-described defect in the pathophysiology of type 2 diabetes is reduced insulin-mediated glycogen synthesis in skeletal muscles (6,7). This defect can already be observed in prediabetic states such as obesity and normal-weight first-degree relatives of type 2 diabetic patients. In lean control subjects, insulin stimulates the fractional velocity (FV) of glycogen synthase (GS) more than twofold. However, in obese diabetic subjects, insulin stimulates the FV of GS to a lower extent (6). The question is: Do cell cultures, established from diabetic subjects, conserve the diabetic phenotype in culture? Previous studies of cell cultures established from diabetic subjects and cultured under basal physiological conditions showed that the basal as well as the insulin-stimulated FV values are reduced (4,5). However, in a recent article, Krutzfeldt et al. (8) could not show any differences in glycogen synthesis between muscle cell cultures established from lean healthy first-degree relatives with high and low insulin sensitivity, and Jackson et al. (9) found that myoblast cultures established from insulin-resistant first degree relatives expressed normal glycogen synthesis. Ciaraldi et al. (10) showed that basal and insulin-stimulated glucose uptake was reduced in cultures established from type 2 diabetic patients. However, Krutzfeldt et al. (8) could not show any differences in glucose transport between muscle cell cultures established from lean healthy first-degree relatives, and Jackson et al. (9) found a reduced insulin-stimulated glucose uptake in myoblast cultures from first-degree relatives, with some of these cultures expressing an increased basal glucose uptake.

Thus, it is unclear whether human satellite cells established from diabetic subjects express traits found in vivo biopsies, such as reduced FV. Furthermore, it is unclear whether the glucose uptake is primarily affected in cultures established from type 2 diabetic subjects. We have recently optimized culture conditions for human satellite cells (1) and shown that cultures established from healthy subjects express GS activity to the same level as seen in in vivo biopsies (2).

The aim of the present study was to evaluate glucose transport and GS activity in human satellite cell cultures established from type 2 diabetic and control subjects. Cultures were exposed to increasing concentrations of insulin for 4 days to clarify whether they express genetic

From the Department of Endocrinology, Odense University Hospital, Odense, Denmark.

Address correspondence and reprint requests to Michael Gaster, MD, PhD, Department of Endocrinology, Odense University Hospital, DK-5000 Odense, Denmark. E-mail (secretary): janne.dyhr@ouh.dk.

Received for publication 24 August 2001 and accepted in revised form 8 January 2002.

DMEM, Dulbecco's modified Eagle's medium; DOG, 2-deoxyglucose; FV, fractional velocity; G6P, glucose-6-phosphate; GS, glycogen synthase; Sc, satellite cells.

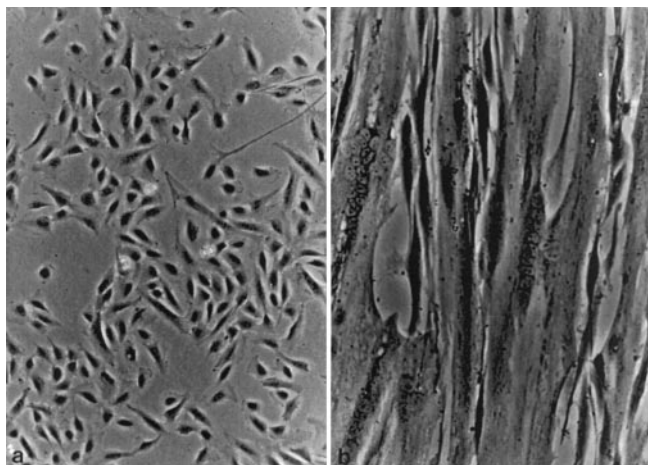


FIG. 1. Phase contrast appearance of human satellite cell culture. Human satellite cell cultures were established, grown, and differentiated as described in RESEARCH DESIGN AND METHODS. Morphological appearance was investigated by phase contrast microscopy. *a*: Mononucleated cells under proliferation ($\times 100$). *b*: Day 8 differentiated cultures cultured under physiological conditions (5.0 mmol/l glucose and 25 pmol/l insulin). The cultures contain many multinucleated myofibers ($\times 200$).

and/or insulin-induced insulin resistance by hyperinsulinemia at the level of GS or glucose transport.

RESEARCH DESIGN AND METHODS

Materials. Dulbecco's modified Eagle's medium (DMEM), FCS, Ultrosor G, penicillin-streptomycin-amphotericin B, and trypsin were obtained from Life Technology (Paisley, Scotland, U.K.). UDP-[14 C]glucose (10.63 GBq/mol) was obtained from DuPont (NEN, Boston, MA). A protein assay kit was purchased from BioRad (Copenhagen, Denmark). Glycogen, pepstatin, leupeptin, phenylmethylsulfonyl fluoride, and extracellular matrix-gel were purchased from Sigma (St. Louis, MO). Biotinylated goat anti-rabbit Ig, biotinylated goat anti-mouse Ig, and horseradish peroxidase-conjugated streptavidin were purchased from DAKO (Glostrup, Denmark). Insulin Actrapid was from Novo Nordisk (Bagsvaerd, Denmark).

Human study subjects. Eight obese type 2 diabetic patients (49.5 ± 1.8 years) and eight young lean control subjects (26.9 ± 0.2 years) participated in the study. Only sedentary male subjects were recruited. None of the diabetic patients received insulin treatment. The patients were without diabetic complications, apart from simplex retinopathy. The control subjects had no family history of diabetes. The groups differed by age ($P < 0.001$), BMI (30.4 ± 1.1 and 23.6 ± 0.7 kg/m 2 for diabetic and lean control subjects, respectively; $P < 0.001$), fasting plasma glucose (9.9 ± 0.6 and 5.3 ± 0.1 mmol/l, respectively; $P < 0.001$), and fasting plasma insulin (71.8 ± 10.6 and 42.0 ± 8.4 pmol/l, respectively; $P < 0.05$). All subjects gave written informed consent, and the local ethics committees of Funen and Vejle County approved the study.

Cell culture. Cell cultures were established as previously described (1,2). Cells were subcultured twice (4–6 weeks of satellite cell proliferation) before final seeding in 100-mm petri dishes. At 75% confluency, the growth medium was replaced by basal medium (DMEM supplemented with 2% FCS, 50 units/ml penicillin, 50 μ g/ml streptomycin, 1.25 μ g/ml amphotericin B, and 25 pmol/l insulin) to induce differentiation. On day 4 after induction of differentiation, established cell cultures were exposed to 4 days of different insulin concentrations (0.1 pmol/l, 1.0 pmol/l to 1.0 μ mol/l at 5.5 mmol/l glucose). All cultures were harvested after 8 days of onset of differentiation. Cells were rinsed twice and incubated for 1 h in basal medium. Cell extracts were obtained after a 1-h acute exposure to basal or high-insulin medium (5.5 mmol/l glucose or 1 μ mol/l insulin).

GS activity. The GS activity, the glucose-6-phosphate (G6P) concentration required for half-maximal activity of GS at 0.3 mmol/l UDP-glucose ($A_{0.5}$), and the Hill coefficient (h) were measured or calculated as described previously (2). The absolute differences between the basal and the insulin-stimulated state for $FV_{0.1}$ and $A_{0.5}$ were designated ($\Delta FV_{0.1}$) and ($\Delta A_{0.5}$), respectively.

Glucose uptake. Cells were grown and differentiated in 12-well plates as described above. Experimental conditions were as follows: 1 pmol/l, 1 nmol/l, and 1 μ mol/l insulin. Before determining glucose uptake, all cell cultures were exposed to basal medium for 1 h. Cultures used for acute insulin stimulation

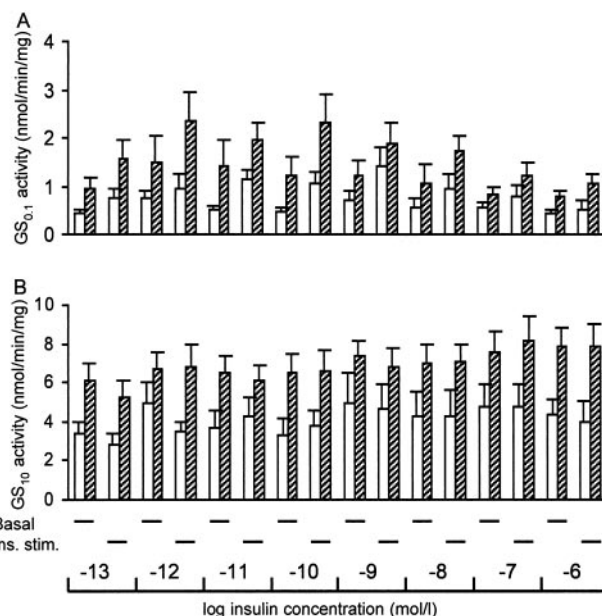


FIG. 2. Effect of preculturing human satellite cell cultures in increasing concentrations of insulin on GS activity measured at 0.1 and 10 mmol/l G6P. Human satellite cell cultures were exposed for 4 days to different insulin conditions, and the GS activity was measured at 0.1 and 10 mmol/l G6P under basal and after acute insulin stimulation (Ins. Stim.) as described in RESEARCH DESIGN AND METHODS. ▨, control cultures; □, diabetic cultures. *A*: The GS activities measured at 0.1 mmol/l in the basal state and in the stimulated state in myotubes precultured at the indicated insulin concentration. *B*: The GS activities measured at 10.0 mmol/l in the basal state and in the stimulated state in myotubes precultured at the indicated insulin concentration. Results are shown as the means \pm SE ($n = 8$) in each group.

were exposed for 1 h to high insulin as described for GS assays. Wells were rapidly washed three times at room temperature with reaction buffer: 150 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l MgSO $_4$, 1.2 mmol/l NaH $_2$ PO $_4$, 10 mmol/l HEPES, and 0.1% BSA, pH 7.4, and then incubated in the same buffer supplemented with 2-deoxyglucose(DOG)/2-[3 H]DOG (10 μ mol/l final concentration, 0.2 μ Ci/well). For the determination of nonspecific DOG uptake, control cultures were incubated with phloretin (100 μ g/ml). The reactions were stopped after 15 min by aspirating the reaction mixture and rapidly rinsing each well four times with PBS at 4°C. Cells were solubilized by the addition of 0.5 ml of 0.1 mol/l NaOH. An aliquot of 50 μ l was removed for protein determination (11). The remaining fluid was placed in a scintillation vial, and scintillation fluid was added. Glucose transport activity is expressed as picomoles of DOG taken up per minute per milligram of total protein.

Intracellular glucose/G6P. Metabolite concentrations were measured in the basal and stimulated state for all conditions. Cells were washed three times with ice-cold PBS and extracted with 5% perchloric acid, frozen, and stored at -80° C. The intracellular glucose/G6P content was measured according to Passonneau and Lowry (12).

Glycogen content. Glycogen content was measured in the basal state of all culture conditions. Cells were washed three times with ice-cold PBS and placed in solution with 0.1 mol/l NaOH. The glycogen content was measured according to Passonneau and Lowry (12). Protein content was measured on aliquots of extract. Results are expressed as milligrams of glycogen per milligram protein.

Statistical analysis. Data in the text and figures are given as the means \pm SE. Statistical analyses were performed with INSTAT 2.01 (GraphPad, San Diego, CA). Nonparametric statistical analyses of data were used: the Mann-Whitney test for unpaired comparisons and Spearman's rank correlation coefficient (r) for ANCOVA. $P < 0.05$ was considered significant.

RESULTS

Cell culture. The appearances under phase-contrast microscope of human satellite cell cultures during proliferation and differentiation are shown in Fig. 1*a* and *b*. Cell cultures were allowed to differentiate for 4 days before cell cultures were exposed to the different insulin concentra-

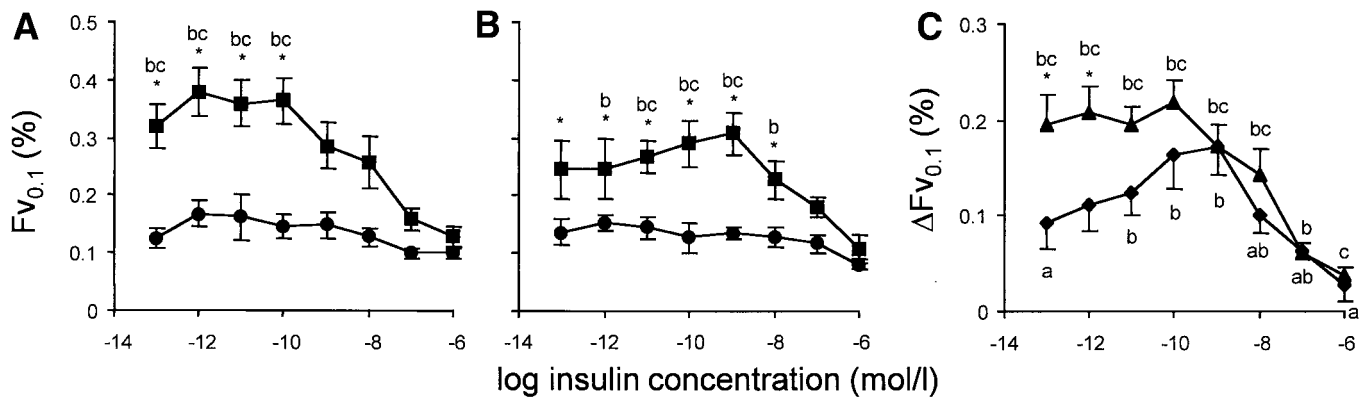


FIG. 3. Effect of preculturing human satellite cell cultures in increasing concentrations of insulin on the fractional velocity ($FV_{0.1}$). Human satellite cell cultures were precultured for 4 days at different insulin conditions, and the fractional activity ($FV_{0.1}$) of GS was determined under basal conditions and after acute insulin stimulation as described in RESEARCH DESIGN AND METHODS. ●, basal activities; ■, acute insulin-stimulated values. **A:** Control cultures. **B:** Cell cultures established from diabetic subjects. **C:** The absolute differences between the basal and the insulin-stimulated state ($\Delta FV_{0.1}$) in control (▲) and diabetic cultures (◆). Results are shown as the means \pm SE ($n = 8$) in each group. * $P < 0.05$ vs. basal; ^a $P < 0.05$ vs. activity at 1 nmol/l insulin; ^b $P < 0.05$ vs. activity at 1 μ mol/l insulin; ^c $P < 0.05$ vs. activity at 100 nmol/l insulin.

tions for 4 days as described in RESEARCH DESIGN AND METHODS.

GS activity. GS activity in cultures established from diabetic and control subjects at day 8 are shown in Fig. 2. GS activity measured at 0.1 and 10 mmol/l G6P was significantly lower in diabetic cultures than in control cultures ($P < 0.0001$). Acute insulin stimulation significantly increased GS activity at 0.1 mmol/l in both diabetic ($P < 0.0001$) and control ($P < 0.0001$) cultures; however, GS activity at 10 mmol/l was unaffected by acute insulin stimulation in both culture types ($P > 0.55$ in diabetic and control cultures). Comparing basal and insulin-stimulated GS activity between cultures exposed to different insulin concentrations did not reveal significant differences in control cultures or those established from type 2 diabetic subjects ($P > 0.05$). An increase in G6P from 0.1 to 10 mmol/l significantly increased GS activity under all conditions ($P < 0.0001$, not shown).

Fractional velocity $FV_{0.1}$. Fig. 3 shows dose-response curves for the impact of insulin on the fractional velocity $FV_{0.1}$ of GS in type 2 diabetic (Fig. 3B) and control cultures (Fig. 3A). Preculturing myotubes for 4 days at increasing

insulin concentrations did not change the basal $FV_{0.1}$ in diabetic or control cultures (Fig. 3A and B). Moreover, the basal $FV_{0.1}$ values in control and diabetic cultures were quite similar ($P > 0.8$ for all conditions). Acute insulin stimulation significantly increased the fractional activity $FV_{0.1}$ of GS in control cultures in the range of 0.1 pmol/l to 0.1 nmol/l ($P < 0.05$) and in diabetic cultures in the range of 0.1 pmol/l to 1.0 nmol/l ($P < 0.05$), whereas higher insulin concentrations diminished the effect of acute insulin stimulation in a dose-dependent pattern. To get further insight into the differences between diabetic and control cultures, we compared the absolute differences between the basal and the insulin-stimulated state ($\Delta FV_{0.1}$) in control cultures and in those established from type 2 diabetic subjects (Fig. 3C). $\Delta FV_{0.1}$ was significantly lower for 0.1–1 pmol/l insulin in the diabetic cultures compared with control cultures ($P < 0.05$), but these differences disappear with increasing insulin concentration as $\Delta FV_{0.1}$ significantly increases in diabetic cultures. Higher insulin concentrations (>1 nmol/l) significantly reduced the $\Delta FV_{0.1}$ in control as well as diabetic cultures ($P < 0.05$) in a parallel manner.

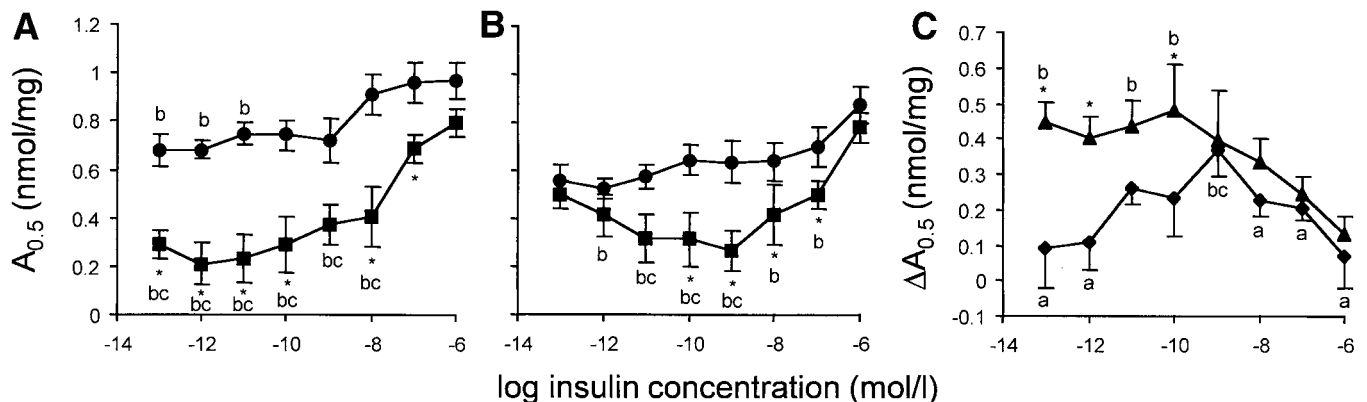


FIG. 4. Effect of preculturing human satellite cell cultures in increasing concentrations of insulin on the activity constant $A_{0.5}$ of GS. Human satellite cell cultures were precultured for 4 days at different insulin conditions, and the activity constant $A_{0.5}$ of GS was determined under basal and after acute insulin stimulation as described in RESEARCH DESIGN AND METHODS. ●, basal activities; ■, acute insulin-stimulated values. **A:** Control cultures. **B:** Cell cultures established from diabetic subjects. **C:** The absolute differences between the basal and the insulin-stimulated state ($\Delta A_{0.5}$) in control (▲) and diabetic cultures (◆). Results are shown as the means \pm SE ($n = 8$) in each group. * $P < 0.05$ vs. basal; ^a $P < 0.05$ vs. activity at 1 nmol/l insulin; ^b $P < 0.05$ vs. activity at 1 μ mol/l insulin; ^c $P < 0.05$ vs. activity at 100 nmol/l insulin.

GS kinetics. Figure 4 shows dose-response curves of the impact of insulin on the activity constant $A_{0.5}$ of GS in type 2 diabetic (Fig. 4B) and control (Fig. 4A) cultures. Chronic high insulin concentrations significantly increased the basal $A_{0.5}$ values compared with low insulin concentrations in cultures established from control subjects (Fig. 4A). Acute insulin stimulation significantly decreases $A_{0.5}$ for GS in cultures precultured at 0.1 pmol/l to 0.1 nmol/l and 10–100 nmol/l insulin, whereas higher chronic insulin concentrations diminished the effect of acute insulin stimulation (Fig. 4A). $A_{0.5}$ values after acute insulin stimulation in the range of 0.1 pmol/l to 10 nmol/l were significantly lower compared with $A_{0.5}$ at insulin concentrations of 0.1–1 μ mol/l. Basal $A_{0.5}$ values for diabetic cultures also seemed to increase, but there was no significant difference between low and high insulin concentrations (Fig. 4B). Acute insulin stimulation significantly decreases $A_{0.5}$ for GS in diabetic cultures precultured at 0.1 nmol/l to 0.1 μ mol/l insulin, whereas higher or lower chronic insulin concentrations diminished the effect of acute insulin stimulation (Fig. 4B). $A_{0.5}$ values after acute insulin stimulation in the range of 1 pmol/l to 1 nmol/l are lower than $A_{0.5}$ values at insulin concentrations of 0.1–1.0 μ mol/l (Fig. 4B). Moreover, the stimulated $A_{0.5}$ values seemed to decrease in the insulin concentration range of 0.1–1 nmol/l and increase thereafter to the level of basal $A_{0.5}$ values. To get further insight into the differences between diabetic and control cultures, we compared the absolute differences between the basal and the insulin-stimulated state ($\Delta A_{0.5}$) (Fig. 4C). $\Delta A_{0.5}$ is lower for the diabetic group than the control group, but this difference disappears because $\Delta A_{0.5}$ in cultures established from type 2 diabetic subjects significantly increased with increasing insulin concentration. Higher insulin concentrations (>1 nmol/l) significantly reduced the $\Delta A_{0.5}$ in control as well as diabetic cultures ($P < 0.05$) in a parallel manner. Furthermore, $\Delta A_{0.5}$ correlated with $\Delta FV_{0.1}$ in control cultures ($r = 0.45$, $n = 64$, $P < 0.01$) and in diabetic cultures ($r = 0.54$, $n = 64$, $P < 0.01$). The Hill coefficient was ~ 1.0 for all culture conditions and did not vary with acute or chronic insulin stimulation in either control cultures or cultures established from diabetic subjects ($P > 0.05$, data not shown).

Glucose uptake. We evaluated the capacity for glucose uptake in control and diabetic cultures precultured under three different insulin concentrations and found that when precultured with 1 pmol/l and 1 nmol/l insulin, the basal glucose uptake was reduced by 30% ($P < 0.05$) in diabetic cultures compared with control cultures (Fig. 5). Type 2 diabetic cells exposed to 1 μ mol/l insulin expressed a basal glucose transport activity similar to corresponding control cultures and significantly greater than the basal glucose transport activity seen under the other insulin conditions ($P < 0.05$). Acute insulin stimulation increased glucose transport by 21% in control cultures precultured at 1 pmol/l insulin, but at higher insulin concentrations we could not detect an effect of insulin on glucose uptake. Acute insulin stimulation increased glucose transport in type 2 cultures to corresponding values at insulin concentrations of 1 pmol/l and 1 nmol/l, but it decreased in type 2 cultures precultured at 1 μ mol/l ($P < 0.05$).

Metabolites. We investigated whether the changes in sensitivity to G6P were accompanied by corresponding

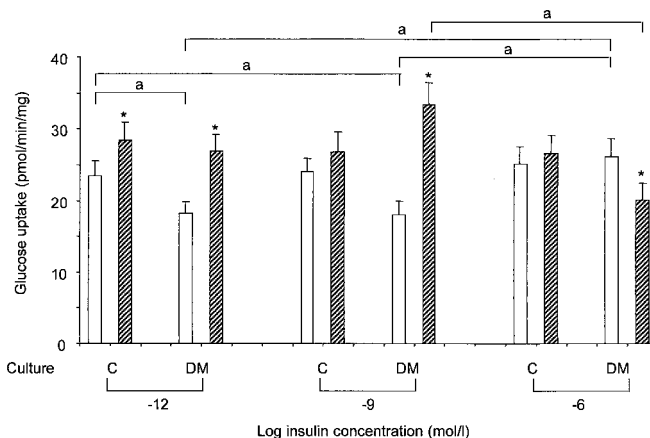


FIG. 5. Effect of preculturing human satellite cell cultures in increasing concentrations of insulin on the basal and insulin-stimulated glucose transport activity in cultures of human myofibers. Human satellite cell cultures were precultured for 4 days at different insulin conditions, and the glucose transport activity was determined under basal and after acute insulin stimulation as described under RESEARCH DESIGN AND METHODS. The glucose transport activity in the basal state (□) and in the stimulated state (▨) are shown for indicated insulin concentrations. Diabetic and control cultures are indicated. Results are shown as means \pm SE ($n = 8$) in each group. * $P < 0.05$ vs. basal. a, $P < 0.05$; C, cultures established from control subjects; DM, cultures established from diabetic subjects.

changes in G6P content and whether some of the differences in GS activities could be explained by changes in glycogen content.

Intracellular glucose. Under all conditions investigated, we found a significant reduction in intracellular glucose content in type 2 cells compared with control cultures (Fig. 6A).

G6P. We could not find significant changes in G6P content as either a function of increasing chronic insulin concentrations or between values before and after acute insulin stimulation. Moreover, we did not find significant differences between control and diabetic cultures (Fig. 6B).

Glycogen content. Glycogen content was significantly decreased in cultures established from diabetic patients compared with those established from control subjects (Fig. 6C). However, we could not find differences between different culture conditions of the two groups. The glycogen content was significantly negatively correlated to the basal $FV_{0.1}$ ($r = -0.33$, $P = 0.01$) and stimulated $FV_{0.1}$ ($r = -0.27$, $P = 0.04$) in cultures established from control subjects, and it was significantly positively correlated to the basal $FV_{0.1}$ ($r = 0.29$, $P = 0.02$) and stimulated $FV_{0.1}$ ($r = 0.27$, $P = 0.02$) in cultures established from diabetic subjects.

DISCUSSION

The present study shows that the diabetic phenotype can be conserved in human satellite cell cultures established from type 2 diabetic subjects and demonstrates that glucose uptake as well as insulin-stimulated GS activity is primarily affected in satellite cell cultures established from patients with type 2 diabetes. Furthermore, these variables could be secondarily modified by chronic hyperinsulinemia. Our dose-response curve at chronic high insulin levels allowed us to differentiate between a primary defect (probably genetic) and the induction of secondary insulin resistance in myotube cultures due to hyperinsulinemia.

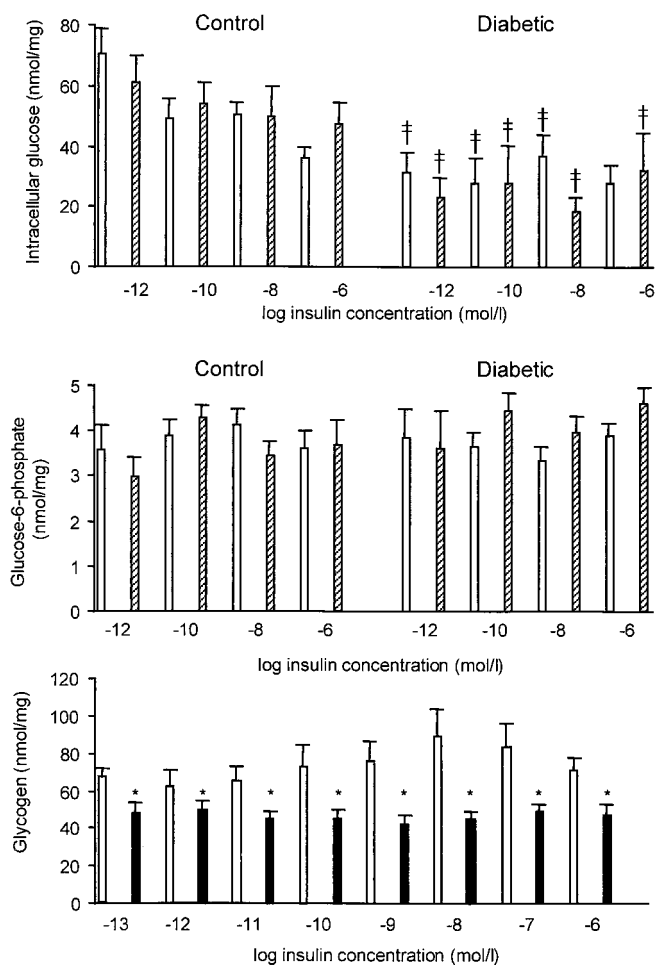


FIG. 6. Effect of the different experimental conditions on the glycogen, intracellular glucose, and G6P content in cultures of human myofibers. Human satellite cell cultures were precultured for 4 days to different insulin concentrations, and the content of intracellular glucose and G6P was determined in the basal and insulin-stimulated state and glycogen was determined in the basal state in cultures as described in RESEARCH DESIGN AND METHODS. *A:* The intracellular glucose content in the basal state (\square) and in the stimulated state (▨) at indicated insulin concentrations. Results are shown as the means \pm SE ($n = 8$) in each group. $\ddagger P < 0.05$ vs. basal. *B:* The G6P in the basal state (\square) and in the stimulated state (▨) at indicated insulin concentration. Results are shown as means \pm SE ($n = 8$) in each group. *C:* The glycogen content in the basal state in control cultures (\square) and in diabetic cultures (▨) at the indicated insulin concentrations. Results are shown as the means \pm SE ($n = 8$) in each group. $*P < 0.05$ vs. corresponding control.

Moreover, the experiments show that hyperinsulinemia can compensate for the genetic defects, at least in part.

Insulin resistance in skeletal muscle in vivo is associated with impaired glucose transport and glycogen synthesis (6,13,14). The molecular differences and the mechanisms responsible for these findings are unclear. It is still uncertain whether the observed changes represent adaptive compensation at the cellular level or the direct expression of a primary genetic trait. In this context, cultures of primary human myotubes offer excellent material for performing studies under standardized conditions. First, satellite cultures express traits known from in vivo muscles (1–3,5,15). Second, cells can be cultured under both basal physiological conditions and insulin resistance-inducing conditions. Recently, we described optimized conditions for satellite cell proliferation and differentia-

tion (1). The main difference between the previously described satellite cell cultures and the cultures we used in the present study are that we exposed our culture to the experimental conditions after differentiation to myotubes had been terminated, at day 4 (1). This ensures that sister cultures from the same subjects are similar in cell composition and differentiation state, and it avoids the influence of experimental conditions on the differentiation process (1).

In this study, we compared a group of “supernormal” young lean subjects with the most common form of type 2 diabetes in obese elderly diabetic subjects in order to optimize our conditions for finding significant differences. It could be speculated that the differences in age may add to obtained results, but age per se seems not to be a significant cause of insulin resistance (16). Satellite cells are quiescent cells that have to be activated in vivo before proliferating and differentiating into myotubes. In our study, isolated quiescent satellite cells were allowed to replicate for 4–6 weeks in a new environment. This new environment will, like the in vivo environment or high insulin concentration in our experiment, influence cell metabolism in a new way, thereby diminishing previous metabolic influences. Moreover, the satellite cells were subsequently allowed to differentiate to myotubes, which changed their protein expression and metabolism. Based on the above consideration, the contribution of previous in vivo influences on our results, i.e., from obesity, may be rather small. However, further investigations are needed to clarify whether previous metabolic influences at the level of quiescent satellite cells in vivo can induce irreversible metabolic changes in cultured human myotubes.

The most well-described defect in the pathophysiology of type 2 diabetes is reduced insulin-mediated glycogen synthesis in skeletal muscles (6,7). In this study, we have shown evidence that skeletal muscle cells established from type 2 diabetic subjects, grown and fused under basal physiological culture conditions, retain defects in insulin-stimulated GS activity, indicating that the insulin-resistant phenotype is intrinsic to the cells. That $\Delta FV_{0.1}$ was significantly lower in diabetic cultures under basal conditions indicates that the finding may be genetic in origin. Previous studies of cell cultures from diabetic subjects cultured under basal physiological conditions showed that the basal as well as the insulin-stimulated $FV_{0.1}$ values are reduced (4,5). These results are in agreement with in vivo data reported by our group (17).

As a new finding, we describe here that impaired GS activity in cell cultures established from type 2 diabetic subjects can be compensated for by increasing insulin concentrations in a dose-dependent manner. This is in agreement with the situation in vivo, where defects in GS activity are compensated for by hyperinsulinemia and hyperglycemia until glycogen synthesis becomes “normal.” In this study, we extended this picture further, since the compensatory effect of hyperinsulinemia on GS in diabetic cultures can be eliminated at supraphysiological insulin concentrations, which induce insulin resistance in diabetic as well as control cultures. The U-shaped curve for type 2 diabetic cultures and the parallel curves for high insulin concentration in control cultures indicate that the mechanism for the primary

defect is different from the mechanism responsible for the induced reduction in acute insulin stimulation of GS.

GS is covalently modified by phosphorylation at nine serine residues by several protein kinases. Insulin activates GS by either inhibiting GS kinase-3 or stimulating protein phosphatase-1. This dephosphorylation is accompanied by a change in GS's affinity for G6P and UDP-glucose. Previously, we have found a close relationship between $FV_{0.1}$, $A_{0.5}$, and the sensitivity of GS to its substrate uridindiphospho-glucose ($Km_{0.1}$) (2). Alterations in $\Delta FV_{0.1}$ in the present study are parallel to similar changes in sensitivity to G6P ($\Delta A_{0.5}$), indicating that phosphorylation/dephosphorylation-dependent mechanisms are involved in the changes of $FV_{0.1}$. The stimulatory effect of G6P at physiological concentrations is due to a conformational change of the phosphorylated form of the enzyme, which facilitates its dephosphorylation. However, we found that the G6P concentrations in type 2 diabetic culture and control cultures were comparable, indicating that type 2 diabetic cultures at euglycemia not are compensating for reduced $FV_{0.1}$ by increasing the G6P concentration.

The glycogen content has been shown to regulate the activity of GS (18). The low glycogen content in diabetes does not seem to be part of the mechanism responsible for insulin resistance because we found a significant positive correlation between glycogen content and GS $FV_{0.1}$ in diabetic cultures. In contrast, increasing glycogen content seems to inhibit GS activity in control cultures. Vestergaard et al. (19,20) have previously shown that total GS activity and GS mRNA were reduced in subjects with type 2 diabetes, but this was not followed by a reduced amount of GS protein. Myotubes established from type 2 diabetic subjects express a reduced GS mRNA and protein compared with control subjects (5). The protein level of GS will be proportional to the maximal GS activity. We found a significantly reduced GS activity at 10 mmol/l G6P in diabetic cultures, indicative of a reduced amount of GS protein.

In accordance with other studies, we found an increased glucose uptake under acute insulin stimulation in cultures established from control subjects precultured under basal physiological insulin concentrations, but when precultured at a higher insulin concentration, we could not see an effect on acute insulin stimulation indicative of induced insulin resistance (10). In accordance, Jackson et al. (9) showed that preculturing at 0.1 $\mu\text{mol/l}$ insulin decreased the effect of acute insulin stimulation on glucose transport. We found a reduced basal glucose uptake in type 2 diabetic cultures when precultured at low insulin concentrations, which is in line with previous observations by Ciaraldi et al. (10). In contrast, type 2 diabetic cultures precultured at very high insulin concentrations expressed an increased basal glucose uptake and a reduced insulin stimulated uptake. Little is known about which transporter mediates these alterations in glucose uptake in human satellite cell cultures, in which GLUT1, GLUT3, and GLUT4 have been described (15,21,22). However, this increase in basal glucose transport under very high insulin concentrations seems to be explained by an increased GLUT1 expression (10).

To determine the relative importance of glucose trans-

port and GS to insulin-stimulated muscle glucose metabolism, we measured the intracellular concentrations of glucose, G6P, and glycogen in cell cultures established from controls and subjects with type 2 diabetes. In healthy humans, skeletal muscle accounts for 70–80% of the insulin-stimulated glucose uptake in vivo (23), and most of the glucose is stored as glycogen (24). We found a reduced glycogen content for all insulin concentrations in diabetic cultures, which is in line with in vivo observations of diabetic muscles and in accordance with our findings of a reduced GS activity in diabetic cultures. If hexokinase activity is reduced relative to glucose transport activity in diabetes, one would predict a substantial increase in intracellular glucose, whereas if glucose transport activity is primary reduced in diabetic cultures, we would expect a decreased intracellular glucose concentration. We found a reduced intracellular glucose content, indicative for a lowered glucose transport activity, which is in agreement with our finding of a reduced basal glucose uptake in diabetic cultures. The decreased glucose transport in diabetic cultures will decrease the intracellular glucose and G6P content, but because the GS activity is also reduced, it is possible to obtain G6P concentrations that are nearly identical in control and type 2 diabetic cell cultures.

Taken together, our data suggest that insulin resistance in patients with type 2 diabetes contain at least two important defects: a reduced glucose transport under basal conditions and a reduced GS activity under acute insulin stimulation, implicating a reduced glucose uptake in the fasting state and a diminished insulin-mediated storage of glucose as glycogen after a meal.

Both glucose transport and glycogen synthesis are stimulated by insulin, and defects in both processes have been postulated to take part in the development of insulin resistance in type 2 diabetic subjects in vivo. Nuclear magnetic resonance studies in vivo show that defective muscle glycogen synthesis is a major cause for insulin resistance and propose that the rate-limiting step is glucose transport (rev. in 25). To identify the rate-limiting step for glycogen synthesis in type 2 diabetic subjects compared with control subjects, the experimental system has to include at least two conditions: 1) similar extracellular conditions under which glucose transport/phosphorylation can be studied and 2) identical glucose uptake fluxes, which will allow the identification of downstream defects. Our experiments fulfill these criteria. Diabetic myotubes expressed a reduced basal glucose uptake compared with control cultures when precultured at equal low-insulin concentrations, and the increase in fractional velocity of GS was reduced after acute insulin stimulation at identical glucose uptake rates in diabetic cultures precultured at low insulin concentrations. Thus, based on above experiments, both glucose transport and GS are rate determining, and the importance of each variable depends on previous and actual concentrations of glucose and insulin.

ACKNOWLEDGMENTS

The Danish Medical Research council; the Novo-Nordisk Foundation; the Danish Diabetes Association; and the

Clinical Research Institute, Odense University Hospital provided financial support for this study.

Irene Lynfort, Karin Dyrgård, and Lone Hansen provided excellent technical assistance. Peter Steahr is thanked for muscle biopsies.

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